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Molecular and Translational Vascular Medicine

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We dedicate this book to our families,
Michelle, Hans, and Nolan Homeister,
and Tina, Connor, and Declan Willis,
who provided the inspiration and support
that enabled us to complete this task.

Jonathon W. Homeister
Monte S. Willis

Preface

The primary goal of this textbook is to provide an overview of cutting-edge and emerging topics in translational vascular medicine as seen by experts from diverse clinical and biomedical research backgrounds. The premise is to highlight the areas of investigation that are currently (or will be) the topics of keen interest to those practicing cardiovascular medicine or researching translational vascular biology. This book includes chapters on translational topics in vascular medicine that are commonly overlooked in research reviews and vascular medicine texts. Throughout the book, the authors communicate complex molecular mechanisms using easy-to-follow text that is complemented with valuable figures that illustrate the key topics. This textbook is intended for physicians, residents, fellows, and graduate students from a variety of specialties related to vascular disease.

This book is divided into two broad themes associated with molecular and translational vascular medicine. The first part includes chapters on recent advances toward understanding the pathogenesis of common vascular diseases and presents the unique contributions of molecular imaging to our understanding of their pathogenesis, as well as their diagnosis and treatment. Several chapters review recent insights into the genetics and molecular and cellular biology specific to aneurysms (Chap. 1), the vasculitides (Chap. 2), and venous thrombosis (Chap. 3). These are followed by a chapter more broadly applicable to vascular diseases that reviews recent progress in understanding the role of the ubiquitin proteasome system in modulating the pathogenesis of endothelial dysfunction (Chap. 4). The first part concludes with a chapter unique to most vascular medicine texts on the development of imaging modalities, and cellular and molecular probes, that hold promise to image the molecular and cellular pathogenesis of, and perhaps treat vascular diseases (Chap. 5).

The second part focuses on the evolving molecular- and cellular-based therapies under development and being used to diagnose and treat vascular disease. The first two chapters discuss recent insights into the mechanisms of vasculogenesis and angiogenesis and translation of this knowledge to treat critical limb ischemia (Chaps. 6 and 7). Next is a review of the pathogenesis of restenosis after arterial injury and the latest therapeutic options to modulate it (Chap. 8). This is followed

by a more detailed discussion of the evolving use of nanoparticle technology in vascular medicine as it applies to drug-eluting stents, as well as its potential to diagnose and treat other vascular diseases (Chap. 9). We conclude this part with a discussion of the current status and potential of emerging endothelial progenitor cell-based therapies for cardiovascular diseases (Chap. 10).

We believe that this book comprises a unique compilation of reviews that cover many of the exciting and current topics in translational vascular research and medicine. The translational focus of the book made it an enjoyable project, and we hope you find it informative and valuable.

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Part I
Recent Advances in Understanding and Imaging the Molecular and Genetic Basis of Vascular Diseases

Chapter 1
The Molecular Biology and Genetics of Aneurysms

Helena Kuivaniemi, Gerard Tromp, David J. Carey, and James R. Elmore

Introduction

Aortic aneurysms and dissections are leading causes of death in the USA with nearly 13,000 deaths reported in 2007 [1]. If left untreated, the aortic wall continues to weaken and becomes unable to withstand the forces of the luminal blood pressure resulting in progressive dilatation and rupture. Along the length of the aorta, significant heterogeneity occurs in the distribution of aneurysmal disease. The prevalence of abdominal aortic aneurysms (AAAs), located in the infrarenal section of the aorta, is at least three times higher than that of the thoracic aortic aneurysms and dissection (TAAD) [2, 3]. These two forms of aneurysms differ in many aspects (Table 1.1), and are therefore discussed here separately. The main emphasis of this chapter is in the non-syndromic forms of aortic aneurysms. We discuss only briefly the rare syndromic forms of aortic aneurysms associated with Marfan syndrome, Ehler–Danlos syndrome, and Loeys–Dietz syndrome (Table 1.2), when there is a phenotypic and genotypic overlap with the non-syndromic forms of aortic aneurysms. Readers interested in more information on these syndromes are referred to recent reviews published elsewhere [13–15].

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Table 1.1 Differences between thoracic and abdominal aorta

Feature	Thoracic aorta	Abdominal aorta
Structure of aorta	Elastic type with more collagen and elastin than in the lower parts	Elastic type with less collagen and elastin than in the upper parts
Embryologic origin	Aortic root: secondary heart field Aortic arch: neural crest Descending aorta: somites	Splanchnic mesoderm
Rupture model	Dissection	Expansion
Susceptibility to aneurysms	Low	High
Susceptibility to atherosclerosis	Low	High
Prevalence of aneurysms	1–2%	Male (>65 years): up to 10% Female (>65 years): 1–2%
Male:female ratio of aneurysm patients	2:1	6:1
Age of aneurysm patients at diagnosis (years)	Syndromic (Marfan) 24.8 Familial 56.8 Sporadic 64.3	Male >65 Female >75
Proportion of patients with syndromic form	~20%	<1%
Chromosomal abnormalities	45,X (Turner syndrome)	

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Regional Differences in Embryologic Origin and Disease Susceptibility in the Aorta

Aortic aneurysms tend to manifest at specific sites with approximately 90% developing between the renal arteries and iliac bifurcation. Aneurysms develop less frequently in the ascending and descending thoracic aorta, and differ from AAAs in prevalence, risk factors, genetics, and histology [2, 3]. Family-based studies on TAADs and AAAs have demonstrated that most families have either TAAD or AAA, but not both [16–19]. Furthermore, based on recent genetic studies there is only a limited overlap in the genetic loci identified for TAAD and AAA (Fig. 1.1; Tables 1.1 and 1.2). These findings suggest distinct genetic mechanisms of aneurysms formed in the thoracic and abdominal regions of the aorta.

Several factors can contribute to the regional variation to disease susceptibility along the length of the aorta: (1) structural differences; (2) differences in mRNA expression; and (3) differences in embryonic origin. There are intrinsic structural differences between the abdominal and thoracic aorta. The abdominal aorta has a narrower diameter and contains several major branch points, both of which result in turbulent blood flow and decreased shear stress. Areas of low shear stress are more

Table 1.2 Genetic loci of syndromic and non-syndromic forms of aortic aneurysms

Chromosomal region	Disease phenotype	Inheritance	Phenotype MIM ID	Gene	Gene/locus MIM ID	SNP ID (risk allele) for associated allele	Comments	Reference
2q31	EDSIV	AD	130050	*COL3A1*	120180		Rare form	
3p12.3	AAA		100070			rs7635818(C)	Identified in GWAS	
3p21	TAAD	AD		*MYLK*			~1% of FTAAD	[5]
3p22	LDS2B	AD	610380	*TGFBR2*	190182		Rare form	
3p22	TAAD	AD		*TGFBR2*	190182		~4% of FTAAD	[6]
4q31	AAA		609782				AAA2 locus; Identified in DNA linkage study	
5q13-q14	TAAD	AD	607087				AAT2 locus; Identified in DNA linkage study; ~10–30% of FTAAD	[7]
9p21	AAA		611891			rs10757278(G)	AAA3 locus	
9q22	LDS1A	AD	609192	*TGFBR1*	190181		Rare form	
9q22	LDS2A	AD	608967	*TGFBR1*	190181		Rare form	
9q22	TAAD	AD		*TGFBR1*	190181		1% of FTAAD: also known as AAT5 locus	[7]
9q33	AAA			*DAB2IP*	609205	rs7025486(A)	Identified in GWAS	[8]
10q23.3	TAAD	AD	611788	*ACTA2*	102620		10–14% of FTAADs; some patients have livedo reticularis and iris flocculi	
10q23.3	MYMY5		614042	*ACTA2*	102620		Rare form of MYMY	
10q23.3	MSSMDS		613834	*ACTA2*	102620			
11q23.3-q24	TAAD	AD	607086				Rare form of FTAAD; AAT1 locus	[9]
12q13-q14	TAAD	AD					Identified in DNA linkage study	[10]

(continued)

Table 1.2 (continued)

Chromosomal region	Disease phenotype	Inheritance	Phenotype MIM ID	Gene	Gene/locus MIM ID	SNP ID (risk allele) for associated allele	Comments	Reference
15q21.1	MFS	AD	154700	*FBN1*	134797			
15q21.1	TAAD	AD		*FBN1*	134797		Rare form of FTAAD	[11]
15q21.1	TAAD			*FBN1*		rs2118181(G)	Identified in GWAS with STAAD	[61]
15q22.33	TAAD	AD		*SMAD3*	603109		Identified in exome sequencing; ~2% of FTAAD	[12]
15q22.33	LDS1C/AOS	AD	613795	*SMAD3*	603109			
16p13.11	TAAD with PDA	AD	132900	*MYH11*	160745		~1% of FTAADs	
19q13	AAA		100070				AAA1 locus: identified in DNA linkage study	

MIM Mendelian inheritance in man database available at http://omim.org; Reference a publication describing the finding if no MIM record is available; *EDSIV* Ehlers–Danlos syndrome type IV; *LDS1A* Loeys–Dietz syndrome, type 1A; *TAAD* thoracic aortic aneurysm and dissection; *FTAAD* familial thoracic aortic aneurysm and dissection; *STAAD* sporadic thoracic aortic aneurysm and dissection; *AAA* abdominal aortic aneurysm; *MYMY5* Moyamoya disease, subtype 5; *MSSMDS* multisystemic smooth muscle dysfunction syndrome; *LDS1C/AOS* Loeys–Dietz syndrome, type 1C, also known as aneurysms-osteoarthritis syndrome; *PDA* patent ductus arteriosus; *MFS* Marfan syndrome; *AD* autosomal dominant; *COL3A1* type III procollagen; *MYLK* myosin light chain kinase; *TGFBR1* and *2*, transforming growth factor β receptors 1 and 2; *DAB2IP* DAB2 interacting protein; *ACTA2* smooth muscle alpha-actin 2; *FB1* fibrillin 1; *SMAD3* SMAD family member 3; *MYH11* smooth muscle myosin heavy chain. Official approved gene symbols for this table and throughout the chapter were obtained from www.gene.ucl.ac.uk/nomenclature

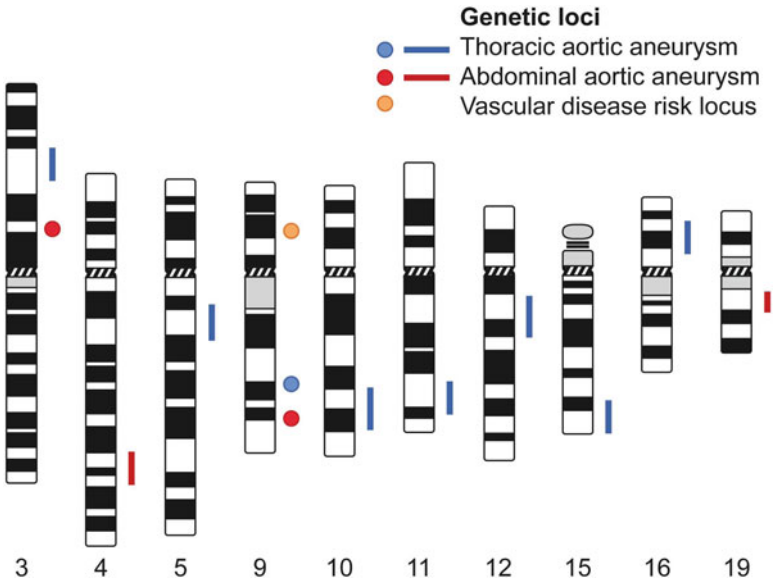

Fig. 1.1 Genetic loci associated with TAAD and AAA. The underlying genetic loci contributing to aortic aneurysms demonstrate that different loci result in different clinical manifestations. *Vertical lines* indicate genomic regions identified by DNA linkage analysis, whereas *dots* indicate SNPs associated with aneurysms, or genes with mutations. For details, see Table 1.2. Reproduced from [24] with permission from Springer Science + Business Media

susceptible to atherosclerotic lesions. In addition, the abdominal aortic wall is thinner than that of the thoracic aorta, contains fewer elastic lamellae, and has decreased amounts of the structural proteins, elastin and collagen. These structural differences may confer greater susceptibility to injury and dilatation to the abdominal aorta.

Recent studies have demonstrated that different regions of the aorta differ in their mRNA expression profiles. One such group of genes is the homeobox (HOX) transcription factors, which are known to control embryonic spatial patterning and regulate proliferation and differentiation of vascular cells. In microarray analyses of multiple adult cell types, a correlation was found between HOX gene expression and the anatomic site of origin [4, 20]. It has also been shown that there are distinct HOX gene expression domains within the vasculature [21]. One recent study used microarray-based expression profiling to identify genes whose expression differed along the aorta in baboons. Several HOX genes were found to have spatial expression changes [22]. Further studies on one of the HOX genes, *HOXA4*, demonstrated that the thoracic aorta had significantly higher HOXA4 protein levels compared to abdominal aorta based on Western blot analyses carried out on 24 paired human samples. Immunohistochemical staining for HOXA4 showed nuclear and perinuclear staining in endothelial and smooth muscle cells in aorta. The *HOXA4* transcript levels were significantly decreased in human AAAs compared to age-matched non-aneurysmal controls. Cultured human aortic endothelial cells and smooth muscle cells stimulated with interferon gamma (INFG; an important inflammatory cytokine in

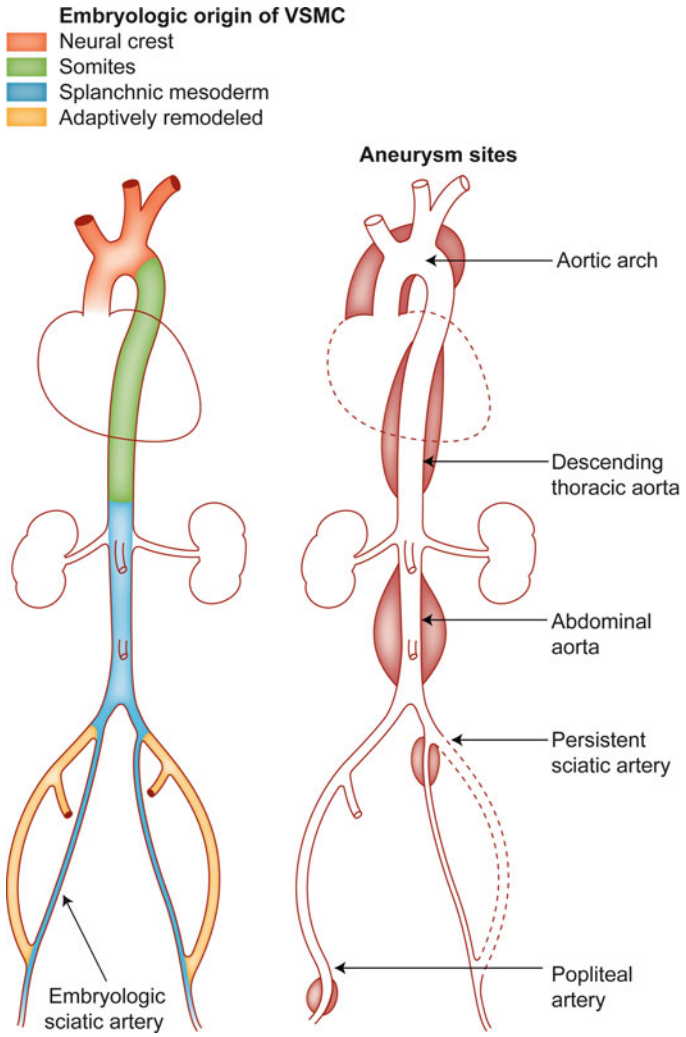

Fig. 1.2 Embryologic origin and disease susceptibility vary along the length of the aorta and connecting arteries. Different parts of the aorta arise from different embryologic origins. Disease susceptibility also varies with the region, with the infrarenal abdominal aorta being more prone to atherosclerosis and aneurysm formation than the thoracic aorta. Reproduced from [24] with permission from Springer Science+Business Media

AAA pathogenesis) showed decreased levels of HOXA4 protein [22]. Based on these studies it is plausible to hypothesize that there are other genes with similar spatial expression patterns that could contribute to regional disease susceptibility.

Different segments of the aorta have distinct embryologic origins [4, 23, 24]. The aorta is composed of cells originating from the neural crest, mesenchyme, and splanchnic mesoderm, and there appears to be a clear difference in the origins of the different segments (Table 1.1 and Fig. 1.2). This provides an explanation why vascular smooth

muscle cells isolated from the thoracic region of aorta respond differently than smooth muscle cells isolated from the abdominal segment of the aorta when treated with transforming growth factor β (*TGFβ*) and various cytokines in cell cultures [25]. This might also explain why mutations in the *TGFβ* receptors (see below) lead to diseases of the thoracic aorta, but not the abdominal aorta.

The early embryo develops a common set of precursor vessels from primitive primordial cells which differentiate into arteries, veins, and lymphatic vessels. Primitive arteries, the paired dorsal aortae, are surrounded by smooth muscle cells of mesodermal origin. Extensive remodeling of the primitive aortic arches results in the adult aortic root and arch and some of the branching vessels. Recent embryological studies revealed that the smooth muscle cells of the adult aortic root and arch as well as the thoracic aorta are derived from a second wave of smooth muscle cells that migrate from the neural crest and replace the original primitive smooth muscle cells of splanchnic mesodermal origin [26]. These neural crest-derived smooth muscle cells likely have a different epigenetic programming that allows them to adaptively remodel the thoracic aorta to withstand the higher pulse pressure and ejection volume by laying down more elastic lamellae during development and growth. In contrast, the epigenetic programming of the smooth muscle cells in the abdominal aorta remains more similar to that of the original primitive arterial smooth muscle cells.

The hypothesis that embryologic origin defines at least partially disease susceptibility was strengthened by experiments in animals in which transplantation of the abdominal aorta into the thoracic aorta led to increased susceptibility to atherosclerosis in the thoracic region, whereas transplantation of the thoracic aorta into the abdominal region led to decrease in susceptibility to atherosclerosis of the abdominal region [27]. These studies showed that it was not merely the local blood flow dynamics, but rather the inherent disease susceptibility of different cell populations that contributed to disease development.

Additional support regarding the consequences of embryological origin derives from the unusual susceptibility of the popliteal artery to aneurysm. It is, after the abdominal aorta, the most frequent site of arterial aneurysm [28–30]. The popliteal artery is derived embryologically from the sciatic artery, which itself is derived from the primitive axial artery [31]. During normal development, the femoral artery branches off the sciatic close to its origin, and grows into a larger vessel that anastamoses with the sciatic artery just above the knee, leaving the popliteal artery as a remnant of the original sciatic artery (Fig. 1.2). When the "primitive" sciatic artery persists, the femoral artery does not develop [31]. This "primitive" sciatic artery is very prone to aneurysm development; it is estimated that about half of the cases of persistent sciatic artery develop aneurysms [31].

Clinical Features of Aortic Aneurysms

AAAs are frequently diagnosed only when they rupture since they are usually asymptomatic up to the time of hemorrhage [2, 3]. The result is high morbidity and mortality in patients with ruptured aneurysms. Current diagnosis of AAAs depends

more on radiological imaging than on physical examination due to the obesity epidemic [2, 3]. Screening for AAAs is rarely performed so that most identified aneurysms are found incidentally during medical imaging for other conditions. Improved diagnosis is critical in identifying small AAAs. Additionally, novel medical and surgical treatment strategies must be further developed to prevent rupture.

The diameter of a normal abdominal aorta is approximately 2 cm. Dilatation of the infrarenal abdominal aorta to a diameter of 3 cm or larger is the classic definition of an AAA [2, 3]. From a clinical perspective, this single size makes it easy for clinicians although the exact definition of an aneurysm is a 50% increase in size compared to the normal diameter for that patient [2, 3]. Ectasia of the abdominal aorta is dilatation of the aorta but to a size of less than 3 cm.

Clinical risk factors for AAA development include older age, male sex, Caucasian race, smoking history, chronic obstructive pulmonary disease, hyperlipidemia, and coronary artery disease [32]. The disease affects primarily men; our experience at Geisinger Clinic reveals that 78% of AAA patients are male and 89% are current or former smokers; these values are typical of what is observed generally (unpublished Geisinger data from the authors). Family history of AAA is a well-known risk factor related to the development of AAA (see below); among the Geisinger AAA patients, 21% report having a relative with an aortic aneurysm. The incidence of AAA is declining in countries where incidence of smoking is decreasing [33].

Based on large cohorts, the rate in which AAAs expand is an average of 3.2 mm per year [34]. The growth rate, which varies depending on the size of the aneurysm, with the smaller aneurysms expanding more slowly, has been estimated to be approximately 10% of the AAA size per year [35]. The ultimate concern with expansion is the rupture of AAAs. Five centimeter AAAs have an estimated rupture rate of 12% per year and 6 cm or larger AAAs have an estimated rupture rate of 14% per year based on the studies following patients unfit for surgery [36]. In this study, 35% of patients developed a ruptured AAA from the time of diagnosis at a median of 18 months (5–5.9 cm AAA: 28% rupture rate; 6 cm or larger AAA: 41% rupture rate).

Patients treated with surgery for ruptured AAAs have an estimated mortality rate of 50%. Since many patients with ruptured AAA never receive medical attention, the overall mortality is estimated to be nearly 90% due to the sudden deaths that occur at home [37].

Prevention of AAA rupture has focused on surgical replacement of the aorta using an open surgical technique or an endovascular approach utilizing stent grafts. Open surgical replacement began in the early 1950s and has an excellent long-term track record for treating aortic aneurysms with mortality rates of approximately 5% [32]. Endovascular AAA repair (EVAR) began with its commercial release in the USA in 1999. The obvious advantages of EVAR are small incisions (or no incisions with a percutaneous approach) and an overall mortality rate of approximately 1% [32]. The long-term follow-up studies of open AAA versus EVAR are showing no sustained difference in the mortality rate despite the initial benefit seen with EVAR [32].

To be considered successful, pharmacological therapy for AAAs requires only an inhibition of the growth rate of small aneurysms in order to avoid or delay the need for surgery [38]. The use of statins as a pharmacological therapy for AAA is controversial, with some studies suggesting inhibition [39] and other studies showing no effect on limiting aneurysm expansion [40]. Doxycycline, a matrix metallopeptidase (MMP) inhibitor, has been used in a small preliminary study and was able to limit aneurysm growth [41]. β-blockers were used in a randomized trial and did not limit aneurysm growth [42]. Angiotensin-converting enzyme (ACE) inhibitors have yet to be reported in a randomized human trial [38]. Smoking cessation, blood pressure control, and statin use for control of lipid levels are currently accepted recommendations for patients with AAAs [32].

Elective AAA repair is indicated when aneurysm diameter is larger than 5 cm in females or larger than 5.5 cm in males [32]. Although these sizes for AAA repair are based on the results of randomized studies (see below), in the USA many smaller aneurysms are repaired due to patient anxiety, chronic low back pain, or physician preference [43]. Noncompliance with necessary follow-up testing is an accepted indication to repair small aneurysms as is any symptomatic or ruptured aneurysm. The United Kingdom Small Aneurysm Trial [44] randomized patients with aneurysms between 4 and 5.5 cm to either immediate open surgery or continued surveillance. Interestingly, 62% of patients in the surveillance arm ultimately required surgery during the mean follow-up interval of 8 years. There were no statistical differences in survival between the groups, but there was a higher operative mortality than expected in both groups. A similar trial, sponsored by the veterans administration in the USA, compared open surgery to surveillance of small aneurysms and was called the aneurysm detection and management (ADAM) study [34]. Patients with aneurysms of sizes between 4 and 5.4 cm were randomized and once again no difference was found between the two groups of patients. Both studies supported the same conclusion that smaller aneurysms could be followed safely.

EVAR has been compared to open surgery in two European randomized clinical trials called endovascular versus open repair of AAA (EVAR-1) [45] and the Dutch randomized endovascular aneurysm repair (DREAM) [46]. In each trial, those patients who were treated with EVAR fared better in the short term with lower morbidity and mortality. Long-term mortality equilibrated in these studies with more interventions required in the stent graft patients. Meta-analysis of AAA stent grafts in the USA has documented lower mortality as compared to open AAA surgery. American surgeons have continued to place stent grafts due to the fact they have lower short-term mortality and with the hope there will be continued clinical improvements with stents that will improve long-term outcomes.

Thoracoabdominal aortic aneurysms and TAAD represent approximately 5% of aortic aneurysms [47]. In comparison to AAA, the average descending thoracic aorta is approximately 2.5–2.7 cm, which means an aneurysm is defined by a size of 3.75–4.0 cm (50% increase). TAADs are generally considered for repair when their size is between 5 and 6 cm. Clinical risk factors for development of an aneurysm are similar between thoracic and AAA, with the exception that TAAD patients are more likely to be female and younger than AAA patients (Table 1.1). Current treatment

of TAAD is similar to AAA and includes both open surgical as well as thoracic endovascular aortic repair (TEVAR) [19] The stent graft procedure is the preferred treatment modality for TAAD, given higher morbidity and mortality of open thoracic surgery [48]. Thoracoabdominal aneurysm repair is more complex with the visceral arterial branches arising from the aneurysmal segment. Fenestrated stent grafts are used in the USA experimentally for thoracoabdominal aneurysms with the standard therapy still being the traditional open surgery.

Interesting subgroups of aortic aneurysm patients include those with dissection, and patients with connective tissue disorders such as Marfan syndrome and Ehlers–Danlos syndrome type IV [49]. Patients with Marfan syndrome present with ascending aortic aneurysm, aortic rupture, and thoracic aortic dissection. The characteristic clinical features include tall stature, along with ocular and skeletal abnormalities. Marfan syndrome is caused by mutations in the fibrillin 1 (*FBN1*) gene [13]. Patients with Ehlers–Danlos syndrome type IV, also referred to as the vascular subtype of Ehlers–Danlos syndrome, present with rupture of hollow organs such as bowel, uterus and aorta or medium-sized arteries. These patients also have joint hypermobility and skin hyperextensibility. Mutations in *COL3A1* gene leading to defective type III collagen protein cause this syndrome [50]. Aortic repair operations in these patients are challenging due to tissue friability [51].

Pharmaceutical treatment has been used for many years for the Marfan syndrome, in which the standard of care includes the use of β-blockers to control blood pressure. Promising results have also been obtained by the use of the angiotensin receptor blocker, losartan, which is currently in randomized trials in the USA [52] as well as in Europe [53]. Angiotensin receptor blockers are effective antagonists of TGFβ signaling, an underlying biological pathway for both the Marfan syndrome and TAAD [19, 47]. It is therefore possible that angiotensin receptor blockers may also be effective for TAAD.

Genetics and Molecular Biology of TAAD

A number of studies have shown that aortic aneurysms are frequently familial, even when they are not associated with heritable disorders such as the Marfan syndrome or the type IV variant of the Ehlers–Danlos syndrome. In 1967, Hanley and Jones [54] reported on a family with non-syndromic TAAD noting that the patients did not fit the diagnostic criteria of the Marfan syndrome. This was followed by numerous additional reports on single families or small collections of families with non-syndromic TAAD [55]. More recently an analysis of larger collections of non-syndromic TAAD patients has established that about 20% have a positive family history for aneurysms and are called the familial TAAD (FTAAD) [56].

The genetics of TAADs (Tables 1.1 and 1.2) appears to differ from that of other types of aneurysms in that they are more often single gene disorders where a mutation in one single gene explains the disease and is highly penetrant showing autosomal dominant inheritance pattern [19, 55]. Altogether ten susceptibility loci for

TAAD are currently known and were identified through genome-wide DNA linkage studies and candidate gene studies (Table 1.2 and Fig. 1.1). These ten loci, however, explain the familial aggregation of TAAD in only about 20% of the families that have been studied, suggesting that additional loci will be found [19, 55].

Seven genes (Table 1.2) have been found to harbor mutations in patients with TAAD. These are the genes for: (1) myosin light chain kinase (*MYLK*); (2) transforming growth factor β receptor 1 (*TGFBR1*); (3) transforming growth factor β receptor 2 (*TGFBR2*); (4) smooth muscle myosin heavy chain 11 (*MYH11*); (5) smooth muscle alpha-actin 2 (*ACTA2*); (6) fibrillin 1 (*FBN1*); and (7) SMAD family member 3 (*SMAD3*) [5, 12, 55, 57, 58]. Molecular testing in the form of sequence analysis is currently available for five of these genes, and a list of providers of the tests can be found in the Gene Tests Laboratory directory at http://www.ncbi.nlm.nih.gov/sites/GeneTests/. Patients with confirmed mutations should be evaluated carefully and considered for elective repair of aneurysms at early stages [19]. In the near future, it is expected that whole-genome sequencing will make the molecular diagnostics more straightforward, since one sequencing reaction will reveal all the variants present in a person's genome. Interpretation and annotation of such large-scale sequencing data are currently challenging and results require validation by other methods.

There is a considerable overlap between the syndromic and non-syndromic forms of TAAD at the molecular level (Table 1.2). Two TAAD genes, *TGFBR1* and *TGFBR2*, are the same as the genes harboring mutations in patients with the Loeys–Dietz syndrome, a rare autosomal dominant syndrome with a variety of clinical features, including hypertelorism, craniosynostosis, structural brain abnormalities, mental retardation, congenital heart disease, bifid uvula with or without cleft palate, and generalized arterial tortuosity with ascending aortic aneurysm and dissection [55]. The TAAD patients harboring mutations in *TGFBR1* and *TGFBR2* do not have these clinical manifestations, and, therefore, cannot be classified as Loeys–Dietz syndrome patients [55].

In addition, some TAAD patients have mutations in the *FBN1* gene, which also harbors mutations in patients with the Marfan syndrome [11]. Other examples of genetic and phenotypic overlap are patients with the subtype 5 of the Moyamoya disease and patients with a new syndrome called multisystemic smooth muscle dysfunction syndrome [59] who have mutations in the *ACTA2* gene, as well as patients with the aneurysms-osteoarthritis syndrome (AOS), also known as the Loeys–Dietz syndrome, type 1C, with mutations in the *SMAD3* gene (Table 1.2).

Some patients with TAAD have other clinical manifestations in addition to the aortic aneurysms and dissections. For example, the 16p13.11 TAAD locus was identified using a large 178-member French family with TAAD and *patent ductus arteriosus* (PDA) [60]. Similarly, patients with mutations in the *ACTA2* gene sometimes have characteristic skin rash (*livedo reticularis*) or eye manifestations (*iris flocculi*).

Genetic studies have provided important information on the pathobiology of TAAD by revealing that dysregulation of TGF signaling is one of the biological mechanisms leading to TAAD. Mutations in four genes which are part of the TGFB

signaling pathway, *TGFBR1*, *TGFBR2*, *FBN1*, and *SMAD3*, have been found in TAAD patients. Another molecular mechanism involved in TAAD and revealed by the genetic studies is smooth muscle contractility, since three genes, *MYLK*, *MYH11*, and *ACTA2* from this functional class, harbor mutations in patients with TAAD [55].

TAAD patients without known family history for the disease, sporadic TAAD (STAAD) cases, are also likely to have genetic risk factors based on a recent genome-wide association study (GWAS) in which a total of 1,313 STAAD cases and 1,500 controls were analyzed [61]. A major susceptibility locus for TAAD was identified at the 15q21.1 locus with multiple associated SNPs identified in a large region of linkage disequilibrium (LD) encompassing *FBN1*, suggesting that STAAD shares pathobiological mechanisms with the Marfan syndrome (see above) [13].

Microarray-based expression profiling has been used to study the differences between atherosclerotic and lesion-free parts of thoracic aorta of organ transplants [62], as well as blood [63] and tissue [64–66] samples from patients with TAAD. These studies revealed a number of genes and microRNAs whose expression levels varied between TAAD cases and controls [62–66].

Genetics of AAA

About 20% of AAA patients have a first-degree relative with the disease [17]. Population-based studies have demonstrated that a family history of AAA is an important risk factor for AAA with an odds ratio (OR) of 1.96 (95% CI: 1.68–2.28) [17]. The lifetime prevalence of AAA among siblings of AAA patients is estimated to be about eightfold higher than in the general population [67]. A recent twin study also strongly supported the involvement of genetic factors in AAA [68].

Several studies have also reported on collections of AAA families [17], the largest one with 233 multiplex families, in which there were at least two related individuals with AAA [69]. In this study, 72% of the AAA families had an autosomal recessive inheritance pattern, while 25% showed an autosomal dominant pattern of inheritance. Two segregation analyses revealed statistically significant evidence for a genetic model with a major gene effect in AAA [70, 71]. Segregation analysis involves the application of formal mathematical and statistical models of genetic inheritance on epidemiological family data where it is suspected that genetics contributes to the disease prevalence. The models permit testing whether purely environmental or stochastic occurrence of disease fits better than genetic models and, if a genetic model fits, whether any of the classic inheritance models (recessive, dominant, additive) fits best [72]. A major gene effect model indicates that there is support for a genetic effect which has moderate intrafamilial correlation, where not everyone with the risk allele will show the disease phenotype, a phenomenon referred to as reduced penetrance.

Several approaches have been used to study the genetics of AAA [18]. Since the first candidate gene studies were published over 20 years ago, nearly 100 genetic

association studies using SNPs in biologically relevant genes have been reported on AAA. The studies investigated SNPs in genes of the extracellular matrix, the cardiovascular system, the immune system, and signaling pathways [73]. Very few studies were large enough to draw firm conclusions and very few results could be replicated in another sample set. Several candidate genes were also sequenced in AAA patients, but no mutations contributing to the disease were identified [73].

There are two unbiased genome-wide approaches for identifying the genes that contribute to a disease phenotype: DNA linkage analysis and association analysis. DNA linkage analysis relies on analysis of the genotypes of family members and investigates the coinheritance of genetic markers through observed meioses. That is, for each zygote the parent genotypes are known so that it can be determined which markers were derived from which parent. With three or more generations it is possible to determine the colocation of genetic markers with some precision. If the disease gene is considered to be a marker, its collocation (linkage) with other markers can be determined. DNA linkage is very powerful, but the information about the position of the disease gene is not very precise (the interval in the genome is large). Association analysis studies populations of apparently unrelated individuals, but makes the assumption that the population has a limited number of ancestors in the past (last common ancestor) and is therefore a study of unobserved meioses. A requirement for allelic association to succeed is that a substantial fraction of the diseased population inherited the same allele in the same disease gene identical by descent (and consequently also identical by state). It is then possible to test for the difference of the disease allele frequency in unaffected and affected individuals (association).

The first DNA linkage study [74] for AAA used 235 affected relative pairs (ARPs) from 119 AAA families and found significant linkage on chromosomes 4q31 (LOD score = 3.73, $p = 0.0012$) and 19q13 (LOD score = 4.75, $p = 0.00014$) [74]. The analysis used a conditional logistic regression model for affected relative pairs to detect linkage, which allowed inclusion of additional variables (covariates) into the analysis. In this study, sex and the number of affected relatives were used as covariates. The analysis model also included the interactions between the sex and number of affected relatives. A problem for all genetic analyses is the presence of genetic heterogeneity, i.e., multiple genes (loci) or alleles contributing to the disease phenotype. Linkage analyses are robust to allelic heterogeneity at the same gene locus, but suffer from loss of power in the presence of locus heterogeneity. Use of conditional logistic regression models with covariates in genetic analyses allows for genetic heterogeneity if the covariates are surrogate markers for the subgroups corresponding to the individual loci (genes). These genomic regions were designated as AAA2 and AAA1 susceptibility loci, respectively, in online Mendelian inheritance in man (OMIM) (Table 1.2 and Fig. 1.1). The 19q13 locus was found also in another DNA linkage study for AAA using Dutch AAA families [75]. The chromosome 4 and 19 candidate regions contain a large number of plausible and physiologically relevant positional candidate genes including low density lipoprotein receptor-related protein 3 (*LRP3*), peptidase D (*PEPD*), hepsin (*HPN*), interleukin 15 (*IL15*), GRB2-associated binding protein 1 (*GAB1*), and endothelin receptor

type A (*EDNRA*). Recently a genetic association study for ten of the chromosome 19 candidate genes was carried out and putative associations with SNPs located in the *PEPD* gene were identified [76]. Further analysis of the *PEPD* gene with DNA sequencing, however, failed to identify mutations responsible for AAA [76]. The results demonstrate the difficulty in making educated guesses about targets in candidate gene studies. In the near future, whole-exome and whole-genome sequencing will be used to identify the sequence variants in the linkage regions contributing to the disease.

Two GWASs for AAAs have been reported [8, 77], and additional studies are currently in progress [78]. The markers used for GWASs are single nucleotide polymorphisms (SNPs). Most SNPs have two alleles which are identified by their state as one of the four bases in deoxyribonucleic acid (DNA): A, adenosine; C, cytosine; G, guanine; and T, thymidine. Association is determined by the excess of one of the two alleles among the affected population when compared to the reference population (controls). Odds ratios are reported for the risk allele which is reported as one of the four bases (above).

The first GWAS used pooled DNA samples and a case–control design to find an AAA-associated haplotype on chromosome 3p12.3 (Table 1.2 and Fig. 1.1) [77]. One SNP in this region (rs7635818) was genotyped in a total of 502 cases and 736 controls from the original study population ($p=0.017$) and 448 cases and 410 controls from an independent replication sample ($p=0.013$; combined $p=0.0028$; combined odds ratio = 1.33). An even stronger association with AAA was observed in a subset of smokers (391 cases, 241 controls, $p=0.00041$, odds ratio = 1.80), which represent the highest risk group for AAA. The AAA-associated haplotype is located 200 kbp upstream of the transcription start site for the contactin 3 gene (*CNTN3*), a member of a family of cell adhesion molecules. It is a plausible candidate gene, but further studies are needed to establish what role CNTN3 plays in AAA pathophysiology. A group from New Zealand followed up these findings by genotyping rs763518 in 567 cases and 552 controls but could not confirm the association. The group also tested three SNPs in intron 2 of *CNTC3* and found a weak association with AAA [79].

The second GWAS on AAA was carried out with 1,292 AAA patients and 30,503 controls from Iceland and the Netherlands [8]. The SNPs with the most significant associations were then genotyped in a replication cohort of 3,267 AAA patients and 7,451 controls. The A allele of an SNP (rs7025486) on 9q33 was associated with AAA, with an odds ratio of 1.21 and $p=4.6\times10^{-10}$. The same SNP was also associated with early onset myocardial infarction (odds ratio = 1.18, $p=3.1\times10^{-5}$), peripheral artery disease (PAD) (odds ratio = 1.14, $p=3.9\times10^{-5}$), and pulmonary embolism (odds ratio = 1.20, $p=0.00030$), but not with intracranial aneurysm (IA) or ischemic stroke. No association was observed between this SNP and common risk factors for arterial and venous diseases such as smoking, lipid levels, obesity, type 2 diabetes, or hypertension. This SNP is located within the *DAB2IP* gene, which encodes an inhibitor of cell growth and survival [8].

To date, the strongest association between AAA and a genetic variant was found when 2,836 AAA cases and 16,732 controls were tested at SNP rs10757278 discovered in a separate GWAS on coronary artery disease [80]. The SNP (rs10757278),

located on chromosome 9p21, was associated with AAA ($p = 1.2 \times 10^{-12}$; OR = 1.31, 95% CI: 1.22–1.42) and has been designated the AAA3 locus in OMIM (Table 1.2). It was also associated with PAD, and IAs, but not with atherosclerotic stroke. This was the first genetic variant common to AAA, IAs, and other cardiovascular diseases suggesting shared pathophysiology in these vascular diseases. The SNP is located only 10 kbp away from rs10811661, an SNP known to be associated with type 2 diabetes. The two SNPs are not associated with one another, and AAA or IA is not associated with rs10811661. This finding is in agreement with epidemiological data demonstrating that diabetes is not a risk factor for AAA or IAs [81].

As with many other associations discovered by GWASs, it was not immediately evident what gene in the vicinity of rs10757278 contributed the risk. In a recent study, the entire region was sequenced in 47 individuals to try to fine-map the region and identify the specific variant contributing to disease [82]. Although a more comprehensive set of SNPs was identified, none showed stronger association than the original GWAS signals [82].

The best candidate gene in the chromosome 9 region associated with AAA is a noncoding RNA gene *CDKN2BAS*, also known as *ANRIL*. The gene produces at least three alternatively spliced transcripts, with the major transcript produced at about tenfold the level of the two minor transcripts in peripheral blood monocytes, as well as in atherosclerotic plaques. Expression levels of the minor transcripts in the peripheral blood monocytes are correlated with atherosclerotic plaque load in the carotid arteries, whereas the major transcripts (produced at ten times the level of minor transcripts) and those of other genes in the locus are not correlated [83]. Recent animal experiments provide some insight into the potential mechanism by which *CDKN2BAS* contributes to vascular diseases. Deletion of a 70 kbp region encompassing rs10757278 and portions of *CDKN2BAS* gene, but not the two closest protein coding genes *CDKN2A* and *CDKN2B*, affects cardiac and vascular expression of *CDKN2A* and *CDKN2B*. Cultured smooth muscle cells from the aortae of mice homozygous for the 70 kb deletion proliferated about twice as fast as those from controls of the same strain, and did not show signs of senescence [84]. A hyperproliferative vascular smooth muscle cell phenotype is consistent with risk for atherosclerosis [85, 86] and possibly occlusive disease, but the mechanism by which it contributes to aneurysms, which are characterized by smooth muscle cell apoptosis and loss rather than proliferation, requires further study.

Animal Models of Aortic Aneurysms

Several animal models are available that reproducibly create aneurysms that are similar in many respect to human aneurysms [87–89]. These models have been extremely valuable in elucidating the cellular and molecular pathophysiology of aneurysms, in identifying potential therapeutic targets for novel pharmacological treatment of aneurysms, and, more recently, in developing new imaging modalities to provide better diagnosis of aneurysms. A limitation of most models is that they induce aneurysm formation by introducing an artificial external perturbation, and

so are not informative with respect to the factors that initiate aneurysm formation in humans.

Specific genetic mutations can lead to spontaneous aneurysm formation or rupture of arteries. These have generally been found in genes that are involved in the maintenance of the extracellular matrix structure. These include defects in the lysyl oxidase (*Lox*) gene, which is required for elastin and collagen cross linking; *Fbn1*, a structural matrix protein involved in the Marfan syndrome in humans; or mutations that affect cardiovascular phenotype globally, e.g., low density lipoprotein receptor (*Ldlr*) or apolipoprotein E (*Apoe*) null animals. These are useful models to study monogenic vascular diseases, but they are not informative with respect to the pathophysiology of the common form of AAA, which is a complex multifactorial disease.

The first experimental animal model of AAA to be widely used is based on an infusion of a solution of pancreatic elastase into an isolated segment of the rat infrarenal aorta. The elastase percolates through the vessel wall and initiates elastin breakdown and a cascade of inflammatory infiltration and activation at the infusion site. Within 1–2 weeks an aneurysm is produced, which is histologically similar to human AAA, with extensive elastin fragmentation and progressive dilatation of the affected region of the aorta. This method was later adapted to produce experimental aneurysms in mice, which have the advantage of the availability of genetically altered strains that allow the role of specific molecular pathways to be investigated. The elastase infusion model of aneurysm formation has also been used successfully in swine [90]; while not widely used as an animal model, swine have the advantage of being more amenable to testing of endovascular devices than rodents.

An alternative method used to experimentally induce aneurysm formation is the application of calcium chloride to the extraluminal surface of the aorta. The calcium chloride produces localized tissue damage and inflammation; within 4–6 weeks an aneurysm is produced at the site of calcium chloride application, along with the typical hallmarks of aneurysm formation, including elastin breakdown, macrophage infiltration, and upregulation of MMPs and other proteolytic enzymes (Fig. 1.3). It was reported recently that a combination of intraluminal elastase and extraluminal calcium chloride in rat aortas produced experimental aneurysms more reproducibly than either method alone [92].

Although these models of induced aneurysm formation faithfully reproduce essentially all of the main features of human aneurysms, a striking difference is the time course of aneurysm formation, which in the animal models is compressed into a period of 2 weeks or less. From an experimental perspective, this has obvious advantages. On the other hand, it may be producing a distorted picture of the temporal sequence of events that lead from aneurysm initiation to progression in humans.

Another widely used model of aneurysm formation is based on the long-term administration of the vasoactive peptide angiotensin-II (AngII) into mice by means of an implanted osmotic pump. This method was originally developed in mouse strains that were hyperlipidemic due to ablation of apolipoprotein E or the *low density lipoprotein* receptor [87]. AngII infusion was later shown to induce aneurysm formation in wild-type mice, albeit at a lower frequency [87]. This is consistent with the

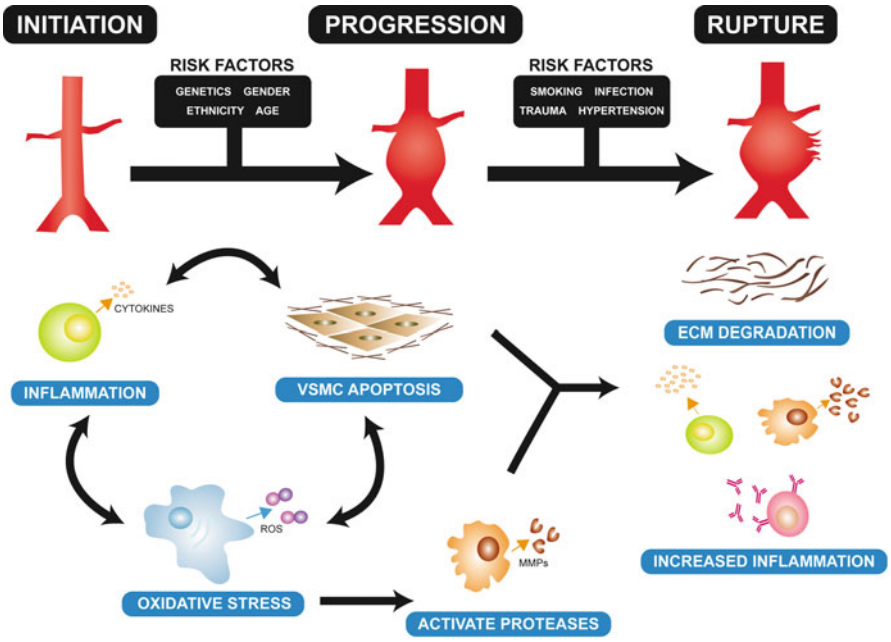

Fig. 1.3 Summary of the AAA pathogenesis. Several biological processes and risk factors have been identified that contribute to AAA pathogenesis. Critical pathways involved in AAA pathogenesis include immunological processes, oxidative stress, depletion of vascular smooth muscle cells through the process of apoptosis, and the destruction of the extracellular matrix by matrix metalloproteinases. A general model for AAA formation is that in response to an unknown initiating stimulus activated immune and inflammatory cells infiltrate the vessel wall; these cells release proinflammatory cytokines and extracellular proteases that cause matrix degradation and elastin fragmentation; proinflammatory cytokines and matrix degradation products lead to recruitment of additional inflammatory cells, which leads to more cytokines and protease activity and further destructive tissue remodeling, in a positive feedback loop. See text for detailed discussion. *VSMC* vascular smooth muscle cell, *ECM* extracellular matrix, *ROS* reactive oxygen species, *MMPs* matrix metalloproteinases. Reproduced with permission from [91]. Copyright © 2011 Prous Science, S.A.U. or its licensors. All rights reserved

observation in humans that hyperlipidemia is not essential to aneurysm formation but is a positive risk factor.

Long-term AngII infusion produces aneurysms in both the thoracic and abdominal portions of the aorta. The characteristics of the aneurysms produced in these two regions are distinct, however (see above). This difference to some extent mirrors the distinct characteristics of thoracic and AAAs in humans, and may result from the different embryologic origins of the vascular smooth muscle cells in the two regions of the vasculature (e.g., neural crest vs. somites). TGF-β signaling appears to be a key mediator of TAAD formation, but not AAA, in the AngII infusion model. This is consistent with different responses of vascular smooth muscle cells from these

regions to TGFβ stimulation (reviewed in [93]), and again is consistent with the distinct nature of aneurysms formed in the ascending and descending limbs of the aorta in humans, where TGFβ signaling has been shown to be important for TAAD but not AAA formation.

Two additional mouse models of aortic aneurysms have been developed; one using a high fat diet in *Apoe* knockout mice and the other using Balb/C donor allografts in a recipient of a different strain [87, 94], but they have not been used widely making it difficult to judge their merits compared to the other models.

The genetic background of mice has been found to influence results in the elastase-perfusion aneurysm model. Mice with a 129/SvJ or 129/SvEv background are resistant to aneurysm formation, whereas FVB and B/6 mice are susceptible in the elastase-perfusion model [95]. Another interesting finding is that results using genetic mouse models sometimes differ based on the model used to induce aneurysm formation. For example, *Mmp12* null mice showed decreased susceptibility to aneurysm formation in the calcium chloride model but not in the elastase model [96, 97]. Similarly, *Timp2* null mice showed decreased susceptibility to aneurysm formation in the calcium chloride model, whereas *Timp1* null mice showed increased susceptibility to aneurysm formation in the elastase model [98, 99].

Recent advances in generating conditional mutant mice in which genes are selectively ablated in specific cells (e.g., smooth muscle cells) may provide useful information about the molecular pathways contributing to the embryological development of the vasculature and disease pathogenesis. This technique was recently used to delete the integrin-linked kinase (*Ilk*) gene in smooth muscle cells in mice [100]. The mice developed TAAD and PDA and the results identified a new molecular pathway linking Ilk signaling to the contractile smooth muscle cell phenotype. Further evidence that Ilk signaling is important for TAAD came from the findings that *Ilk* mRNA levels were found to be lower in mice with aortic aneurysms induced in the AngII infusion model [100].

Another interesting application of these animal models is their use in developing and testing novel imaging technologies to more precisely characterize aneurysms in situ, with a long-term goal of developing better means to identify aneurysms with a high risk of rupture. For example, magnetic resonance imaging using a novel contrast reagent, P947, has confirmed the presence of MMPs at sites of focal inflammation in the elastase-infusion model of AAA [101]. PET imaging using a novel macrophage-targeted nanoparticle revealed the presence of macrophages at sites of aneurysm formation in the AngII mouse aneurysm model [102]. Further development of these and other imaging techniques could identify distinct cellular and molecular signatures that are better predictors of rupture risk than aneurysm diameter alone in humans.

Another interesting application of animal models is to understand the mechanistic basis of the effect of cigarette smoking on aneurysm risk. A synergistic effect of cigarette smoke on aneurysm formation was demonstrated in the elastase-infusion model in mice. Exposure to cigarette smoke for 2 weeks prior to initiation of aneurysm formation produced larger aneurysms in animals that received a low, sub-

maximal dose of the elastase. The mechanism by which cigarette smoke affected aneurysm expansion is not known, but in this study the effect of cigarette smoke was independent of an effect on *Mmp9* or *Mmp12* expression [103].

The pronounced sex bias of AAA in humans has also been observed in rodent models using the AngII and the elastase-perfusion models of AAA. In the AngII mouse model castration of male mice reduces aneurysm formation to levels observed in female mice, suggesting that male hormones are a key risk factor. Administration of dihydrotestosterone increased aneurysm incidence in both male and female mice [104]. In the AngII model, the effect of male sex hormones appears to be mediated by aortic expression of the type 1a receptor of AngII (Agtr1a). This is based on the observation that Agtr1a expression is higher at sites of aneurysm formation in male but not in female mice. Castration of male mice decreased Agtr1a expression in the abdominal aorta, and its expression was restored by administration of dihydrotestosterone [104]. Although intriguing, the generalizability of these findings to the sex bias seen in human AAAs is unclear, since there is no evidence of a direct role for AngII or its receptors in human AAA formation. Furthermore, recent epidemiological studies found that lower free testosterone and higher luteinizing hormone levels were associated with AAA in older men [105].

Animal models have also been used to identify shared molecular pathways for two or more cardiovascular diseases with the hope that such research could provide drug targets for more than one disease. Mice lacking the Kruppel-like factor 15 (*Klf15*), a zinc finger transcription factor, developed both cardiomyopathy mimicking human heart failure, and aortic aneurysms in the AngII model via a shared mechanism dependent on p300 acetyltransferase and p53 [106]. The mice could be rescued by deleting p53 or inhibiting p300 [106]. These findings are consistent with lower levels of KLF15 mRNA in the hearts and aorta of patients who have either heart failure or AAA [106, 107].

Pathobiology of AAA

General Model of AAA Pathobiology

A hallmark of aortic aneurysms is extensive extracellular matrix degradation and especially loss of intact elastin fibrils (Fig. 1.3) [17, 89, 91, 108]. Another consistent feature is loss of viable smooth muscle cells from the medial layer of the aorta; the smooth muscle cells are often replaced by a poorly organized, collagen-rich connective tissue. This tissue remodeling alters the physical properties of the vessel wall which makes it less able to maintain the vessel's normal shape in response to aortic blood pressure. Many aspects of the cellular and molecular processes that produce these changes are now understood, as a result of extensive analysis of both human AAA tissue and animal models [17, 89, 91, 108]. It is now generally accepted that extracellular matrix degradation is due to increased localized proteolytic activity at sites of aneurysm formation. Increased levels of MMPs, especially MMP9 and

MMP12, are consistently observed in human AAA tissue and in experimental aneurysms in animal models. Furthermore, increased proteolytic activity and matrix degradation are consistently associated with the focal presence of immune and inflammatory cells, including macrophages, B cells, and T cells [17, 89, 91, 108]. Thus, a general model for aneurysm formation that has evolved is that in response to an unknown initiating event, activated immune and inflammatory cells infiltrate the vascular wall. These cells then release proinflammatory cytokines and extracellular proteases, which cause matrix degradation and elastin fragmentation. The released proinflammatory cytokines and possibly matrix degradation products then lead to recruitment of additional immune and inflammatory cells, which leads to more proinflammatory cytokines and proteolytic activity and further matrix destruction, in a positive feedback loop (Fig. 1.3).

Intraluminal Thrombus

Intraluminal thrombus is a common, but not universal, finding in human AAA and in animal models [109]. It has been proposed that the thrombus provides a substrate for platelet recruitment and activation, activation of plasminogen and other proteolytic enzymes, and generation of reactive oxygen species, all of which contribute to the breakdown of the vascular wall at the site of aneurysm formation [110]. Administration of AZD6140, an antagonist of purinergic receptor P2Y1 (P2ry1), reduced thrombus development at the site of an aneurysm, as well as leukocyte infiltration of the mural thrombus, MMP9 expression, and elastin degradation in a rat model [111]. P2Y1 (P2ry1) is a G-protein-coupled receptor that regulates platelet activation. In this model the aneurysms were induced in the rats by grafting de-cellularized pig aortas into the rat aortas [111]. Although these data support a role for platelet activation in aneurysm formation, perhaps through effects on intraluminal thrombus, some studies on human AAA have not found evidence for this [109].

Role of Chymase-Positive Mast Cells

Chymase-positive mast cells have been shown to be present in human AAA tissue, and also accumulate at sites of aneurysm formation in rat and mouse models [113]. A role for mast cells in AAA formation is suggested by the finding that in mast cell-deficient rats, aneurysms produced by calcium chloride treatment are smaller and show less activated MMP9 and elastin degradation. Similar results were produced by treatment with tranilast, an inhibitor of mast cell degranulation [113]. Mice with a knockout of the mouse chymase homolog, mast cell protease-4 (*Mcp4*), show reduced AAA expansion and less elastin degradation than wild-type mice in the elastase infusion model. In vitro studies showed that Mcp4 regulates the activation of cathepsin proteases. This suggests that chymase may be a key upstream regulator

of a proteolytic cascade that includes cathepsins and MMPs; activation of this cascade in response to unknown initiating inflammatory events results in progressive matrix degradation leading to aneurysm formation [114].

Role of Matrix Metalloproteinases

The membrane-anchored MMP14, also known as MT1-MMP, is expressed by infiltrating macrophages, and is elevated at sites of aneurysm formation in the calcium chloride mouse model [115]. Transplantation of bone marrow cells from *Mmp14*-null mice into wild-type mice almost completely blocked formation of aneurysms induced by calcium chloride administration. Macrophage infiltration was still observed, suggesting that MMP14 had a direct effect on aneurysm formation. Further evidence for this comes from the observation that in vitro macrophage MMP14 has significant elastolytic activity, even in the absence of MMP9 [115].

Inflammatory Signaling

A number of studies have used animal models to investigate the role of inflammatory signaling in aneurysm formation [17, 87, 89, 94, 112, 113, 114, 116–119]. Together, the findings consistently identified localized inflammatory activation at sites of aneurysm formation which is critical for aneurysm expansion. Inflammation is also strongly correlated with the presence of proteolytic enzymes and elastin fiber degradation, which are generally accepted as proximal features of the cellular and molecular changes associated with aneurysm expansion. Animal studies have identified a multitude of inflammatory signaling molecules that appear to contribute to aneurysm formation, including tumor necrosis factor [TNF; also known as TNF-α], prostaglandins, leukotrienes, and peroxisome proliferator-activated receptor γ (PPARG) [116, 120–122]. Modulation of these pathways by pharmacological interventions or genetic manipulation has been reported to attenuate aneurysm formation in animal models (see below).

Prostaglandin E Signaling

The prostaglandin E receptor 4 (PTGER4) binds prostaglandin E2 and attenuates inflammatory signaling. Bone marrow transplantation of *Ptger4*-deficient cells resulted in increased inflammation and enhanced aneurysm formation in the AngII mouse model [120].

Peroxisome Proliferator-Activated Receptor γ

Vascular SMC-specific knockout of *Pparg* resulted in increased levels of activated cathepsin S and enhanced elastin degradation in the calcium chloride animal model [122]. Conversely, administration of the PPARG agonist rosiglitazone to mice treated with AngII to induce aneurysm formation showed fewer ruptures and reduced rates of aneurysm expansion; this was independent of effects of the drug on blood glucose levels. Rosiglitazone caused reduced expression of Agtr1a, suggesting a potential mechanism for the observed effects on aneurysm formation. Because there is no direct evidence linking AngII or this receptor to AAA formation, however, the implications for treatment of human AAA are unknown [123].

Tumor Necrosis Factor α

The proinflammatory growth factor TNF is present at elevated levels in human AAA tissue. Mice lacking *Tnf* are resistant to aneurysm formation and show less macrophage infiltration and reduced levels of MMP9 [116]. Consistent with these results, administration of the TNF antagonist, infliximab, had a similar effect on aneurysm formation [116].

Leukotrienes

Leukotriene B4 receptor 1 (LTB4R1), also known as BLT1, is a G-protein-coupled receptor that is activated by binding the proinflammatory lipid mediator leukotriene B4 and is expressed in subclasses of leukocytes. *Ltb4r1* null mice treated with AngII to induce aneurysm formation had fewer and smaller aneurysms, reduced leukocyte infiltration, and lower MMP activity than wild-type mice [121].

Inhibiting Inflammatory Signaling in AAA: NFκB, Statins, and Resveratrol

NFκB and members of the E 26 (ETS)-family are nuclear transcription factors that are common points of convergence for many proinflammatory signaling pathways. NFκB and ETS transcription factors in general are activated in human AAA tissue [88] and in animal models of experimental AAA, where they are detected in infiltrating macrophages and other cells. *In silico* analysis of the promoter regions of the genes upregulated in human AAA tissue [107] demonstrated enrichment for binding sites of NFκB and ETS family of transcription factors [88]. Administration of a chimeric decoy oligodeoxynucleotide that targets NFκB and ETS transcription

factors to preformed experimental aneurysms in rabbits caused a decrease in aneurysm size, suppression of MMP9 activation, and preservation of elastin fibers at sites of aneurysm formation [117]. Similarly, when the decoy oligodeoxynucleotides were administered by intraperitoneal injection to rats in which experimental aneurysms were induced by elastase infusion, the treatment led to attenuated aneurysm expansion, less MMP9 and MMP12 activation, and preservation of elastin fibers [124].

Statins, in addition to their well-known effects on cholesterol biosynthesis, also have broad anti-inflammatory activity. Statin administration attenuated aneurysm formation in several different animal models, including the rat and mouse elastase infusion model and the AngII mouse model [125, 126]. Resveratrol, a polyphenol found in red wine and known to have anti-inflammatory and antioxidant effects, prevented the formation of AAA in the calcium chloride mouse model [127]. The effects on aneurysm formation in both the statin and resveratrol studies correlated with decreases in inflammatory cell invasion and activation.

Microarray Analysis of AAA Pathobiology

Genome-wide microarray-based expression studies provide a comprehensive and unbiased approach to identify biological pathways involved in disease pathobiology [128]. Several such studies have been carried out to analyze human aneurysmal tissue [107, 129] and peripheral blood [130, 131] samples obtained from patients with AAA as well as tissue samples from animal models of AAA [118, 119, 126]. Many genes previously implicated in the pathogenesis of AAA such as *MMP1*, *MMP9*, *IL1B*, and *PLAU* were among the differentially expressed genes. There were also novel genes, such as *RUNX3* (runt-related transcription factor 3), which were substantially different between AAA and control aorta. Kyoto encyclopedia of genes and genomes (KEGG) annotation, which categorizes genes by biological pathway to determine which pathways are statistically enriched when compared to a standard reference, showed that two of the most significantly enriched pathways were the *leukocyte transendothelial migration* and the *natural killer cell cytotoxicity* pathway [107]. These findings are biologically relevant to AAA as the infiltration of immune cells to AAA is well documented [16, 17]. The microarray-based expression analysis extended these findings by demonstrating broad coordinated gene expression in immunological pathways (Fig. 1.3).

Microarray-based expression studies also provided strong evidence that complement cascade, which acts at the interface between innate and adaptive immunity by augmenting antibody responses and enhancing immunologic memory, plays a role in the pathobiology of human AAA [132]. The pathway was activated in human AAA, particularly via the lectin and classical pathways. Furthermore, there was an overrepresentation of binding sites for the transcription factor STAT5A on the promoter regions of the complement cascade genes suggesting coordinated regulation of their expression [132].

Role of Infectious Disease in the Pathobiology of AAA

Microorganisms including *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Helicobacter pylori*, human cytomegalovirus, *herpes simplex virus*, and different oral bacteria have been studied as possible triggers for the development of AAAs, but the data are inconclusive [133]. In the past, infection by *Treponema pallidum* caused thoracic aortic aneurysms in the late stage of syphilitic disease. *Borrelia*, which is responsible for the Lyme disease and is a member of the same spirochete family, was investigated in a recent study with AAA patients. In this study, ~34% of German AAA patients had antibodies against *Borrelia*, which was significantly more than the 3–17% of general population in Germany or even the 16% of PAD patients used as controls [133]. Since *Borrelia* DNA could not be detected in the AAA wall even with sensitive PCR-based methods, it is plausible to hypothesize that AAAs are triggered by *Borrelia* via molecular mimicry in which similar epitopes are present in the surface of *Borrelia* and components of the aortic wall. Molecular mimicry has also been suggested in studies which identified a candidate autoantigen called aortic aneurysm-associated protein-40 (AAAP-40) in the aortic wall; AAAP-40 protein has sequence homology with *T. pallidum*, *herpes simplex virus*, and human cytomegalovirus [16].

Role of Atherosclerosis in AAA

In the past it was thought that atherosclerosis causes AAA; "atherosclerotic aneurysm" is still a commonly used term in textbooks to describe AAA. Several pieces of evidence, however, suggest that even though atherosclerosis and AAA share some characteristics, they are separate disease entities. There is divergence in risk factors between AAA and aortoiliac occlusive disease (AOD). Most notable is the negative association with type 2 diabetes [81], a known risk factor of atherosclerotic disease. Additionally, unlike AAA, AOD has an approximately equal number of affected males and females. Histological examination of AAA further supports the hypothesis that these two diseases are different. Findings characteristic of AAA are inflammation in the entire aortic wall, especially adventitia, loss of smooth muscle cells, and disruption of elastic fibers in the tunica media (Fig. 1.3) [3]. In contrast, AOD is primarily an intimal disease, with some invasion deeper into the vessel wall. Molecular and genetic studies have shown large differences in gene expression patterns and genetic risk factors [62, 128, 134]. In conclusion, AAA and AOD are distinct in that they differ histologically, have common, but also different, epidemiological and genetic risk factors, and have different gene expression profiles.

Conclusions and Future Direction of Research

In this chapter, we have summarized the current knowledge on molecular biology and genetics of aortic aneurysms. Although great advances have been made in the past 20 years, complete understanding of the pathobiology of aortic aneurysms is still lacking, and a number of key questions related to the factors determining predisposition to aneurysm and early detection of them, as well as the treatment of this deadly disease remain: (1) who will get an aneurysm, (2) what factors determine how fast aneurysms grow, and (3) what factors cause aneurysms to rupture. Answers to these questions will allow us to more reliably identify individuals who will develop an aneurysm and, more importantly, which aneurysms will rupture. Furthermore, they will lead to new ways to treat aneurysms, including novel nonsurgical approaches. AAA is a complex disease, and obtaining answers to these rather simple questions will also be complex, and require innovative, interdisciplinary approaches that integrate information from epidemiological [135, 136], genetic [18, 73], molecular biology [128], and bioengineering [137] studies on humans and animal models [87].

A critical issue related to treatment of aortic aneurysms is the need to develop means to slow the growth of an aneurysm or prevent a dissection. It would be far more desirable to treat elderly aneurysm patients with medicines that slow the growth of their aneurysm, and consequently reduce the likelihood of rupture than to subject them to surgical repair. Unfortunately, despite a large number of studies testing a variety of agents in animal models, no pharmacological treatment for human aortic aneurysms is yet in clinical practice.

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Chapter 2
The Molecular Biology and Treatment of Systemic Vasculitis in Children

Despina Eleftheriou and Paul A. Brogan

Introduction

Systemic vasculitis is characterized by blood vessel inflammation, which may lead to tissue injury from vascular stenosis, occlusion, aneurysm or rupture [1]. Apart from relatively common vasculitides such as Henoch–Schönlein Purpura (HSP) and Kawasaki disease (KD), most of the primary vasculitic syndromes are rare in childhood, but when present are associated with significant morbidity and mortality [2, 3]. The cause of the majority of childhood vasculitides is unknown, although it is likely that a complex interaction between environmental factors, such as infections and inherited host responses, triggers the disease and determines the vasculitis phenotype [4]. This chapter summarizes the findings of recent studies relating to the pathogenesis of systemic vasculitis, and considers HSP, KD, antineutrophil cytoplasmic antibodies (ANCAs)-associated vasculitis, polyarteritis nodosa and Takayasu arteritis (TA). Rarer forms of vasculitis are beyond the scope of this chapter, and the reader is referred elsewhere [5]. In addition, we discuss current therapeutic approaches and ongoing challenges in the field of paediatric vasculitis research.

Henoch–Schönlein Purpura

HSP is the most common childhood primary systemic vasculitis [2]. HSP typically affects children between the ages of 3–10 years [6]. Gardner-Medwin et al. reported an estimated annual incidence of 20.4 per 100,000 children in the UK [2].

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Modifications of the classification criteria defining HSP described by Ozen et al. in 2005 [7] have recently been made following a formal validation study [8]. According to the new EULAR/PRINTO/PRES definition, a patient is classified as having HSP in the presence of purpura or petechie with lower limb predominance (mandatory criterion) plus one out of four of the following criteria [8]:

1. Abdominal pain
2. Histopathology showing typical leucocytoclastic vasculitis with predominant IgA deposit or proliferative glomerulonephritis with predominant IgA deposit
3. Arthritis or arthralgia
4. Renal involvement (proteinuria or haematuria or presence of red blood cell casts)

In cases with purpura with atypical distribution, a demonstration of IgA is required at biopsy. This new definition provides sensitivity and specificity for classification of HSP (using other forms of vasculitis as controls) of 100% and 87%, respectively [8].

Pathogenesis

As many as 50% of occurrences in paediatric patients are preceded by an upper respiratory tract infection [4, 9]. Several agents have been implicated, including group A streptococci, varicella, hepatitis B, Epstein–Barr virus, parvovirus B19, *Mycoplasma*, *Campylobacter*, and *Yersinia* [4]. Of note, Masuda et al. showed that nephritis-associated plasmin receptor (NAPlr), a group A streptococcal antigen, may have a pathogenetic role in a subset of patients with HSP nephritis [10]. Among 33 children with biopsy proven HSP nephritis, 30% had segmental or global mesangial deposition of NAPlr antigen, comparing to 3% in other children with non-HSP nephritis glomerular diseases (half of these children had IgA nephropathy) [10]. The exact pathophysiologic mechanism, if any, and the relationship between NAPlr and HSP nephritis need, however, further investigation. So far no single infectious agent has been consistently identified, and it is likely that genetically controlled host responses determine whether or not an individual develops HSP in response to infectious triggers. But despite the fact that the cause of HSP is unknown, it is likely that IgA has a pivotal role in the pathogenesis of the disease, a hypothesis supported by the almost universal deposition of IgA in lesional vascular tissue [11]. Skin or renal biopsies demonstrate the deposition of IgA (mainly IgA1) in the wall of dermal capillaries and post-capillary venules and mesangium [11]. In addition, serum IgA levels have been reported to be increased during the acute phase of the disease, and a proportion of patients have circulating IgA-containing immune complexes and cryoglobulins [12]. Some studies have found IgA antineutrophil cytoplasmic antibodies (IgA-ANCAs) in a proportion of patients with HSP, while others have shown an increase in IgA-rheumatoid factor or IgA-anticardiolipin antibodies [11]. Recently, galactose deficiency of O-linked glycans in the hinge region of IgA1 has

been reported in adults with IgA nephropathy and children with HSP [13]. These aberrantly glycosylated IgA1 proteins form immune complexes that deposit in the mesangium; their binding to mesangial cells stimulates cellular proliferation and overexpression of extracellular matrix components resulting in the typical renal lesions associated with HSP [13]. Recently, Hiasano et al. showed complement activation through both the alternative and lectin pathways in patients with HSP nephritis and demonstrated that this complement activation is promoted in situ in the glomerulus [14]. The formed IgA immune complexes, through the activation of complement, lead to the formation of chemotactic factors (such as C5a), which in turn recruit polymorphonuclear leucocytes to the site of deposition [15, 16]. The polymorphonuclear leucocytes thus recruited by chemotactic factors cause inflammation and necrosis of vessel walls with concomitant thrombosis [11]. This subsequently results in extravasation of erythrocytes from haemorrhage in the affected organs and is manifested histologically as leucocytoclastic vasculitis [11]. The term leucocytoclasis refers to the breakdown of white blood cells in lesional tissue, particularly the characteristic nuclear debris ("nuclear dust") observed, and is not specific for HSP.

Genetics

Several genetic polymorphisms have been linked with HSP in various population cohorts, often with consistent results across multiple studies (summarized in Table 2.1) [32]. Many of these polymorphisms relate to cytokines or cell adhesion molecules involved in the modulation of inflammatory responses and endothelial cell activation [4, 32]. The connection between HSP and HLA alleles is the most convincing genetic association. In three cohorts from Italy, northwest Spain and Turkey, DRB1*01 and DRB*11 have each been positively associated (OR 1.5–2.5), and DRB*07 negatively associated, with HSP in two of the three studies [17, 18, 33]. HLAB35 was associated with HSP in a Turkish cohort [19], but was only associated with nephritis in a Spanish cohort [20]. Null alleles in either of the complement factor C4 genes (C4A or C4B) have shown associations with HSP in multiple cohorts of different ethnicities [29], but different findings regarding associations with C4A or C4B alleles, association with heterozygosity or only homozygosity for a null allele, and close linkage of the C4 genes to HLA have resulted in debate about the significance of these findings. Polymorphisms in the angiotensin-converting enzyme (ACE) gene have been associated with risk of HSP in two cohorts (OR 2.3–2.7) [24, 25]; several additional studies have focused on association of ACE alleles with risk of nephritis but without consistent findings. A high carriage rate of mutations in MEFV was recently reported in Turkish children with HSP (OR 2.06) [27]. On the whole, however, studies of this nature have been hampered by relatively small patient numbers and thus lack the power to be definitive or necessarily applicable to all racial groups.

Table 2.1 Positive genetic associations in Henoch Schönlein purpura

Molecule/genetic polymorphism	Role of polymorphism	Reference
Human leucocyte antigens (HLAs)	Positivity for HLA-B35 predisposes to renal involvement in a Spanish cohort HLA-B35 predisposes to HSP in a Turkish cohort DRB1*01 and *11 positively associated and DRB*07 negatively associated with risk of HSP	[17–20]
Interleukin-8 (IL-8)	Polymorphism associated with renal involvement	[21]
Interleukin-1 receptor antagonist (IL-1Ra)	Polymorphism predisposes to renal involvement	[22]
Interleukin-1β (IL-1 β)	Polymorphism predisposes to renal involvement	[23]
Angiotensin converting enzyme (ACE)	Increased risk of HSP	[24, 25]
Vascular endothelial growth factor (VEGF) and its receptor (KDR)	VEGF polymorphisms predispose to renal involvement	[26]
Familial Mediterranean fever genotypes (MEFV gene mutation)	Mutations in MEFV found more commonly in Israeli and Turkish children with HSP	[27, 28]
Complement C4A and C4B	Increased risk of HSP	[29]
PAX2 (paired box gene 2)	Polymorphisms in PAX2 predispose to renal involvement in HSP	[30]
Nitric oxide and associated molecules	Inducible nitric oxide synthase 2A promoter polymorphism predisposes to renal involvement	[31]

Clinical Features

Skin involvement is typically with purpura, which is generally symmetrical, affecting the lower limbs and buttocks in the majority of cases, the upper extremities being involved less frequently [34]. The abdomen, chest and face are generally unaffected [34]. Angioedema and urticaria can also occur [34]. Around two thirds of the children have joint manifestations at presentation [34]. Three quarters of the children develop abdominal symptoms ranging from mild colic to severe pain with ileus and vomiting [34]. Haematemesis and melena are sometimes observed [34]. Other complications include intestinal perforation and intussusception [34]. Acute pancreatitis is also described, although is a rare complication [34]. Other organs less frequently involved include the central nervous system (cerebral vasculitis), gonads (orchitis may be confused with torsion of the testis) and the lungs (pulmonary haemorrhage) [34]. Reports of HSP nephritis indicate that between 20% and 61% of cases are affected with this complication. Renal involvement can present with varying degrees of severity [34]. This includes isolated microscopic haematuria, proteinuria with microscopic or macroscopic haematuria,

acute nephritic syndrome (haematuria with at least two of hypertension, raised plasma creatinine and oliguria), nephrotic syndrome (usually with microscopic haematuria) or a mixed nephritic–nephrotic picture [34].

Treatment

The large majority of cases of HSP require symptomatic treatment only [34]. Non-steroidal anti-inflammatory drugs (NSAIDs) may be used to treat arthralgia associated with HSP [34]. Controversies concerning the use of corticosteroids in the treatment of HSP exist with regard to whether or not they can (1) reduce severity or duration of disease, (2) decrease the risk of glomerulonephritis, and (3) prevent relapses of the disease [35, 36]. Chartapisak et al. recently systematically reviewed all published randomized controlled trials (RCTs) for the prevention or treatment of renal involvement in HSP [37]. Meta-analyses of four RCTs, which evaluated prednisone therapy at presentation of HSP, showed that there was no significant difference in the risk of development or persistence of renal involvement at 1, 3, 6 and 12 months with prednisone compared with placebo or no specific treatment [37]. In the largest of these trials, which enrolled children between January 2001 and January 2005, the primary outcome (urinary protein/creatinine ratio at 1 year) was measured in 290 children [38]. This is the largest study to date showing no significant benefit of prednisone over placebo in preventing persistent renal disease [38]. That said, there could still be a role for early use of corticosteroids in patients with severe extrarenal symptoms such as abdominal pain and arthralgia, as suggested by the findings of a study performed by Ronkainen et al. [36]. Prednisone (1 mg/kg/day for 2 weeks, with weaning over the subsequent 2 weeks) was effective in reducing the intensity of abdominal pain and joint pain [36]. Prednisone did not prevent the development of renal symptoms but was effective in treating them if present; renal symptoms resolved in 61% of the prednisone patients after treatment, compared with 34% of the placebo patients [36]. Of note, Nikibakhsh et al. reported recently on the successful treatment with mycophenolate mofetil (MMF) of recurrent skin, articular and gastrointestinal symptoms in children with who failed to respond to systemic steroid therapy [39].

For patients with rapidly progressive glomerulonephritis with crescentic change on biopsy, uncontrolled data suggest that treatment may comprise aggressive therapy with corticosteroid, cyclophosphamide and possibly plasma exchange [34], as with other causes of crescentic nephritis. Other therapies such as cyclosporin, azathioprine and cyclophosphamide have been reported to be effective [40–42]. As HSP is the most common cause of rapidly progressive glomerulonephritis in childhood, more aggressive therapeutic approaches such as plasma exchange have been employed in some cases [43]. These treatment options, while important in select cases, are not yet supported by RCTs. In addition, there are no robust clinical trials to guide therapy for HSP nephritis that is not rapidly progressive (patients may exhibit less than 50% crescents on renal biopsy, sub-optimal GFR; heavy proteinuria which is not necessarily nephrotic range) [34]. Many would advocate corticosteroids [34].

Others advocate the addition of cyclophosphamide to corticosteroids in HSP nephritis with biopsy showing diffuse proliferative lesions or sclerosis, but with <50% crescentic change with ongoing heavy proteinuria [34]. In patients with greater than 6 months duration of proteinuria, an ACE inhibitor may be indicated to limit secondary glomerular injury, although again the evidence to support this therapy is lacking.

Outcome

The majority of children with HSP make a full and uneventful recovery with no evidence of ongoing significant renal disease [34]. Renal involvement is the most serious long-term complication of HSP [34]. Narchi et al. systematically reviewed all published literature with regards to long-term renal impairment in children with HSP [44]. Persistent renal involvement (hypertension, reduced renal function, nephrotic or nephritic syndrome) occurred in 1.8% of children overall, but the incidence varied with the severity of the kidney disease at presentation, occurring in 5% of children with isolated haematuria and/or proteinuria but in 20% who had acute nephritis and/or nephrotic syndrome in the acute phase [44].

Kawasaki Disease

KD is an acute self-limiting systemic vasculitis predominantly affecting young children [2]. It is distributed worldwide, with a male preponderance, an ethnic bias towards Asian children, some seasonality and occasional epidemics [45–49]. It is the second most common vasculitic illness of childhood and the most common cause of acquired heart disease in children in the UK and the USA [2, 50, 51]. The incidence in Japan is 138/100,000 [52] in children younger than 5 years, whereas in the USA it is 17.1 [53] and in the UK 8.1 [54].

Pathogenesis

The aetiology of KD remains unknown, but currently it is felt that some ubiquitous infectious agent produces an abnormal immunological response in a genetically susceptible subject that results in the characteristic clinical picture [55, 56]. Pronounced seasonality and clustering of KD cases have led to the hunt for infectious agents as a cause [55, 56]. However, so far no single agent has been identified, a fact most recently highlighted by the negative results that emerged from studies examining the potential link between coronavirus infection and KD in Taiwan [57]. One debate regarding the cause of KD has centred around the mechanism of immune

activation: conventional antigen versus superantigen (SAg) [55, 58]. SAgs are a group of proteins that share the ability to stimulate a large proportion of T cells (up to 30% of the T-cell repertoire compared with one in a million T cells for conventional antigens) by binding to a portion of the T-cell receptor β chain (TCRVβ) in association with the major histocompatibility complex (MHC) class II molecules with no requirement for antigen processing [59]. SAgs have been identified in a variety of microorganisms, including many of the bacteria and viruses isolated from children with KD [55, 59, 60]. In 1992, Abe et al. were the first to describe the selective expansion of Vβ2 and Vβ8.1T cells in KD [61], indicating T-cell Vβ skewing—the hallmark of a SAg-mediated process. Since then, many similar studies have examined T cell Vβ repertoires in KD, or examined the prevalence of serological conversion or colonization with SAg-producing organisms [62, 63]. An SAg is also responsible for induction of coronary artery disease in a murine model of KD (discussed in detail in the "In Vivo Experimental Data in KD" section) [55, 59, 60]. However, Rowley et al. recently reported three fatal cases of KD and observed IgA plasma cell infiltration into the vascular wall during the acute phase of the illness [64]. By examining the clonality of this IgA response using reverse transcriptase (RT)-PCR in lesional vascular tissue, these researchers observed that the IgA response was oligoclonal, suggesting a conventional Ag process rather than a SAg-driven one [64]. Although the debate continues regarding the mechanism of initial immune activation, different mechanisms are most likely involved with a final common pathway of immune activation responsible for this clinical syndrome. Regardless of how T cells get activated, the massive immune response characteristic of KD is translated into systemic inflammation manifested clinically as fever and the cardiac features of KD [55].

In Vivo Experimental Data in KD

Experimental mice develop coronary arteritis in response to intra-peritoneal injections of *Lactobacillus casei* wall extract (LCWE) with the resultant vasculitis being similar to KD in children [65, 66]. Young mice (age 4–5 weeks) are more susceptible to LCWE-induced disease compared with older mice [65–69]. The peripheral immune activation within hours of LCWE injection is followed by local infiltration into cardiac tissue at day 3 with the inflammatory infiltrate comprising mainly T cells [65–69]. This inflammatory response peaks at day 28 post injection and is accompanied by elastin breakdown with disruption of the intima and media, as well as aneurysm formation at day 42 [65–69]. Additionally, an SAg found within LCWE contributes significantly to the development of vascular disease [60]. The common features between this murine model and the human disease include an infectious trigger leading to immune activation; disease susceptibility in the young; a time course similar to that seen clinically in KD; similar pathology of coronary arteritis; and response to intravenous immunoglobulin (IVIG) treatment [55]. The proposed disease model supported by the in vivo experimental data in this mouse model

begins with immune activation by a microbe with superantigenic activity [55]. The SAg found in the LCWE preferentially expands T-lymphocytes expressing TCRVβ 2, 4, 6 and 14 positive T cells, and this superantigenic activity is directly correlated with the ability to induce coronary arteritis in mice [60]. Ablation of IFN-γ confirmed that IFN-γ plays an important regulatory role in disease induction in this disease model [55]. Mice with absence of TNF-α activity (blockade of TNF-α) or TNFR1 knockouts) do not develop coronary disease after LCWE stimulation [68]. Of note, the T cells found in affected vessels express SAg-reactive TCRVβ families, an unexpected finding considering the usual fate of SAg-activated T cells, which are actively deleted by apoptosis. Moolani et al. have shown that co-stimulation can rescue SAg-stimulated T cells from apoptosis [70]. Furthermore, the coronary endothelium is transformed into a professional antigen-presenting cell (APC) by upregulation of co-stimulatory molecules driven partially by the tissue-specific expression of Toll-like receptor (TLR) [55]. Increased TLR2 expression in conjunction with TLR2 stimulation by the TLR2 ligand in LCWE leads to increased expression of co-stimulatory molecules facilitating rescue of SAg-activated T cells and continued local production of proinflammatory cytokines [71, 72]. This leads to further exacerbation of the inflammation at the coronary vessel wall [55]. IFN-γ and TNF-α are involved in transcriptional regulation of matrix metalloproteinases (MMPs), with TNF-α upregulating, and IFN-γ inhibiting production of MMP-9 [55, 73]. Following that, the enzymatic activity of MMP-9 leads to elastin breakdown and aneurysm formation [73]. Of note, recently Alvira et al. have shown that in the coronary arteritis associated with KD, TGF-β suppresses elastin degradation by inhibiting plasmin-mediated MMP-9 activation [74]. Thus, strategies to block TGF-β, used in those with Marfan syndrome, are unlikely to be beneficial in KD as they lead to worsening of elastin degradation in this murine model of KD [74]. So in summary, a sustained local immune response together with persistent TNF-α production and leucocyte recruitment lead to upregulation of proteolytic activity, elastin degradation, vessel wall damage and the characteristic coronary artery lesions seen in KD [55].

Genetics

Although the clinical syndrome and occurrence of epidemics suggest an infectious cause for KD, a genetic contribution to risk is suggested by the much higher prevalence of the disease in Japan and Korea than elsewhere, and by increased prevalence within families with an increased relative risk to siblings compared to the general population [75]. Recently, a number of polymorphisms have been identified that appear to be linked with disease susceptibility in KD or the risk of coronary artery aneurysms (CAAs). These polymorphisms are summarized in Table 2.2 [4, 81]. In general, candidate gene studies in KD have been difficult to interpret, since most findings have not been replicated. Indeed, conflicting results have been reported for the few genes that have been evaluated in multiple cohorts.

Table 2.2 Genetic polymorphisms associations with Kawasaki disease

Molecule/genetic polymorphism	Role of polymorphism	Reference
Mannose binding lectin	Ambiguous role for MBL influencing risk of coronary artery aneurysms (CAA)	[76]
Angiotensin-converting enzyme (ACE)	ACE I/D polymorphism increases disease susceptibility	[77]
Matrix metalloproteinases (MMP)	MMP-3 6A/6A Polymorphism results in higher frequency of CAA MMP-1, 3, 7, 12 and 13 in the gene cluster on Chr.11q22 results in CAA in US–UK subjects	[78, 79]
Interleukin 1 receptor antagonist (IL-1Ra)	Polymorphism associated with increased disease susceptibility	[80]
Interleukin 18 (IL-18)	Increases disease susceptibility in Taiwan	[81]
Tumour necrosis factor-alpha (TNF-α)	TNF-α-308A associated with increased intravenous immune globulin (IVIG) resistance	[82]
Interleukin-10 (IL10)	IL-10 gene promoter polymorphisms influence risk of CAA	[82]
Vascular endothelial growth factor (VEGF) and its receptor (KDR)	Polymorphisms of both contribute to increased CAA risk	[83]
Chemokines	Chemokine receptor CCR5 and its ligand CCL3L1 influence disease susceptibility	[84]
Nitric oxide and associated molecules	No association of eNOS and iNOS gene polymorphisms to the development of CAL in Japanese KD patients	[85]
Fcγ receptors	No association for Fcγ RIIa-131H/R, FcγRIIb-232I/T, FcγRIIIa-158V/F and FcγRIIIb-NA1/NA2	[86]
Inositol 1,4,5-trisphosphate 3-kinase C (ITPKC) gene	Increases diseases susceptibility and risk of CAA No association with KD and CAA in Taiwanese children	[75, 87]
Caspase 3 (CASP3)	Associated with CAA in Taiwanese children Susceptibility to KD in both Japanese and US subjects of European ancestry	[88, 89]
COL11A2	Susceptibility to disease and CAA	[90]
Inositol 1,4,5-trisphosphate receptor type 3 (ITPR3)	Increased risk of CAA	[91]

Furthermore, a genome-wide linkage study using microsatellite markers in Japanese families identified a number of potential loci [92]. Finer scale studies of the 19q32.2-32.3 region led to identification of a linked group of single-nucleotide polymorphisms (SNPs) in the inositol 1,4,5-trisphosphate 3-kinase C (ITPKC) gene, associated with KD, with an odds ratio of 1.74 [75, 92]. ITPKC mutation was associated with KD not only in Japanese but also in US Caucasian patients, particularly with the risk for developing coronary artery lesions [75, 92]. Additional data

supported a functional significance for one polymorphism identified: SNP (itpkc_3C) led to reduced splicing of the ITPKC gene product and therefore could result in a lower mRNA concentration [75]. Of note, however, Chi et al. subsequently showed no statistically significant association between the ITPKC gene SNP rs28493229 and KD or coronary artery lesions in Taiwanese children [87]. The first genome-wide association study (GWAS) in KD was notable for assessment of population stratification and for replication of GWAS findings in an independent cohort [93]. GWAS of 109 Caucasian patients, followed by SNP genotyping of the 1,116 most significant SNPs in 583 families, then fine mapping of known genes near some of the 40 SNPs that were successfully replicated, led to identification of eight putative novel susceptibility genes [odds ratio (OR) approximately 1.1–1.5] [93].

Clinical Features

The principal clinical features are fever persisting for 5 days or more, peripheral extremity changes (reddening of the palms and soles, indurative oedema and subsequent desquamation), a polymorphous exanthema, bilateral conjunctival injection/congestion, lips and oral cavity changes (reddening/cracking of lips, strawberry tongue, oral and pharyngeal injection) and cervical lymphadenopathy (acute, non-purulent) [56]. For the diagnosis to be established according to the Diagnostic Guidelines of the Japan Kawasaki Disease Research Committee, five of six criteria should be present [94]. If CAAs are present, fewer features may be necessary for diagnostic purposes [48, 95]. The cardiovascular features are the most important manifestations of the condition with widespread vasculitis affecting predominantly medium-size muscular arteries, especially the coronary arteries [56]. Coronary artery involvement occurs in 15–25% of untreated cases with additional cardiac features in a significant proportion of these, including pericardial effusion, electrocardiographic abnormalities, pericarditis, myocarditis, valvular incompetence, cardiac failure and myocardial infarction [56]. Another clinical sign that maybe relatively specific to KD is the development of erythema and induration at sites of Bacille Calmette–Guérin (BCG) inoculations [46]. Other system involvement can occur, including the gastrointestinal tract, the hepatobiliary tract with hydrops of the gall bladder being well recognised, the central nervous system with seizure and meningeal features, the auditory system with deafness, the skeletal system with arthropathy and the urinary system [56].

Treatment

Early recognition and treatment of KD with aspirin and IVIG have been shown unequivocally by meta-analysis to reduce the occurrence of CAAs [96, 97]. The prevalence of CAA is inversely related to the total dose of IVIG [97], 2 g/kg of IVIG being the optimal dose, usually given as a single infusion [96]. Meta-analysis of

RCTs comparing divided lower doses of IVIG (400 mg/kg/day for 4 consecutive days) versus a single infusion of high-dose IVIG (2 g/kg over 10 h) has clearly shown that even though the 4-day regimen has some benefit, a single dose of 2 g/kg has a greater therapeutic effect in the prevention of CAA [96, 97]. However, IVIG resistance occurs in up to 20% of cases [98]. In those cases most advocate a second dose of IVIG and/or the use of corticosteroids. Regarding corticosteroid use in IVIG resistant to KD, there are apparently conflicting data from clinical trials. Inoue et al. reported on a randomized control trial of 178 KD patients who were assigned to receive IVIG (1 g/kg/day) for two consecutive days, given over 12 h, or IVIG plus prednisolone sodium succinate (2 mg/kg/day) three times daily, given by intravenous (IV) injection until the fever resolved and then orally until the C-reactive protein (CRP) level normalized [98]. Patients in both groups received aspirin (30 mg/kg) and dipyridamole (2 mg/kg/day) [98]. The addition of corticosteroid was associated with reduced CAA compared with IVIG alone: in those receiving IVIG and anti-platelet therapy, 11.4% had CAA at 1 month, compared with 2.2% in those receiving IVIG plus corticosteroids [98]. Also the duration of fever was shorter and CRP decreased more rapidly in the group of patients receiving corticosteroids [98]. In contrast, Newburger et al. in a subsequent multicenter, randomized, double-blind, placebo-controlled trial examined the effect of the addition of a single dose of intravenous methylprednisolone to standard therapy [99]. They found that this corticosteroid regimen did not improve the CAA outcome in these children [99]. These contrasting results suggest that dose and duration of corticosteroids may be critical when considering this as adjunctive therapy in KD. Infliximab, a chimeric monoclonal antibody against TNF-α, has been reported to be effective for the treatment of IVIG-resistant KD [100, 101]. In 13 of 16 patients with failed response to a single dose or IVIG who received infliximab, there was cessation of fever followed by reduction in CRP [100]. More recently, Burns et al. reported on a multi-centre, randomized, prospective trial of second IVIG infusion (2 g/kg) versus infliximab (5 mg/kg) in 24 children with acute KD and fever after initial failed treatment with IVIG [101]. There was cessation of fever within 24 h in 11 of 12 subjects treated with infliximab and in 8 of 12 subjects retreated with IVIG [101]. No significant differences were observed between treatment groups in the change from baseline for laboratory variables, fever or echocardiographic assessment of coronary arteries [101]. These reports are encouraging but further RCTs to establish the optimal management of KD, and in particular IVIG-resistant KD, are needed [102]. In that respect a multi-centre, double-blind, randomized, placebo-controlled trial intended to assess the efficacy of etanercept (a fusion protein combining the TNF receptor 2 and the Fc component of human IgG1) in reducing the IVIG refractory rate during treatment of acute KD is ongoing [103].

In the convalescent phase of the condition, if aneurysms persist, anti-platelet therapy in the form of low-dose aspirin should be continued long term until the aneurysms resolve [56]. In the presence of giant aneurysms (greater than 8 mm), warfarin is recommended in addition to aspirin [104]. Some patients may require coronary angioplasty or a revascularization procedure should ischemic symptoms arise or evidence of obstruction occur [105].

Outcome

The acute mortality of KD in Japan is 1.14 [105]. About 20% of patients who develop CAAs during the acute disease will develop coronary artery stenoses, and the risk is greater with large (giant) aneurysms [105]. However, emerging data suggest that, in spite of seeming recovery, there are long-term cardiovascular sequelae for patients with KD that persist into adult life and that may have important implications [106].

Antineutrophil Cytoplasmic Antibody-Associated Vasculitides

ANCA-associated vasculitides (AAV) are small-vessel vasculitides characterized by necrotizing inflammation of small vessels in association with autoantibodies to neutrophil constituents—in particular, proteinase 3 (PR3) and myeloperoxidase (MPO) [107, 108]. The AAV comprise Wegener's granulomatosis (WG, now also referred to as granulomatous polyangiitis, although for the purposes of this review the term WG is used), microscopic polyangiitis (MPA), including its renal-limited (RL) subset designated as idiopathic necrotizing crescentic glomerulonephritis (iNCGN), and Churg–Strauss syndrome (CSS) [107, 108]. Although rare, AAV do occur in childhood and are associated with significant morbidity and mortality [109].

Pathogenesis

The pathogenesis of AAV is still not fully elucidated, but clinical as well as experimental data strongly suggest a role for autoimmune responses to PR3 and MPO in disease development [110].

In Vitro Studies

The most accepted model of pathogenesis suggests that ANCA activate cytokine-primed neutrophils within the microvasculature, leading to bystander damage to endothelial cells themselves and rapid escalation of inflammation with recruitment of mononuclear cells [111]. Falk et al. demonstrated in 1990 that ANCAs in vitro activate neutrophils to produce reactive oxygen species and release of lytic enzymes [112]. This process requires priming of neutrophils. Priming involves the stimulation of neutrophils with low doses of proinflammatory cytokines that result, among other things, in surface expression of PR3/MPO on the neutrophil membrane but without full neutrophil activation, before their interaction with ANCA [113]. Primed neutrophil activation by ANCA involves interaction with their target antigens on

the neutrophil membrane and also with Fcγ receptors—in particular, FcγRIIa and FcγRIIIb [113]. In addition, Reumaux et al. showed that ANCA-induced neutrophil activation occurs only when neutrophils are attached to a surface and not when floating in the circulation [114]. Furthermore, Radford et al. demonstrated that ANCA can directly activate neutrophils to become firmly adherent to vessel walls, where they may obstruct flow, initiate tissue damage and contribute to the pathogenesis of vasculitis [115]. These effects can be blocked by antibodies to FcγRIIa and by antibodies to CD11b [115]. In more detail, Savage et al. showed that activation of neutrophils by ANCA causes integrin- and cytokine receptor-mediated adherence to cultured endothelial cells and transmigration across the endothelial layer [116]. In addition, activation of neutrophils with ANCA causes a conformational change in beta-2 integrins that enhances ligand binding [116]. A role for adhesion molecules in the interaction between ANCA-activated neutrophils and vessels also is supported by immunohistologic evidence of upregulated adhesion molecules in glomerular lesions in renal biopsy specimens from patients with AAV [117]. In addition to binding to the surface of endothelial cells, both PR3 and MPO are internalized into endothelial cells, where they have different pathologic effects [118]. For example, after internalization, PR3 causes endothelial cell apoptosis, whereas MPO causes generation of intracellular oxidants [118]. These differences in MPO and PR3 interaction with endothelial cells could influence the patterns of tissue injury induced when these antigens react with ANCA at the endothelial cell surface [111]. Furthermore, there is new evidence that after neutrophil activation by ANCA, the neutrophils are driven down an accelerated apoptotic death pathway by reactive oxygen species [119]. These neutrophils develop the morphologic features of apoptosis, but there is dysregulated coordination of cell surface changes that normally accompany apoptosis, including delay in phosphatidylserine expression [119], which could contribute to failure of these apoptotic cells to be recognized and safely removed by phagocytes [119]. Apoptotic neutrophils eventually disintegrate, releasing cytotoxic contents within vascular tissue. This process may explain the leucocytoclasia often seen in vasculitic lesions. Also pertaining to safe clearance of apoptotic neutrophils are two studies showing that apoptotic neutrophils can express proteinase-3 and myeloperoxidase at the cell surface, which can act as an opsonin for ANCA [120, 121]. Both apoptotic and ANCA opsonized apoptotic neutrophils can be phagocytosed by macrophages, but whereas the former induce an anti-inflammatory response from the macrophage release of interleukin-10, the latter are taken up more avidly and are proinflammatory by inducing macrophage release of interleukin-1, interleukin-8 and TNF [120, 121].

The signalling cascades that lead to functional responses such as superoxide release are only beginning to be elucidated. Tyrosine kinases and protein kinase C are known to be involved [122]. Now, mitogen-activated protein kinases that require tyrosine phosphorylation for activation also have been implicated, particularly in TNF-mediated priming [123].

Furthermore, in WG the granulomatous inflammation displays several different morphologies. Within a surrounding inflammatory background, poorly formed epithelioid cell granulomas, scattered histiocytic giant cells of Langhans type or

palisading histiocytes around central necrosis may be seen [124]. The mixed inflammatory infiltrate in WG is composed of lymphocytes, plasma cells, neutrophils, eosinophils, monocytes, macrophages, histiocytes and giant cells [124]. Since INF-γ and T cells play pivotal roles in granuloma formation, alterations of the T-cell and cytokine response could contribute to anomalous autoantigen presentation in ectopic lymphoid-like structures and sustain autoimmunity to PR3 [125]. Skewing of the T-cell phenotype with expansion of the CD4+ and CD8+ T cells lacking CD28 expression is seen in WG [126, 127]. Expansion of CD28 negative T cells is already evident in localized WG and further increases in generalized disease [126, 127]. Abundant IFN-γ, CD26 and Th-1 type CC chemokine receptor CCR5 expression are seen in granulomatous lesions of the respiratory tract in localized WG, but appear less strong in generalized WG [128, 129]. Moreover, a fraction of Th2 type IL-4 producing CCR3+ T cells is present in the circulation and tissue lesions in generalized but not in localized WG [128]. These data suggest that an aberrant Th-1 type response favouring granuloma formation might play a role in initiation of WG [130]. Ectopic presentation of the Wegener's autoantigen PR3 and autoimmunity to PR3 might be sustained within inflammatory lesions and by skewed T-cell and cytokine responses [130]. Progression from localized to generalized WG is associated with the appearance of another subset of Th-2 type cells, which could be a consequence of B-cell expansion and T-cell-dependent PR-3 ANCA production during disease progression [130]. In addition, Th17 cells have been recently described as major effector cells in autoimmune diseases [131]. It has been demonstrated that stimulation of peripheral blood mononuclear cells from PR3-ANCA positive patients with WG with the autoantigen PR3 results in production of interleukin (IL)-17 and not INF-γ, demonstrating that the autoimmune effector cells are Th17 cells [132]. In healthy individuals regulatory T cells (Tregs) control the activity of immune effector cells [131]. There is increasing evidence that the balance between Th17 cells and Fox P3-positive regulatory T cells is disturbed in autoimmune inflammatory conditions [131]. In patients with WG in remission, the percentage of Fox P3-positive Tregs was shown to be increased but the cells were functionally deficient [133].

Taken together, in vitro studies support a pathogenic role for the autoimmune responses to PR3 and MPO in AAV. Autoantibodies could be responsible for small-vessel necrotizing vasculitis, whereas dysregulation of T-cell homoeostasis may underlie granulomatous inflammation.

In Vivo Studies

Evidence for a pathogenic role of MPO-ANCA in AAV comes from animal models for MPO-ANCA-associated vasculitis [134]. Xiao et al. immunized mice deficient for MPO with mouse MPO and transferred splenocytes from these immunized mice into immunodeficient or normal mice [134]. The recipient mice developed pauci-immune

necrotizing glomerulonephritis and haemorrhagic pulmonary capillaritis, similar to the clinical manifestations and the histopathology of MPO-ANCA-associated vasculitis [134]. In addition, transfer of IgG alone from MPO-immunized mice resulted in pauci-immune focal necrotizing glomerulonephritis in the recipient, demonstrating the pathogenic potential of anti-MPO antibodies [134]. Additional studies showed that both neutrophils expressing MPO and the alternative pathway of complement besides the antibodies are required to induce AAV as recipient mice deficient for factor B and complement C5 did not develop disease [135]. Also in a rat model of MPO-ANCA vasculitis, in which rats were immunized with human MPO, the pathogenic potential of anti-MPO antibodies was demonstrated [136]. Of note, however, no animal models for PR3-ANCA-associated WG have been generated [110].

Microbial Factors as Triggers of AAV

A series of early observations have suggested that infectious episodes may trigger relapses of AAV [137]. Further studies of upper airway involvement in WG showed good responses to treatment with trimethoprim/sulphamethoxazole [138]. Long-term studies demonstrated that chronic nasal carriage of *Staphylococcus aureus* is a major risk factor for relapse in WG in conjunction with persistence of ANCA, and maintenance treatment with trimethoprim/sulphamethoxazole reduced the occurrence of relapses by 60% in patients with WG [139]. Possible mechanisms whereby *S. aureus* could result in flares of WG include SAg production and T- and B-cell activation, direct tropism of *S. aureus* for endothelial cells, with binding and internalization of the organism by endothelial cells or by priming of neutrophils [140].

Recently, two studies have shed new light on the possible role of microbial factors in the pathogenesis of AAV. In the first study, antibodies to complementary PR3 were detected in serum samples from patients with PR3-ANCA-associated vasculitis [141]. Complementary PR3 is a protein translated from the antisense DNA strand encoding PR3. Such a complementary protein is a mirror of the original protein [141]. As such, antibodies to a complementary protein can induce anti-idiotypic antibodies that react with the original protein [141]. Pendergraft et al. immunized mice with complementary PR3, and these mice then developed antibodies to PR3 [141]. This complementary PR3 shows homology with a number of microbial proteins, including proteins from *S. aureus* [141]. This raises the possibility that infection with *S. aureus* could lead to antibodies cross-reacting with complementary PR3, which, in turn, evoke antibodies to PR3 by idiotypic–anti-idiotypic interaction.

A second study describes antibodies to the lysosomal membrane glycoprotein 2 (hLAMP-2) as a sensitive and specific marker for pauci-immune crescentic glomerulonephritis [142]. hLAMP-2 is present on neutrophils and endothelial cells [142]. Anti-hLAMP-2 antibodies, raised in rabbits, were able to activate neutrophils and induce apoptosis of human microvascular endothelial cells [142]. More importantly,

these antibodies induced pauci-immune focal necrotizing glomerulonephritis when injected into rats [142]. Eight out of nine amino acids of the P41–49 immunodominant epitope of hLAMP-2 were shown to be identical to the P72–80 peptide of FimH, an adhesion molecule of fimbriae of Gram negative bacteria [142]. Immunization of rats with FimH resulted in the generation of antibodies cross-reacting with hLAMP-2 and inducing pauci-immune glomerulonephritis [142]. These observations suggest that infection with Gram negative bacteria could result in a loss of tolerance and could lead to AAV.

Genetics

A number of candidate gene association studies have identified variants associated with an increased incidence of AAV [143]. Most of the genes described so far encode proteins involved in the immune response and are summarized in Table 2.3. Of note, the genes with variants most strongly associated with AAV, the MHC and *PTPN22* genes, also have variants associated with other autoimmune diseases, including rheumatoid arthritis, type 1 diabetes and systemic lupus erythematosus (SLE) [143]. This suggests that genetic risk factors common to other autoimmune diseases also apply to AAV. Different variants within each gene may be associated with different polymorphisms—for example, SLE associates with the IL-2RA SNP rs11594656, while AAV is associated with rs4129506 [143]. A GWAS of AAV is currently ongoing and may be enlightening in that respect.

Furthermore, Ciavatta et al., in an attempt to uncover a potential transcriptional regulatory mechanism for PR3 and MPO disrupted in patients with ANCA vasculitis, examined the PR3 and MPO loci in neutrophils from ANCA patients and healthy control individuals for epigenetic modifications associated with gene silencing [173]. They demonstrated that levels of the chromatin modification H3K27me3, which is associated with gene silencing, were depleted at PR3 and MPO loci in ANCA patients compared with healthy controls [173]. Interestingly, in both patients and controls, DNA was unmethylated at a CpG island in PR3, whereas in healthy controls, DNA was methylated at a CpG island in MPO [173]. Consistent with decreased levels of H3K27me3, JMJD3, the demethylase specific for H3K27me3, was preferentially expressed in ANCA patients versus healthy controls [173]. In addition, the mechanism for recruiting the H3K27 methyltransferase enhancer of zeste homolog 2 (EZH2) to PR3 and MPO loci was shown to be mediated by RUNX3. RUNX3 message was decreased in patients compared with healthy controls, and may also be under epigenetic control [173]. DNA methylation was increased at the RUNX3 promoter in ANCA patients [173]. These data indicate that epigenetic modifications associated with gene silencing are perturbed at ANCA autoantigen-encoding genes, potentially contributing to inappropriate expression of PR3 and MPO in ANCA patients [173].

Table 2.3 Positive genetic association studies in antineutrophil cytoplasmic antibody-associated systemic vasculitis

Molecule/genetic polymorphism	Disease	Reference
HLA DPB1*0401	WG	[144]
HLA DPB1*0401	WG	[145]
HLA B50	WG	[146]
HLA DR9	WG	[146]
HLA DQw7	WG, MPA	[147]
HLA DR3	WG, MPA	[147]
HLA DR1	WG	[148]
HLA DR4	WG, MPA, CSS, RL	[149]
HLA DR6	WG, MPA, CSS, RL	[149]
HLA DRB4	CSS	[150]
HLA DRB3	CSS	[150]
HLA DRB4	CSS	[151]
HLA DRB3	CSS	[151]
PTPN22-620W	WG	[152]
PTPN22-620W	WG, MPA, CSS	[153]
IL-2RA rs41295061	WG, MPA, CSS	[154]
CTLA4 −318T	WG	[155]
CTLA4 +49G	WG, MPA, CSS, RL	[156]
CTLA4 rs3087243	WG, MPA, CSS	[153]
PRTN3 −564G	WG	[157]
AAT Z allele	WG, MPA, RL	[158]
AAT Z allele	WG, MPA, RL	[159]
AAT Z allele	WG	[160]
AAT Z allele	WG	[161]
AAT Z allele	WG	[162]
C3F	WG, MPA	[163]
CD18 Ava II	MPO positive	[164]
IL-10 microsatellite	WG	[165]
IL-10 (−1082) AA genotype	WG, MPA	[166]
IL-10 haplotype	CSS	[167]
LILRA2 intron 6 AA genotype	MPA	[168]
CD226 rs763361	WG	[169]
FCGR2A R131 RR genotype with FCGR3A F158 FF	WG	[170]
FCGR3B copy number high	WG, MPA, CSS	[171]
FCGR3B copy number low	WG	[171]
FCGR3B copy number low	MPA	[171]
FCGR3B copy number low	WG	[172]

Clinical Features

WG typically affects the upper and lower respiratory tract and is associated with glomerulonephritis, although the disease can affect any organ system in the body [34]. From a clinical perspective, it may be useful to think of WG as having two forms: a predominantly granulomatous form with mainly localized disease with a

chronic course; and a florid, acute small vessel vasculitic form characterized by severe pulmonary haemorrhage and/or rapidly progressive vasculitis or other severe vasculitic manifestation [34]. These two broad presentations may coexist or present sequentially in individual patients. Symptoms and signs of upper respiratory tract involvement include epistaxis, otalgia and hearing loss (conductive and sensorineural) [34]. Nasal septal involvement with cartilaginous collapse results in the characteristic saddle nose deformity, although this may not be present at initial presentation [34]. Chronic sinusitis may be observed. Glottic and subglottic polyps and/or large- and medium-sized airway stenoses can result from granulomatous inflammation [34]. Lower respiratory tract manifestations also include granulomatous pulmonary nodules with or without central cavitation and pulmonary haemorrhages that can be relatively asymptomatic but result in evanescent pulmonary shadows on chest X-ray, or catastrophic pulmonary haemorrhage from pulmonary capillaritis associated with respiratory failure and high mortality [34].

The typical renal lesion is a focal segmental necrotizing glomerulonephritis, with pauci-immune crescentic glomerular changes [34]. Clinical manifestations include hypertension, significant proteinuria, nephritic and nephrotic syndrome, and ultimately the protean clinical features renal failure [34]. Other manifestations include orbital involvement with granuloma, retinal vasculitis, peripheral gangrene with tissue loss, and vasculitis of the skin, gut, heart, central nervous system and/or peripheral nerves (mononeuritis multiplex), salivary glands, gonads and breast [34]. Non-specific symptoms such as malaise, fever, weight loss or growth failure, arthralgia and arthritis are relatively common [34].

Treatment of AAV

Renal morbidity and mortality is a major concern in the AAV, hence therapy aimed at preservation of renal function is a recurring theme for the treatment of AAV in adults and children [174]. Treatment for paediatric AAV is broadly similar to the approach in adults, with corticosteroids, cyclophosphamide (usually 6–10 intravenous doses at 500–1,000 mg/m^2 [2] per dose given 3–4 weekly; alternatively given orally at 2 mg/kg/day for 2–3 months), plasma exchange (particularly for pulmonary capillaritis and/or rapidly progressive glomerulonephritis-"pulmonary-renal syndrome") routinely employed to induce remission [3, 175]. Intravenous pulsed cyclophosphamide is increasingly favoured over oral continuous cyclophosphamide in adults because of reduced cumulative dose and less neutropenic sepsis [176, 177] and is thus increasingly used to treat children with AAV as well, albeit without good paediatric evidence. This is followed by low-dose corticosteroids and azathioprine (1.5–3 mg/kg/day) to maintain remission [3, 178]. Anti-platelet doses of aspirin (1–5 mg/kg/day) are empirically employed on the basis of the increased risk of thrombosis associated with the disease process [179]. Methotrexate may have a role for induction of remission in patients with limited WG [180], but is less commonly used as an induction agent in children with AAV. Co-trimoxazole is commonly

added for the treatment of WG, particularly in those with upper respiratory tract involvement, serving both as prophylaxis against opportunistic infection and as a possible disease-modifying agent [139]. Recommendations regarding duration of maintenance therapy are based on adult trial data, suggesting that the strongest predictor of relapse is withdrawal of therapy, and hence maintenance therapy should be continued for several years [174]. As a general therapeutic measure, prophylaxis against osteoporosis, gastrointestinal ulceration and infection (bacterial, protozoal and fungal) is standard for treatment for AAV [174].

As the use of cyclophosphamide contributes to morbidity and mortality [3, 174] with infection playing a prominent role [181], and disease relapses occur in 50% of the patients with AAV as drugs are reduced or withdrawn, newer immunosuppressive agents and immunomodulatory strategies are being explored in both adults and children [3, 174]. Such treatments include MMF and rituximab, which have already been reported to be effective at inducing or maintaining remission in adults with AAV [182, 183]. Of interest, two recent randomized control trials reported on the efficacy of rituximab compared to cyclophosphamide to induce remission in adults with AAV [184, 185]. Jones et al. report on the results of a randomized trial of rituximab versus cyclophosphamide in ANCA-associated renal vasculitis (RITUXIVAS) and Stone et al. report on the results of the rituximab in ANCA-associated vasculitis (RAVE) trial [184, 185]. Similar conclusions are reached in the two studies [184, 185]. Both trials showed that rituximab was efficacious in inducing a remission, as compared with intravenous cyclophosphamide (in the RITUXIVAS trial) or oral cyclophosphamide (in the RAVE trial) [184, 185]. There are, however, a number of important differences between the two trials. In the RITUXIVAS trial, patients who were randomly assigned to the rituximab group also received at least two doses of intravenous cyclophosphamide, whereas in the RAVE trial, patients randomly assigned to the rituximab group did not receive any cyclophosphamide [184, 185]. The trials were similar in that all patients in both trials received both intravenous and oral glucocorticoid therapy [184, 185]. Investigators in the RITUXIVAS trial reported sustained remission for 12 months, whereas outcome data from the RAVE trial were reported only on the 6-month remission-induction period [184, 185]. The RAVE trial data were confounded by the use of glucocorticoid therapy for 5 of the 6 months of follow-up [185]. In addition, both trials raised concerns about the substantial complications from the use of rituximab and other immunomodulating agents in ANCA-associated disease [184, 185]. Fewer adverse events would have been expected in patients treated with rituximab as compared with cyclophosphamide. Unfortunately, in the RAVE trial the rate of adverse events was equivalent in the two study groups [185]. Similarly, in the RITUXIVAS study, 6 of 33 patients in the rituximab group died, as did 2 of 11 patients in the control group [184]. The RAVE trial also showed an unexpectedly elevated number of malignant conditions detected over a relatively short treatment period [185]. These studies suggest that rituximab might be considered as an option for first-line therapy for induction of remission of ANCA-associated disease. It remains unclear whether rituximab should be used with glucocorticoids alone or in combination with intravenous cyclophosphamide.

Biologic therapy is also increasingly used to treat children with small vessel vasculitis, including AAV and ANCA negative vasculitides [186]. Agents used include rituximab (previously mentioned), anti-TNF-α (etanercept, infliximab, and adalimumab), and anakinra (recombinant interleukin 1 receptor antagonist) [186]. These therapies are mainly reserved for those children who have failed standard treatment, or in those patients where cumulative cyclophosphamide and/or corticosteroid toxicity is of particular concern [186]. Of note is the European vasculitis study group (EUVAS) MYCYC trial (UK and Europe), which is comparing induction therapy of WG and MPA using cyclophosphamide (standard therapy) versus MMF (experimental therapy). This is the first EUVAS trial to include children as well as adults and is actively recruiting patients under the age of 17 years in the UK. For a full list of the past and present EUVAS trials for AVV, the reader is directed to: http://www.vasculitis.org/.

Outcome

The AAV still carry considerable disease-related morbidity and mortality, particularly due to progressive renal failure or aggressive respiratory involvement, and therapy-related complications such as sepsis. The mortality for paediatric WG from one recent paediatric series was 12% over a 17-year period of study inclusion [187]. The largest paediatric series of WG reported 40% of cases with chronic renal impairment at 33 months follow up despite therapy [188]. For MPA in children, mortality during paediatric follow up is reportedly less than 14% [189]. For CSS in children, the most recent series quotes a related mortality of 18%, all attributed to disease rather than therapy [190].

Polyarteritis Nodosa

Systemic polyarteritis nodosa (PAN) is rare in childhood. Although the epidemiology is poorly defined, PAN occurs more commonly in children than in adults, as well as being more common than the AAV [56]. Disease manifestations are diverse and complex, ranging from the benign cutaneous form to the severe disseminated multi-systemic form [56].

Pathogenesis

The immunopathogenesis leading to vascular injury in PAN is probably heterogeneous [56]. Based on animal models, the mechanism of vascular inflammation

implicated most often is induction by immune complexes [56]. In addition, there are some data supporting a role for hepatitis B in some patients [191] and reports of a higher frequency of exposure to parvovirus B19 and cytomegalovirus in PAN patients compared to control populations [192, 193]. HIV has also been implicated and PAN-like illnesses have additionally been reported in association with cancers and haematological malignancies [194, 195]. However, associations between PAN and these infections or other conditions are rare in childhood. Streptococcal infection may be an important trigger [195], and indirect evidence suggests that bacterial SAgs may play a role in some cases [56]. In terms of pathogenetic mechanisms, it seems likely that the immunological processes involved are similar to those in other systemic vasculitides and include immune complexes, complement, possibly autoantibodies, cell adhesion molecules, cytokines, growth factors, chemokines, neutrophils and T cells [196, 197]. Of note, immunohistochemical studies performed on biopsied perineural and muscle vessels from homogeneous populations of PAN patients showed that inflammatory infiltrates consist mainly of macrophages and T lymphocytes, particularly of the CD8+ subset [198]. To date, there is no reliable animal model of the disease. The PAN-like disease in cynomolgus macaques, which is very similar to the human disease, occurs only sporadically [199, 200]. Snyder et al. described a PAN-like illness arising spontaneously in beagle dogs, but to date this animal model has not provided insight to the pathogenesis of PAN in humans [201].

Furthermore, it is assumed that there are probably genetic predisposing factors that may make individuals vulnerable to develop PAN, as have also been considered for other vasculitides [202–204]. An example of this is the link with familial Mediterranean fever [56, 205]. Yalcinkaya et al. have recently reported on the prevalence of FMF mutations in 29 children with PAN showing that 38% of the patients were carriers of MEFV mutations [205].

Clinical Features

The new EULAR/PRINTO/PRES classification criteria for PAN are as follows: histopathological evidence of necrotizing vasculitis in medium- or small-sized arteries or angiographic abnormality (aneurysm, stenosis or occlusion) as a mandatory criterion, plus one of the following five—skin involvement, myalgia or muscle tenderness, hypertension, peripheral neuropathy and renal involvement [8]. The main clinical features of PAN are malaise, fever, weight loss, skin rash, myalgia, abdominal pain and arthropathy [56]. Additional features include ischemic heart and testicular pain; renal manifestations such as haematuria, proteinuria and hypertension; and neurologic features such as focal defects, haemiplegia, visual loss, mononeuritis multiplex and organic psychosis. Livido reticularis is also a characteristic feature, and occasionally subcutaneous nodules overlying affected arteries are present.

Treatment

For many years, the treatment of PAN has involved the administration of high-dose steroid with an additional cytotoxic agent such as cyclophosphamide to induce remission [56, 206–208]. Empirically, aspirin has also been given as an anti-platelet agent by some clinicians [209]. Once remission is achieved maintenance therapy with daily or alternate day prednisolone and oral azathioprine is frequently utilized for about 18 months. Adjunctive plasma exchange can be used in life-threatening situations [210]. Biologic agents such as infliximab and rituximab are increasingly used [185, 211–215]. Treatment for cutaneous PAN is typically much less aggressive. Agents commonly utilized include low-dose prednisolone, anti-platelet agents, colchicine, hydroxychloroquine or azathioprine [56]. However, in a few cases cutaneous PAN may progress over time to the systemic form of the disease and therefore require more aggressive therapy [56].

Outcome

Ozen et al. reported on a retrospective series of childhood PAN and improved outcome compared to that reported in adults with only 1 (1.1%) death and 2 (2.2%) patients with end-stage renal disease among 110 patients [195]. Of note, however, in that series 30% of patients were classified as having cutaneous PAN, which typically has a more benign course than systemic PAN [195].

Takayasu Arteritis

TA is a predominantly large vessel vasculitis with a worldwide distribution, although the disease is most common in Asia [216]. Onset of TA is most common during the third decade of life but has been well reported in young children [216].

Pathogenesis

Even though the precise factors responsible for the arterial damage in TA are unknown, it is believed that genetically linked immune responses to unidentified antigens may incite autoimmune damage by cell-mediated or humoral pathways, resulting in the disease and its relapses [216]. In the acute phase of TA, the inflammatory lesions originate in the vasa varum and are characterized by perivascular cuffing mainly composed of γδT lymphocytes, cytotoxic lymphocytes and T helper cells [217]. Luminal stenosis of advential small arteries due to intimal thickening is relatively common [217]. In the chronic stage of TA, intimal fibrosis is often accompanied by well-formed fibrous atherosclerotic plaques and calcification [217].

Furthermore, autoantibodies against aortic endothelial cells have been proposed as a key factor in the pathogenesis of TA [218, 219]. Chauhan et al. reported that patients with TA show circulating anti-aortic endothelial cell antibodies (AAECAs) directed against 60–65 kDa heat-shock proteins (HSPs 60/65) [218, 219]. Sera from AAECA-positive patients with TA were found to induce apoptosis of aortic endothelial cells, suggesting that these antibodies may have a role in the disease pathogenesis [218]. Lastly, while previous reports have suggested a link between TA and tuberculosis, additional studies have not supported this association [220].

Genetics

Familial occurrence of the disease has been extensively reported, leading to a hypothesis for a hereditary basis [221]. The genetic association of TA with HLAB52, and particularly B*5201 that has been observed, with high estimated OR (4.7–10.2), in multiple cohorts of diverse ethnicity (East Asia, South Asia and Mexico) [222]. In addition, a hypothesis was made, based on a Japanese cohort, that an even stronger association can be identified, considering HLA alleles that share the motif of glutamate at position 63 and serine at position 67, which characterizes B*3902 as well as B*5201 [223]. Data supporting this hypothesis were recently reported using a Mexican cohort [222]. Candidate gene studies have also reported associations with interleukin (IL)-12, IL-2 and IL-6 gene polymorphisms in a Turkish cohort but have not been replicated [4, 32].

Clinical Features

Clinical diagnosis of TA is commonly challenging for the clinician. It is estimated that one-third of children present with inactive, so-called burnt-out stage of disease, in which clinical features represent vascular sequelae rather than active vasculitis [216]. The natural history and the time from onset of symptoms to diagnosis are variable. The clinical spectrum at presentation of children with TA differs from that of adults; however, hypertension is the most common symptom in both groups [216]. Cakar et al. recently reported in a series of 19 children with TA that the most common complaints at presentation were headache (84%), abdominal pain (37%), claudication of extremities (32%), fever (26%) and weight loss (10%) [224]. One child presented with visual loss. Examination on admission revealed hypertension (89%), absent pulses (58%) and arterial bruits (42%) in the same cohort [224].

Treatment

Corticosteroids are still the mainstay of treatment for TA [4, 216]. In addition, MTX, azathioprine, MMF and cyclophosphamide have been used in children [4, 216].

Ozen et al. described six children with TA, and treatment with steroid and cyclophosphamide induction followed by MTX was suggested as effective and safe for childhood TA with widespread disease [225]. Anti-TNF therapy may be beneficial [226]. Surgical intervention is frequently required to alleviate end-organ ischemia and hypertension resulting from vascular stenoses [216].

Outcome

The mortality rate in children has been reported as high as 35% [216]. The outcome depends on the vessel involvement and on the severity of hypertension [216].

Novel Biomarkers for Vasculitis Disease Activity: Tracking Endothelial Injury and Repair

Initially considered as a single cell lining of the vascular tree, the endothelium has recently emerged as a dynamic interface responsive to environmental stimuli [227]. As a result, alteration of the endothelium generates a repertoire of biological responses playing a key role in the control of vascular homeostasis such as haemostasis, inflammation or angiogenesis [228]. As a consequence, the endothelium not only displays altered functions but also loses its integrity. Endothelial microparticles (EMPs) released from activated or apoptotic endothelial cells and whole endothelial cells, circulating endothelial cells (CECs), detached from injured vessels constitute a fundamental feature of these injurious responses affecting the vessel wall [229–231]. In response to injury, regenerative mechanisms are activated to restore endothelium integrity [232]. In the past, endothelial repair was considered to solely involve adjacent endothelial cells able to replicate locally and replace the lost cells. Since the original study by Asahara et al., it has become obvious that the recruitment of endothelial progenitor cells (EPCs) represents an additional mechanism for vascular repair [232]. These stem cells are mobilized from the bone marrow and are able to differentiate into mature cells, restoring endothelial integrity at sites of vascular injury [232]. This spectrum of endothelial responses can be considered in a dynamic triad “activation/injury/repair”, which has critically transformed our understanding of endothelial biology.

CECs and EMPs are sensitive biomarkers of vascular injury for monitoring disease activity and response to therapy in children with vasculitis [233]. In addition, preliminary data show altered endothelial repair responses in children with systemic vasculitis, suggesting an unfavourable balance of endothelial injury and repair in childhood vasculitis [234].

Does Vasculitis in Childhood Predispose to Accelerated Atherosclerosis?

Several key aspects of the long-term outcome of vasculitis in the young remain of ongoing concern. Histological findings seen in KD arteries at sites of previous aneurysmal lesions long after disease resolution appear to be indistinguishable from atherosclerosis [235]. Dhillon et al. studied vascular responses to reactive hyperemia in the brachial artery using high-resolution ultrasound [106]. Flow-mediated dilation (an endothelial-dependent response) was reduced in KD patients compared with control subjects many years after the illness, even in patients without detectable early coronary artery involvement. In addition, Cheung et al. studied a cohort of patients with KD with or without coronary aneurysms compared to healthy controls and demonstrated reduced arterial distensibility (an independent risk factor for cardiovascular morbidity and mortality in adults), as assessed using ultrasound pulse wave velocity in the brachio-radial arterial segments and carotid IMT [236]. Similar findings have also been documented in children with PAN [237]. Thus, the long-term outlook for patients with systemic vasculitis must remain guarded at the present time.

Conclusions and Future Directions

A series of significant short- and long-term challenges are looming in the field of paediatric vasculitis research. The development of biomarkers that allow reliable non-invasive monitoring of disease activity and guide therapeutic decisions is of great clinical importance [233, 238]. Furthermore, several key aspects of the long-term cardiovascular risk for children who have systemic vasculitis are described [239]. The emergence of new therapies for the treatment of vasculitis in children provides a real opportunity to limit cyclophosphamide and corticosteroid exposure in the young. These include MMF [182, 183, 240, 241] and biologic agents such as rituximab [184, 185, 187], anti-TNF-α [187, 242] and thalidomide analogues such as lenalidomide [243], amongst others. None of these agents yet has an evidence base to justify their routine use in paediatric vasculitis, although many are increasingly used in this context in individual cases. It is likely that in the future clinical trials in the young will attempt to focus on these agents as alternatives to cyclophosphamide and azathioprine for induction of and/or maintenance of remission of systemic vasculitis. These sorts of trials will require international collaboration if meaningful patient numbers are to be realized, and this remains an important challenge for vasculitis research in children.

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Chapter 3
Recent Insights into the Molecular and Cellular Contributions to Venous Thrombosis

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Clinical Impact of Acute Deep Vein Thrombosis

Venous thromboembolism (VTE) is a significant health care problem in the USA, with an estimated 900,000 cases of acute deep venous thrombosis (DVT) and pulmonary embolism (PE) yearly, causing approximately 300,000 deaths each year [1]. Treatment costs for VTE are in the billions of dollars per year [2]. For the past 150 years, our view of the pathogenesis of VTE centered on Virkow's triad of stasis, changes in the vessel wall (now recognized as injury), and thrombogenic changes in the blood. Stasis is probably permissive, and not a direct cause of VTE, while systemic infection and systemic inflammation may be more causal than previously thought [3, 4].

The late DVT sequelae, postthrombotic syndrome (PTS), affects between 400,000 and 500,000 patients with skin ulcerations and six to seven million patients with other manifestations including stasis pigmentation and stasis dermatitis. It has been reported that up to 28% of patients evaluated after having an iliofemoral DVT develop marked edema and skin changes consistent with venous stasis syndrome within 20 years [5]. Risk factors for PTS include the rate of venous recanalization, the global and anatomical level of venous reflux from dysfunction of the venous valves, and the presence of persistent venous obstruction. Finally, it must be kept in mind that mortality is increased after VTE. This is due to both overall mortality and cardiovascular mortality (especially with idiopathic thrombosis) because of the age and comorbidities of the population of patients who often develop VTE [6–12].

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Overview of Thrombogenesis and Thrombolysis

Initiation of Thrombogenesis

Hemostasis is typically initiated (Fig. 3.1) by damage to the vessel wall and disruption of the endothelium, although it may be initiated in the absence of vessel wall damage, particularly in venous thrombosis [13]. Vessel wall damage simultaneously results in de-encryption (i.e., activation) of local and circulating tissue factor (TF) via protein disulfide isomerase [14]. Tissues also vary with regard to their susceptibility to thrombosis, suggesting that regional tissue-dependent mechanisms may be different. For example, hemostasis in cardiac muscle may be more dependent on the extrinsic pathway, while skeletal muscle may be more dependent on the intrinsic pathway [15], and circulating TF may be more important in venous thrombosis than in arterial thrombosis [13].

The adhesion of platelets to exposed subendothelial collagen is the first step in the formation of an effective hemostatic "platelet plug," as a result of platelet activation. This platelet–vessel wall interaction is mediated by von Willebrand factor (vWF), whose platelet receptor is glycoprotein (Gp) Ib (Fig. 3.1). Similarly, fibrinogen forms bridges between platelets by binding to the GpIIb/IIIa receptor resulting in platelet aggregation [16]. Activation of platelets also leads to the release of the prothrombotic contents of platelet granules, and the expression of membrane-bound receptors for coagulation factors Va and VIIIa, as well as fibrinogen, vWF, and ADP. Platelet activation also leads to the elaboration of arachidonic acid metabolites such as thromboxane A_2, further promoting platelet aggregation (as well as vasoconstriction). Platelet shape changes result in exposure of negatively charged

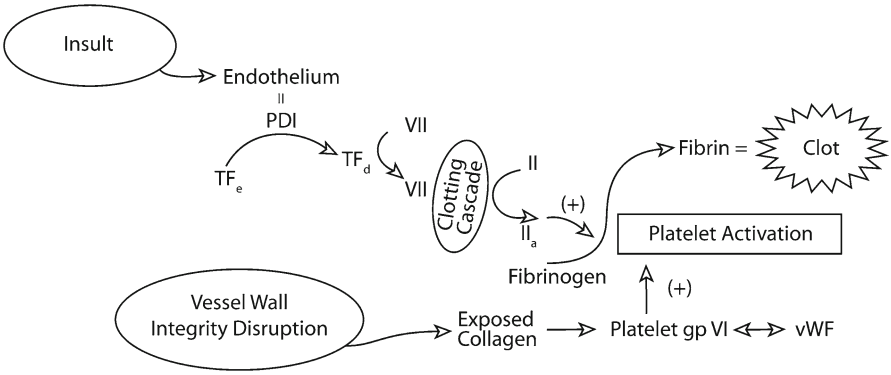

Fig. 3.1 Simplified schematic of coagulation. The insult may be a local or systemic inflammatory state such as infection, which then activates the endothelium. Tissue factor is de-encrypted by protein disulfide isomerase, which then activates factor VII, then factor II (thrombin). Alternatively, direct vascular damage may activate platelets independent of thrombin, ultimately forming fibrin. *PDI* protein disulfide isomerase, *TFe* tissue factor encrypted, *TFd* tissue factor de-encrypted, *gp* glycoprotein

procoagulant phospholipids normally located within caveolae of the platelet membrane [17]. Platelets also release microparticles (MP) rich in TF and other procoagulants, which accelerate and concentrate the thrombus generation.

The extrinsic pathway (Fig. 3.1) begins with de-encrypted TF forming a complex with factor VII, causing activation (VIIa). The TF–VIIa complex then activates factors IX and X to IXa and Xa in the presence of Ca^{2+}. Feedback amplification occurs, as VIIa, IXa, and Xa are all capable of activating VII to VIIa, especially when bound to TF [18]. Factor Xa is also capable of activating factors V to Va. Factors Xa, Va, and II (prothrombin) form on the platelet phospholipid surface in the presence of Ca^{2+} to initiate the prothrombinase complex, which catalyzes the formation of thrombin from prothrombin. Thrombin feedback amplifies the system not only by activating factors V to Va but also by activating factors VIII (normally circulating bound to vWF) to VIIIa and XI to XIa. After activation, factor VIIIa dissociates from vWF and assembles with factors IXa and X on the platelet surface in the presence of Ca^{2+} to form the Xase complex, which catalyzes the activation of factor X to Xa.

Thrombin (factor II) is central to coagulation through its cleavage and release of fibrinopeptide A (FPA) from the α chain of fibrinogen and fibrinopeptide B (FPB) from the β chain of fibrinogen. This causes fibrin monomer polymerization and cross-linking, stabilizing the thrombus and the initial platelet plug. Thrombin also activates factor XIII to XIIIa, which catalyzes this cross-linking of fibrin, as well as that of other plasma proteins, such as fibronectin and α2-antitrypsin, resulting in their incorporation into the clot and increasing resistance to thrombolysis [19]. In addition, factor XIIIa activates platelets, as well as factors V and VIII, further amplifying thrombin production.

Coagulation can also be activated through the intrinsic pathway with activation of factor XI to XIa, which subsequently converts factors IX to IXa [14], promoting formation of the Xase complex and ultimately thrombin. The physiologic contribution of the intrinsic pathway is probably not as important in the venous system.

Natural Anticoagulants

Physiologic anticoagulants balance thrombin formation and limit thrombotic activity to sites of vascular injury (see Esmon for a detailed review [20]). Antithrombin (AT) is a central anticoagulant protein that binds to thrombin at the site of thrombosis and interferes with coagulation by three major mechanisms. First, inhibition of thrombin prevents the removal of FPA and FPB from fibrinogen, limiting fibrin formation. Second, thrombin becomes unavailable for factors V and VIII activation, slowing the coagulation cascade. Third, thrombin-mediated platelet activation and aggregation is inhibited. In the presence of heparin, inhibition of thrombin by AT is accelerated, resulting in systemic anticoagulation. AT has been shown to directly inhibit factors VIIa, IXa, Xa, XIa, and XIIa. Thus, patients with a genetic deficiency of AT are at much higher risk of developing VTE than the normal population.

A second natural anticoagulant mechanism is activated protein C (APC), which is produced on the surface of intact endothelium when thrombin binds to its receptor, thrombomodulin (TM) and endothelial protein C receptor (EPCR). The thrombin–TM complex inhibits the actions of thrombin, and also activates protein C to APC. APC in the presence of its cofactor, protein S, inactivates factors Va and VIIIa, therefore reducing Xase and prothrombinase activity [21].

The third innate anticoagulant is TF pathway inhibitor (TFPI). This protein binds the TF–VIIa complex, thus inhibiting the activation of factors X to Xa and formation of the prothrombinase complex. Finally, heparin cofactor II is another inhibitor of thrombin, but whose action is in the extravascular compartment. The activity of heparin cofactor II is augmented by glycosaminoglycans, including both heparin and dermatan sulfate, but its deficiency is not associated with increased VTE risk [22].

Activation and Inhibition of Thrombolysis

Thrombus formation is balanced by controlled thrombolysis in order to localize intravascular thrombosis (see Vassalli et al. for a detailed review of thrombolysis [23]). The central fibrinolytic enzyme is plasmin, a serine protease generated by the proteolytic cleavage of the proenzyme, plasminogen. Its main substrates include fibrin, fibrinogen, and other coagulation factors. Plasmin also interferes with vWF-mediated platelet adhesion by mediating proteolysis of GpIb [24].

Activation of plasminogen occurs by several mechanisms. In the presence of thrombin, vascular endothelial cells produce and release tissue plasminogen activator (tPA) as well as α_2-antiplasmin, a natural inhibitor of excess fibrin-bound plasmin. As clot is formed, plasminogen, tPA, and α_2-antiplasmin become incorporated into the fibrin clot. In contrast to free circulating tPA, fibrin-bound tPA is an efficient activator of plasminogen.

Another endogenous activator of plasminogen is the urokinase-type plasminogen activator (uPA), also produced by endothelial cells, but it has less affinity for fibrin. The activation of uPA in vivo is not completely understood. However, it is hypothesized that plasmin in small amounts (produced through tPA) activates uPA, leading to further plasminogen activation and amplification of fibrinolysis [25]. It is likely that uPA is more important for plasmin activation in the venous system than tPA [26].

The third mechanism of plasminogen activation involves factors of the contact activation system: activated forms of factors XII, kallikrein, and XI that can each independently convert plasminogen to plasmin. These activated factors may also catalyze the release of bradykinin from high molecular weight kininogen, which further augments tPA secretion. Finally, APC has been found to proteolytically inactivate plasminogen activator inhibitor type 1 (PAI-1), an inhibitor of plasmin activators released by endothelial cells in the presence of thrombin [20].

The degradation of fibrin polymers by plasmin ultimately results in the creation of fragment E and two molecules of fragment D, which are released as a covalently linked dimer (D-dimer) [27]. Detection of D-dimer in the circulation is a marker for ongoing thrombus metabolism and has been shown to accurately predict ongoing risk of recurrent VTE [28].

Interestingly, the activity of the fibrinolytic system within the vein wall is reduced in the area of the valvular cusps as compared with the nonvalvular area [29]. Deep veins of the lower limb have the lowest fibrinolytic activity in soleal sinuses, as well as in the popliteal and femoral vein regions, as compared with the other anatomic locations. This observation underlies a popular hypothesis as to why DVT most commonly originates in the lower limb. However, no in vivo real-time imaging studies in humans have ever shown how and where DVT actually forms.

In plasma, PAI-1 is the primary inhibitor of plasminogen activators and is likely most important in the venous system [23, 30, 31]. The primary function of PAI-1 is to inhibit plasminogen activators from converting plasminogen to plasmin, which is responsible for initiating fibrinolysis. It is secreted in an active form from liver and endothelial cells and is stabilized by binding to vitronectin (and inhibits thrombin in this form). PAI-1 is stored in the alpha-granules of quiescent platelets [32].

Mouse Models of Venous Thrombosis

Why a Mouse?

While there is no reported spontaneous venous thrombosis in animals, several experimental mouse models (Table 3.1) exist for venous thrombosis research including: photochemical [33], inferior vena cava (IVC) stasis [34–36], IVC stenosis [37], mechanical trauma [38, 39], and electrolytic models [40, 41]. Day et al. provide a review of the current options [42].

In terms of thrombotic pathways, the mouse's physiological characteristics, which are similar to humans, make it a useful experimental tool. The mouse's small size is convenient, making them inexpensive to house. They are relative easy to breed and are well characterized genetically, allowing for gene addition (transgenic mice) or deletion (knockout mice).

Photochemical Injury Using Rose Bengal Dye

The photochemical injury model uses Rose Bengal dye administrated at a dose of 50 mg/kg. The dye is activated by a green light laser (540 nm) placed 6 cm from the vein injury site for 15 min [33, 42, 43]. Using the jugular vein, this technique produces an occlusive thrombosis within the vein with a mean time to occlusion of

Table 3.1 Mouse models to study deep venous thrombosis

Model	Brief model description	Advantages	Disadvantages
IVC photochemical injury model	Intravascular Rose Bengal dye activated by a green light laser (540 nm) for 15 min	Produces a subtle endothelial injury that activates the vascular endothelium	Produces inconsistent thrombosis (personal communication, DDM)
IVC ligation or stasis model	A nonreactive suture ligature constricts the IVC just below the renal veins to produce complete blood stasis	The IVC yields quantifiable amounts of vein wall tissue and thrombus. It has proven useful for evaluating interactions between the vein wall and the occlusive thrombus for accessing the progression from acute to chronic thrombosis	Lack of blood flow inhibits the maximal effect of administered systemic therapeutic agents on the thrombus and vein wall
IVC stenosis model	A nonreactive suture is placed around the IVC to reduce blood flow	Reduced flow combined with temporary endothelial compression Produces a laminar thrombus allowing the study of cellular kinetics	The degree of stenosis is inconsistent, leading to variable thrombus sizes
Mechanical injury model	External mechanical force to damage the endothelium. Fiber-optic technology is used to transilluminate the vein to visualize and record thrombosis	Measures the kinetics of temporal thrombus growth and resolution in the femoral vein	Only allows study of early stages of thrombosis. Expensive and specialized optical equipment are required to visualize thrombus generation and resolution in this model
Electrical stimulation of femoral vein	Electric current passed through a needle within the femoral vein injures the vein wall and results in thrombus formation	Useful for evaluating venous thrombogenesis. Thrombosis is induced at the site of the electrode and grows in a sequential fashion	Small yield of thrombus and tissue mass for protein and gene expression analysis in comparison to the IVC thrombosis models
Electrical stimulation of IVC vein	Electric current passed through a needle within the IVC injures the vein wall and results in thrombus formation in the presence of blood flow	Produces consistent thrombus size, enough sample quantity per mouse to be able to study thrombogenesis, thrombus resolution, and also new agents for DVT treatment	Requires 15 min for the electrolytic process

26.5 ± 9 min, or 39.7 ± 1 min if the dose of Rose Bengal is 25 mg/kg [33]. In the IVC, this technique produces a subtle endothelial injury that activates the vascular endothelium, but produces inconsistent thrombosis (author personal communication, DDM).

The IVC Ligation Model or Stasis Model

The stasis or IVC ligation mouse model of venous thrombosis involves a total occlusion or ligation of the IVC below the renal veins with a Prolene 7-0 (a nonreactive suture) to produce complete blood stasis. Back branches are cauterized and side branches are also ligated. In most species, a consolidate thrombus forms by 3 h postligation and yields quantifiable amounts of vein wall tissue and thrombus. In addition, the ligation model can be utilized to evaluate interactions between the vein wall and the occlusive thrombus and to assess the progression from acute to chronic inflammation. This model is well established and broadly used in mice [34, 35, 42, 44–46]. The major disadvantage with the stasis model is the lack of blood flow, which jeopardizes the maximal effect of administered systemic therapeutic agents on thrombi.

The IVC Stenosis Model

In the mouse stenosis model, a 4-0 silk suture is placed around the IVC and tied down on a piece of 5-0 prolene, which is then removed creating a stenosis. The reduction in blood flow, when combined with external endothelial compression with a neurosurgical vascular clip for 15 s, produces a laminar thrombus allowing the study of cellular kinetics and therapeutic agents [37, 47]. Although the technique is simple and produces a large thrombus, the degree of stenosis is inconsistent, causing large standard deviations in thrombus size that make this model less appropriate for pharmacological investigation (personal observation).

The Mechanical Injury Model

The mechanical injury model measures the kinetics of temporal thrombus growth and resolution in the mouse femoral vein. This model uses an external mechanical force to damage the endothelium. Fiber optic lights transilluminates the vein to visualize the thrombus. The main disadvantages of this model are that it only allows the study of early stages of thrombosis and the yield of thrombus, and vein wall mass is too small for most analysis. In addition, this model is expensive, since specialized optical equipment is required to visualize thrombus generation and resolution [38, 39, 48].

Electrolytic Vein Models

Mouse Femoral Vein

Venous thrombogenesis can be evaluated in a temporal fashion using a murine femoral vein electrical stimulation thrombus model [40]. Thrombosis is induced at the site of the electrode and grows in a sequential fashion. Cooley et al. [40] speculated that thrombus formation in their model is the result of either direct electrical injury (resistance heating) or a free radical-induced injury to the vein wall. When compared to IVC thrombosis models, the main limitation of this model is that the yield of thrombus and vein wall mass is too small for protein and gene expression analysis.

Mouse IVC

The electrolytic inferior vena cava model (EIM) is a newer mouse model of venous thrombosis recently published by Diaz et al. [41]. In this model, a 25G stainless-steel needle attached to a silver-coated copper wire is inserted into the exposed caudal IVC and positioned against the anterior wall (anode). Another wire is implanted subcutaneously to complete the circuit (cathode). A current of 250 μA is applied over 15 min using a Grass S48 square wave stimulator and a constant current unit. The direct current results in the formation of toxic products of electrolysis that activate the endothelial surface of the IVC, promoting a thrombogenic environment and subsequent thrombus formation. Importantly, it was demonstrated that heat does not participate in thrombus formation in the EIM model. This is the first IVC thrombosis mouse model that consistently produces thrombosis and produces a thrombus sample large enough to adequately study thrombogenesis, thrombus resolution, and pharmacologic applications in the field of venous thrombosis (Fig. 3.2).

The Role of Inflammation in Thrombosis

An acute to chronic inflammatory response occurs in the vein wall and thrombus after the thrombus forms. This response leads to thrombus amplification, organization, and recanalization, and occurs at the expense of vein wall and vein valve damage [49]. The question that still remains is whether inflammation participates as an acute trigger of thrombosis, starting a series of thrombotic mechanisms, or if inflammation after thrombosis is a consequence.

During acute thrombosis, the primary inflammatory cells that migrate into the vein wall and thrombus are neutrophils. Cell adhesion molecules (CAMs) expressed on endothelial cells facilitate this phenomenon [50]. Neutrophil extravasation through an intact layer of endothelial cells has been described in early stages of

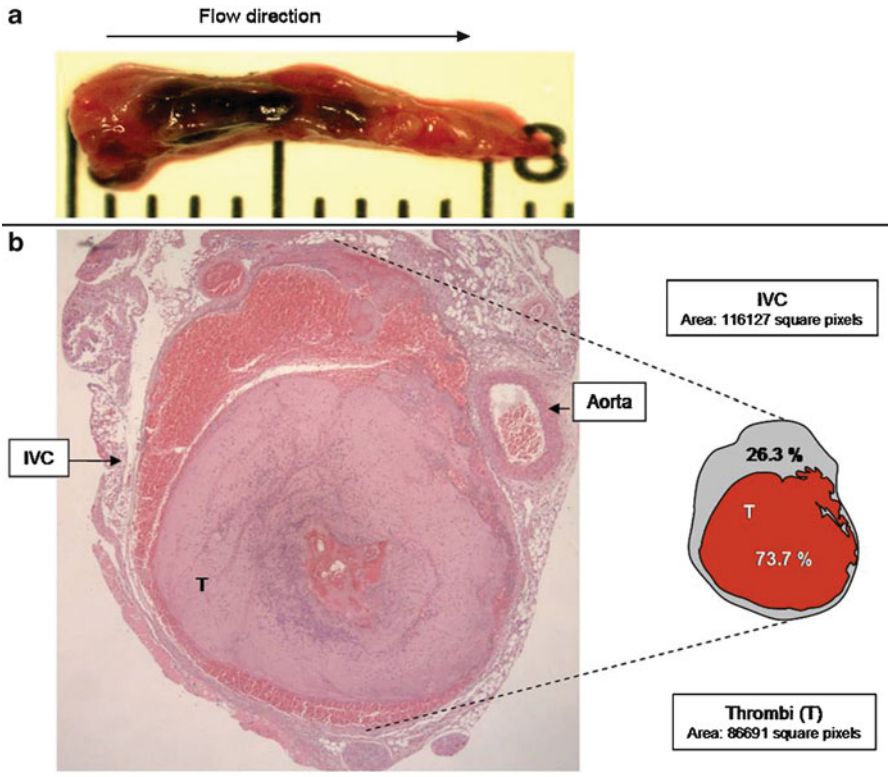

Fig. 3.2 Electrolytic injury model (EIM)-induced thrombosis of the inferior vena cava. Thrombus harvested after EIM. (**a**) The thrombus shape suggests thrombus formation in the presence of constant flow. (**b**) A hematoxylin and eosin stained histologic section of the inferior vena cava and adjacent structures demonstrates 30% patency 2 days after EIM

venous thrombosis, linking acute inflammation and acute thrombosis [51–55]. Selectins (P- and E-selectin), which are CAMs that modulate leukocyte–endothelial interactions, are integrally involved in this process. Indeed, proinflammatory cytokines increase CAM expression in veins [56].

Cytokines, chemokines, and inflammatory factors such as interleukin-1 (IL-1) facilitate the inflammatory response in both the vein wall and the thrombus. Both proinflammatory and anti-inflammatory mediators are involved in the ultimate vein wall and thrombus response. Other interactions between inflammation and coagulation exist. For example, inflammation is associated with increased expression of fibrinogen, TF and membrane phospholipids, and increased platelet reactivity. Inflammation is also associated with decreased expression of thrombomodulin and vascular heparans, a decreased half-life for APC, and decreased fibrinolysis (by increasing PAI-1) [57].

CAMs and MPs in Venous Thrombosis

P-selectin is a central adhesion molecule involved in the interactions between inflammatory cells and vessels and has been linked to cardiovascular events in both the arterial and venous circulations [58]. This molecule is present in the alpha-granules of platelets and the Weibel–Palade bodies of endothelial cells. After activation by local vascular injury or platelet activation, it is translocated to the plasma membrane of these cells, mediating the initial inflammatory response [59]. Recombinant P-selectin glycoprotein ligand 1 (rPSGL-1)-Ig chimera binds and inhibits cell-associated P-selectin. Thrombin-activated platelets expressing P-selectin bind to neutrophils, and rPSGL-1-Ig effectively blocks this binding by approximately 90% [60]. Recently, a synergism between leukocytes and platelets has been identified [61], such that TF can be transferred from leukocytes to platelets in a P-selectin-mediated fashion, and even platelets have been demonstrated to express functional PSGL-1, allowing for a P-selectin-mediated mechanism for platelet rolling [53, 62].

In a mouse model of stasis DVT, P-selectin is upregulated as early as 6 h after thrombus induction, while E-selectin is upregulated at day 6 after thrombosis, with increases in gene expression preceding the protein elevations [34]. To further define the importance of the selectins to the thrombo-inflammatory response, genetically modified P- or E- selectin knockout (KO) mice were challenged with stasis DVT. Deletion of E-selectin and combined P-selectin/E-selectin deletion were associated with decreased thrombosis and significantly reduced vein wall inflammatory response [34]. A significant decrease in fibrin content is found 2 days after thrombus formation in P-selectin/E-selectin-deficient animals compared to control mice, and at 6 days after thrombus formation in E-selectin, P-selectin, and P-selectin/E-selectin-deficient animals. Mice deficient in both selectins had the smallest thrombi [63]. Together, these data suggest that selectins contribute to both thrombus generation and thrombus amplification.

When P-selectin binds to its receptor, PSGL-1, MPs are produced that can promote thrombus formation (Fig. 3.3). MPs are fragments of phospholipid cell membranes that promote coagulation and modulate a number of inflammatory cell–vessel wall interactions. For example, platelet-derived MPs are involved in the pathogenesis of heparin-induced thrombocytopenia, possibly by promoting thrombosis after the antibody binding to the platelets [65]. Platelet MPs rich in P-selectin have been found to allow flowing neutrophils to aggregate by a non-L-selectin mechanism [66]. Less is known about leukocyte-derived MPs, although they are associated with endothelial cell activation and cytokine gene induction [67]. Additionally, MPs derived from endothelial cells induce monocyte TF antigen expression and release [68].

MPs are procoagulant and are recruited back to the developing thrombi, where they amplify coagulation [69, 70]. The PSGL-1 present on the surface of MPs can bind to P-selectin on activated platelets and endothelial cells, allowing the MPs to interact directly at the point of thrombus initiation, accumulation, and propagation. Fluorescently labeled MPs have been shown to be taken up into thrombi within 1 min

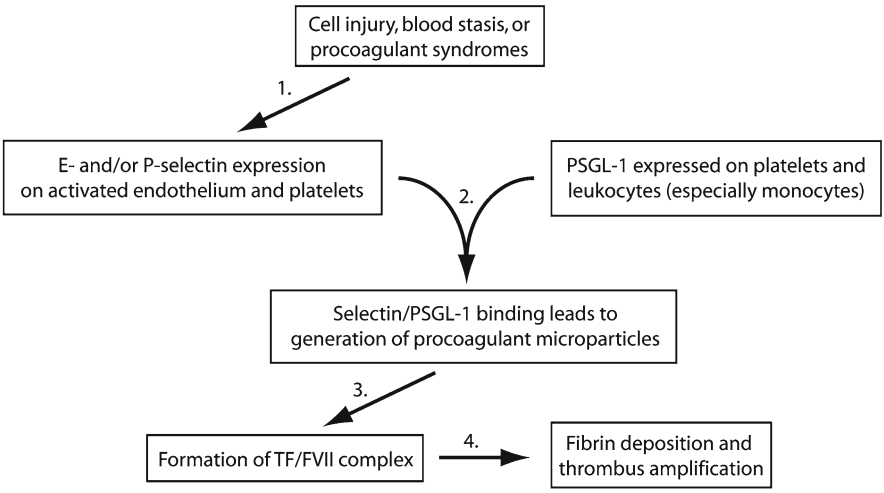

Fig. 3.3 Proposed contribution of microparticles to venous thrombosis. (1) Injury, stasis, procoagulant syndromes, or other stimuli upregulate endothelial selectin adhesion molecules that (2) bind to P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes and platelets. This results in the production of procoagulant microparticles, especially from monocytes but also from platelets and endothelial cells. (3–4) The microparticles then are concentrated in the forming thrombus where they contribute to amplification of the coagulation cascade and thrombus formation. *TF* tissue factor, *FVII* factor VII. Based on data from [64]

of ferric chloride-induced venule thrombosis in a mouse cremaster muscle model [71]. The colocalization of fibrin, platelets, and leukocytes in the developing thrombus supports this hypothesis, as do the recent observations of the importance of P-selectin-mediated monocyte–platelet interactions to the generation of TF [72, 73].

The delta CT mouse carries transgenic expression of the extracellular (soluble) portion of P-selectin and therefore has fourfold elevated levels of soluble P-selectin (sP-selectin). A stasis model of DVT has been used in this mouse to determine the effect of sP-selectin on thrombogenesis and MPs. A 50–60% increase in thrombus size was found at days 2 and 6 after thrombosis, and this increase was associated with elevated procoagulant MPs in the circulation, most prominently of leukocyte origin. Consistently, mice deficient in P- and E-selectin had significantly smaller thrombi, and decreased levels of MPs derived from leukocytes after thrombus development [35].

E-selectin polymorphism in humans has been associated with enhanced endotoxin-triggered, TF-mediated coagulation, atherosclerosis, myocardial infarction and restenosis after angioplasty and may be associated with recurrent VTE [74–82]. Jilma et al. more recently demonstrated that homozygosity of the single nucleotide polymorphism *Ser*128*Arg* in the E-selectin gene in humans may be associated with recurrent VTE [74]. This polymorphism alters ligand affinity, enhances myeloid cellular tethering, and regulates leukocyte–endothelial cell interactions in vitro [74–77]. When 585 patients with a first idiopathic VTE were prospectively

examined, 102 patients (17%) were heterozygous for the Ser128Arg mutation and 11 (2%) were homozygous. Of the total patient population, 90 patients (15% of 585 patients) demonstrated recurrent VTE. Homozygosity for this mutation appeared to increase the cumulative likelihood for early recurrent VTE and was considered an independent predictor of recurrent VTE (hazard ratio = 4.1) [74].

Endothelium and Vessel Wall Hemostasis

Most of the thrombosis–thrombolysis processes occur in juxtaposition to the endothelium, and hence the endothelium is one of the pivotal regulators of thrombotic homeostasis. Under normal conditions, endothelial cells maintain a vasodilatory and local fibrinolytic state in which coagulation, platelet adhesion, and activation are suppressed. Specifically, the nonthrombogenic endothelial surface is maintained through several mechanisms, including: (1) endothelial production of TM and subsequent activation of Protein C; (2) endothelial expression of heparan sulfate and dermatin sulfate, which accelerate AT and heparin cofactor II activity; (3) constitutive expression of TFPI, and (4) local production of tPA and uPA. In addition, the production of nitric oxide (NO) and prostacyclin by the endothelium inhibits the adhesion and activation of leukocytes and produces vasodilation [83]. TF production is also inhibited by NO [84].

During states of endothelial cell injury, a prothrombotic and proinflammatory state is supported by the endothelial surface [85]. Release of platelet activating factor (PAF) and endothelin-1 promotes vasoconstriction [85], while production of vWF, TF, PAI-1, and factor V augments thrombosis. Indeed, vWF is expressed to a greater extent on the endothelium of veins as compared with arteries, and tPA is less commonly expressed in venous endothelium [86].

In an elegant study by Zhou and colleagues, stasis thrombosis in the rat induced early endothelial damage with subendothelial collagen exposure and P-selectin expression within 6 h [87]. These experiments suggest mechanical injury results in a shift to a procoagulant milieu that drives ongoing thrombosis. Interestingly, the early thrombus matrix does facilitate the recruitment of CD34(+) cells, which may promote endothelial healing [88].

Mechanisms That Resolve Venous Thrombosis

Regardless of the location and extent of an acute DVT, the process of resolution has only recently become well understood (Fig. 3.4). The natural fibrinolytic mechanisms break down the thrombus over time and at variable rates [90, 91], with resultant vein wall damage. Venous thrombus resolution has a distinct time line and mimics wound healing, involving profibrotic growth factors, collagen deposition,

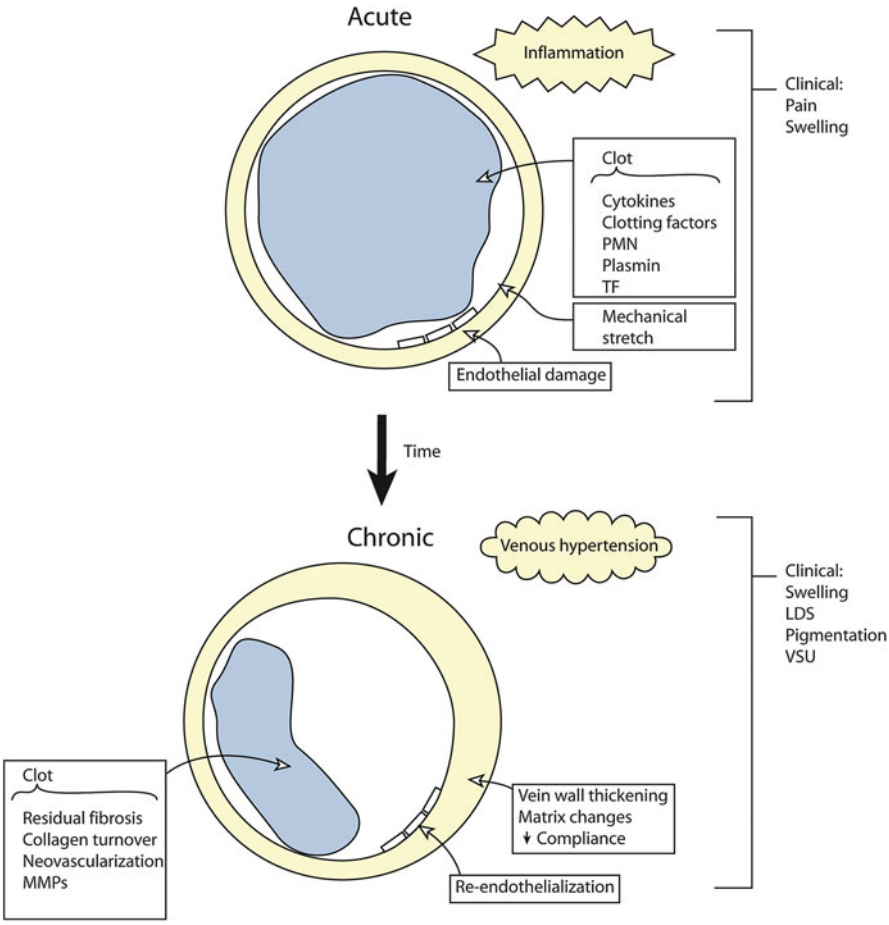

Fig. 3.4 Hypothesized model of rodent venous thrombosis resolution. Early acute thrombus resolution involves release from the large clot of interleukin-1, necrotic cellular debris, platelet factors that drive neutrophil influx, and plasminogen activators, such as uPA. Concurrently, MMP9 is released, and plasmin is upregulated. At the same time, loss of endothelium integrity exposes the subendothelial matrix proteins that may further potentiate thrombosis. In the media, collagenolysis and elastinolysis occur, in addition to direct mechanical stretch. Acute clinical symptoms in the affected limb include pain and swelling. Later (usually after day 8), vein wall medial thickening occurs, resulting in decreased compliance and possibly decreased vasoreactivity. Re-endothelialization commences but is incomplete until a much later time point (>14 days). Thrombus neovascularization and organization are associated with resolution. Within the vein wall, matrix turnover occurs with increased MMP2 expression, as well as collagen-I and -III production. Interleukin-13 and TGFβ are two profibrotic growth factors that may be involved with late vein wall remodeling. Symptoms of chronic thrombosis include limb swelling, lipodermatosclerosis (LDS), dermal hyperpigmentation, and venous stasis dermal ulceration (VSU). Reprinted from [89] with permission from Elsevier

Table 3.2 Characteristics of experimental venous thrombosis resolution

	Acute (<8 days)	Chronic (>8 days)
Effector cell	Neutrophil	Fibroblast, monocyte, VSMC
Chemokines/cytokine	IL-8, IL-1β, IL-6	MCP-1, SLC
Growth factors	–	VEGF, βFGF, TGFβ
Neovascularization of clot	Minimal; some channels express laminin	Channels express vWF
Matrix remodeling	MMP9, elastase?	uPA–plasmin, MMP2
Vein wall collagen type	Collagen III	Collagen I

IL interleukin, *MCP-1* monocyte chemoattractant-1, *SLC* secondary lymphoid chemokine, *VEGF* vascular endothelial growth factor, *βFGF* basic fibroblast growth factor, *TGF-β* transforming growth factor-beta, *MMP* matrix metalloproteinase, *VSMC* vascular smooth muscle cell

matrix metalloproteinase (MMP) expression, and neovascularization (Table 3.2). Similarly, the fact that leukocytes invade the thrombus in a specific sequence suggests their importance in normal thrombus resolution [92]. The first cell type in the thrombus is the neutrophil (PMN). Although PMNs may cause vein wall injury, they appear essential for early thrombus resolution by promoting both fibrinolysis and collagenolysis [55, 93]. In a rat model of stasis DVT, neutropenia was associated with larger thrombi at 2 and 7 days, and was correlated with increased thrombus fibrosis and significantly lower thrombus levels of both uPA and MMP9 [94].

Leukocyte-derived uPA has been shown to modulate the uPA–plasmin axis in venous thrombosis [26]. In contrast, other work suggests that early (4 <day) venous thrombosis resolution may be independent of uPA. For example, in genetically deleted uPA mice, an early gamma interferon (IFNγ)-dependent increase in MMP2 activity was found [95]. Mice lacking MMP2 had larger early venous thrombosis, despite no change in local plasmin levels.

The monocyte is likely the most important cell for the later stages of DVT resolution [96]. Monocyte influx into the thrombus peaks at day 8 after thrombogenesis and correlates with elevated levels of monocyte chemotactic protein-1 (MCP-1), one of the primary CC chemokines that directs monocyte chemotaxis and activation [97]. MCP-1 has also been associated with DVT resolution [98]. When mice lacking the MCP-1 receptor CC receptor-2 are challenged with stasis thrombosis, late impairment of thrombus resolution is observed, probably due to impaired IFNγ-inducible vein wall proteinase activity that may be independent of monocytes [99].

Venous thrombosis is most always a sterile inflammatory nidus. Sterile inflammation and the process of resolution have only recently been investigated, but many parallels between the venous thrombosis and solid organ necrosis have been observed [100]. Experimental stasis venous thrombosis in the mouse suggests a role for TLR9 in venous thrombosis resolution, as deletion of TLR9 is associated with larger thrombus, increased thrombus leukocytes, and a decreased Th1 cytokine immune environment [101]. Interestingly, stimulation of TLR9 with an oligodeoxynucleotide (ODN) aptamer is associated with smaller thrombi in mice, suggesting that modulation of TLR9 signaling may have a possible clinical application.

Venous thrombosis resolution also involves neovascularization [52, 102]. Neovascularization occurs at later time points, usually after day 6. It is likely that local hypoxia drives hypoxia inducing factor 1alpha [HIF-1α] upregulation and production of vascular endothelial growth factor (VEGF), with generation of endothelial lined channels [103, 104]. Interestingly, circulating progenitor cells play a role in venous thrombosis resolution, but endothelial progenitor cells may not be important for neovascularization itself [105]. That is, while neovascular channels are observed in resolving experimental venous thrombosis, the progenitor cells did not specifically line these channels, suggesting another role for them in the resolution process.

Proinflammatory and Profibrotic Mediators in Vein Wall Remodeling

As the thrombus resolves, a number of proinflammatory factors are released into the local environment, including interleukin (IL)-1-α, IL-1-β, and tumor necrosis factor-alpha [TNFα] [92]. Gene expression of the anti-inflammatory cytokine IL-10 is upregulated at day 2, and remains so through day 9 after thrombosis in a stasis model of venous thrombosis, suggesting that it serves as a counterbalance to the inflammatory response. Additionally, vein wall IL-10 protein levels are elevated before mRNA upregulation, suggesting an initial increase from preformed IL-10 followed by IL-10 synthesis [34]. The cellular sources of these different mediators have not been specifically defined, but likely include leukocytes and smooth muscle-like cells within the resolving thrombus and the adjacent vein wall.

The leukocyte kinetics in the vein wall after DVT are similar to that observed in the thrombus, with an early influx of PMNs, followed by monocytes. Based on experimental studies, elastinolysis and collagenolysis occur early, as measured by an increase in vein wall stiffness, persists through 14 days, and is accompanied by elevated MMP2 and MMP9 activities [106].

An elevation of profibrotic mediators, including transforming growth factor-beta [TGFβ], interleukin (IL)-13, and monocyte chemotactic protein-1 (MCP-1), is associated with the early biomechanical injury from DVT [106, 107]. These mediators are present within the vein wall and thrombus and may drive the fibrotic response. Although exogenous MCP-1 may hasten DVT resolution, it does have profibrotic activity in vivo. The profibrotic growth factor TGFβ is also present in the thrombus and is activated with normal thrombolysis [108]. This factor may be a key to promoting vein wall fibrosis. Consistently, fibrosis with a significant increase in vein wall collagen has been observed after experimental stasis thrombogenesis [109]. An increase in collagens I and III gene expression, as well as an increase in MMP-2 and MMP-9 gene expression and activity, correlates with this increase in fibrosis [109].

Also, increased plasma levels of interleukin-6 (IL-6), a major inflammatory cytokine, have been demonstrated during venous thrombosis [110] and PTS [111].

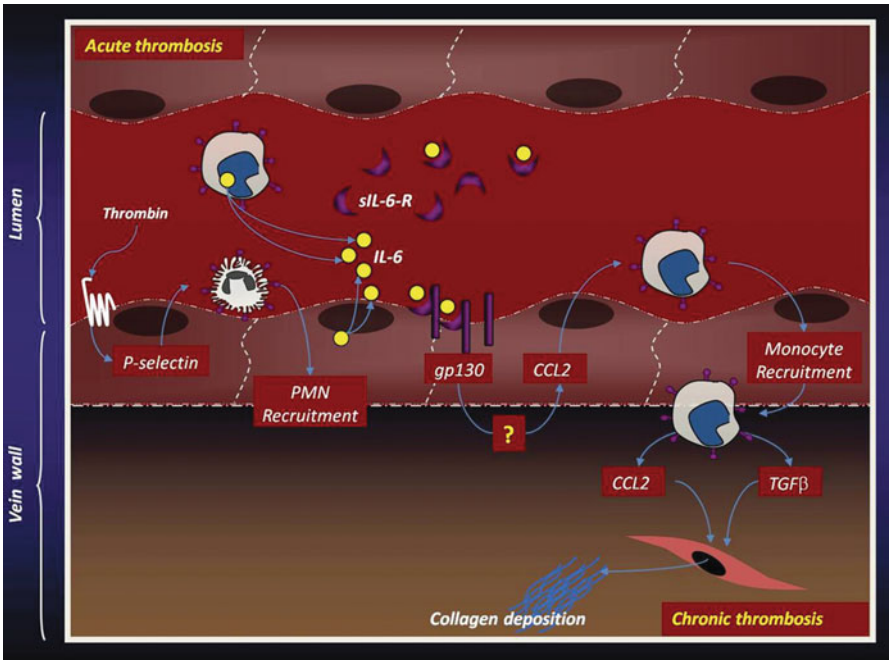

Fig. 3.5 Proposed mechanism of IL-6-induced vein wall fibrosis following venous thrombosis. During venous thrombosis, endothelial cells express P-selectin and neutrophils are recruited. IL-6 is produced by activated venous endothelial cells and causes upregulation of vein wall CCL2, leading to monocyte recruitment. Recruited monocytes secrete cytokines and growth factors that stimulate fibroblasts and smooth muscle cells to produce and deposit collagen. *PMN* polymorphonuclear neutrophil, *gp130* glycoprotein 130, *CCL2* C–C motif chemokine ligand 2, *IL-6* interleukin-6, *sIL-6-R* soluble IL-6 receptor, *TGFβ* transforming growth factor beta. Modified from [46] with permission from Elsevier

While these studies suggest that IL-6 may be used as a marker for venous thrombosis, its role as an active cytokine in the development of PTS has been recently established [46]. IL-6 plays a critical role in the transition from acute to chronic inflammation. Antibody neutralization of IL-6 in the mouse model of venous thrombosis was associated with reduced vein wall MCP-1 gene and protein expression, as well as monocyte infiltration. Moreover, blockade of IL-6 was associated with less vein wall fibrosis at the chronic time point (14 days after thrombosis). A proposed mechanism of IL-6 induced vein wall fibrosis following venous thrombosis is shown in Fig. 3.5.

Further linking inflammation to fibrosis, we have shown that inhibition of the inflammatory response can decrease vein wall fibrosis. In a rat model of stasis DVT, P-selectin inhibition achieved through treatment with either low-molecular-weight heparin (LMWH) or an oral inhibitor of P-selectin 2 days after establishment of thrombosis led to significantly decreased vein wall injury (independent of thrombus size), as measured by vein wall tensiometry (stiffness), intimal thickness score, interleukin (IL)-13 levels, MCP-1 levels, and platelet-derived growth factor-β (PDGFβ) levels [107]. The mechanism accounting for this protective effect is not

yet known, but probably does not involve leukocyte blockade, as no differences in vein wall monocyte influx were observed.

Loss or injury of venous endothelium also contributes to vein wall fibrosis and predisposition to recurrent thrombosis. An experimental model of DVT showed decreased expression of homeostatic endothelial genes such as NO and thrombomodulin as compared with controls, and correlated with loss of vWF-positive cell luminal staining [112]. Other investigators have found that prolonged venous stasis is associated with decreased plasminogen activators, probably related to loss of endothelium [113]. The period of time the endothelium may be impaired is not clear, although preliminary studies suggest only modest recovery by 21 days after thrombosis, as measured by endothelial markers such as CD31 and thrombomodulin.

The role of the vein wall media after thrombotic injury has not been well studied. The vascular smooth muscle cell (VSMC) found in the media contributes significantly to the neointimal process in arterial injury. As the vein wall becomes thickened after thrombosis resolution, the VSMC is likely the cell that produces collagen. Thus, early vein wall injury is associated with active matrix remodeling that seems to promote late fibrosis, not unlike many other tissue responses to inflammation. The specific mechanisms and strategies to reverse the fibrotic process are being actively investigated [114]. Preliminary studies suggest that after thrombotic injury the VSMC changes to a synthetic state from a contractile state, as measured by common antigen markers, and may contribute to the fibrotic response. The $CD45^+/CCR7^+/Col\ I^+$ leukocyte (fibrocyte) may also contribute to both the thrombus and the vein wall response in venous thrombosis resolution. In a nonstasis model of venous thrombosis, CCR7 signaling seems important for both of these processes, although it may be less beneficial in a stasis model (Henke, unpublished data). It may be that the $CCR7^+$ fibrocyte directs the normal matrix response for vein wall healing.

Links Between Dyslipidemia, Inflammation, and Venous Thrombosis

An interface among hyperlipidemia, vascular inflammation, and thrombosis has been suggested from a recent clinical trial showing decreased risk of VTE with use of 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibition [115]. PAI-1 levels are elevated by hyperlipidemia, and high levels of PAI-1 appear to synergize with factor V Leiden genetic abnormalities to cause thrombosis. Studies on the role of elevated levels of PAI-1 in venous thrombosis have not been consistent in suggesting increased thrombotic risk [116, 117], although it is plausible that elevated PAI-1 could suppress fibrinolysis, and thereby increase thrombosis potential. In humans, genetic polymorphisms, particularly the 4G/4G insertion/deletion in the PAI-1 promoter region, affect transcription and correlate with increased risk of VTE. Studies have found an eightfold increased VTE risk in patients with the 4G allele combined with other thrombophilic markers [118], while PE was increased 4.5-fold in 4G/4G patients with protein S deficiency [119].

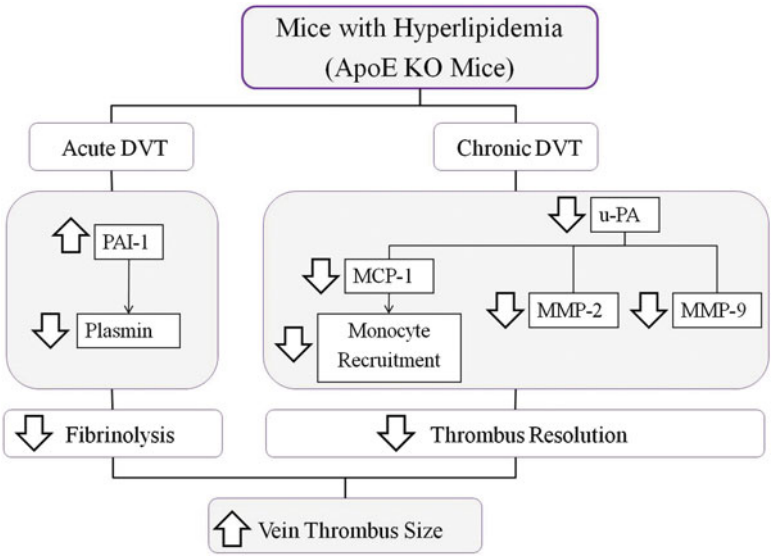

Fig. 3.6 Events that affect venous thrombosis in hyperlipidemic ApoE−/− mice. During acute venous thrombosis in hyperlipidemic ApoE−/− mice, we observed an increase in PAI-1 that contributes to increased thrombus weight due to impaired fibrinolysis. During chronic venous thrombosis in ApoE−/− mice, undetectable levels of uPA lead to decreased expression of MMP-9, MMP-2, and MCP-1, resulting in decreased monocyte recruitment and impaired thrombus resolution

Experimentally, venous thrombi are larger in a stasis model in ApoE−/− mice, as compared with controls, suggesting a role of hyperlipidemia in venous thrombosis. Correspondingly, a significant increase in early circulating PAI-1 activity and a decrease in circulating plasmin activity was documented in ApoE−/− mice compared to controls, suggesting an impaired thrombolytic potential. In addition, ApoE−/− mice had undetectable levels of uPA in both vein wall and thrombus, compared to WT mice at both days 6 and 14 after thrombosis, suggesting impaired late thrombolysis. Loss of uPA leads to a sequence of biological events that result in impaired thrombus resolution through MMPs and MCP-1 [120]. Indeed, MMP2 and MMP9 were significantly decreased at the chronic time points (days 6 and 14) in APOE-deficient mice compared to WT mice, and MCP-1 was significantly decreased at both acute (day 2) and chronic (day 6) time points compared to WT mice. Consistently, monocyte recruitment was significantly reduced at days 6 and 14. In nonhyperlipidemic mice, loss of uPA is associated with increased MMP activity [95]. Thus, in hyperlipidemic mice with venous thrombosis, increased PAI-1 may contribute to an acute increase in thrombus size due to impaired fibrinolysis. In chronic venous thrombosis, decreased uPA is associated with impaired resolution due to decreased monocyte influx and MMP activity (Fig. 3.6). These observations may explain the clinical benefit of control of plasma lipid concentration on vascular venous inflammation.

Aging Alters the Risk for Venous Thrombosis

The incidence of VTE increases markedly with advancing age. In younger individuals, the incidence of venous thrombosis is less than 1 per 10,000 per year; however, this increases to approximately 1% in the elderly. Thus, aging is the most prevalent risk factor for venous thrombosis [121]. Biomarkers associated with thrombosis that increase with age are summarized in Table 3.3.

Increased risk of venous thrombosis in humans is associated with increased plasma levels of markers of intravascular coagulation such as D-dimer and prothrombin fragment [122]. Increases in fibrinogen, factors VIII and IX without a proportional increase in anticoagulant factors may also contribute to the increasing risk of DVT with age. Aging is also positively associated with IL-6 and C-reactive protein (CRP) levels, indicating that inflammation or an inflammatory state is likely an important stimulus for thrombotic events in the aging population [123].

The anatomy of the venous vessel wall is also altered with aging. The aged vein wall shows atrophy of the muscle fibers, increases in collagen, and decreased valve competency due to thickening of the values [124]. It has been hypothesized that the venous valve sinus may be a regulator of the prothrombotic state in that immediate environment. Increased levels of anticoagulant proteins such as endothelial TFPI and thrombomodulin are more active in the valvular sinus than the luminal endothelial layer, but with aging, the endothelium shifts to a lesser anticoagulant state [123]. Consistently, these factors may be decreased in the valve sinus [125].

The expression of PAI-1 is not only elevated in the aged but also induced by a multitude of pathologies associated with the process of aging, such as obesity and insulin resistance [126–128]. PAI-1 has also been shown to significantly increase with age in both plasma and murine tissues, and in aged rats [129, 130]. Recent studies performed in animal models of venous thrombosis have also shown that increased soluble P-selectin and vein wall protein levels of P-selectin in aging mice correspond to increased thrombus burden in these animals. Experimentally, soluble P-selectin and P-selectin vein wall gene expression were significantly

Table 3.3 Deep venous thrombosis (DVT)-associated biomarkers for inflammation, coagulation, and fibrinolysis that increase with age

Marker	Change with age (↑ or ↓)	Role in DVT
CRP	↑	–
IL-6	↑	+
P-selectin	↑	++
Fibrinogen	↑	+
Coagulation factors	↑	++
vWF	↑	+
D-dimer	↑	–
PAI-1	↑	+
Endothelial function	↓	?

CRP C-reactive protein, *IL-6* interleukin 6, *vWF* von Willebrand factor, *PAI-1* plasminogen activator inhibitor-1

higher 2 days post thrombosis in 18-month-old mice compared with 2- and 11-month-old mouse groups, respectively (unpublished observation). In a previous aging study, older mice (11 months) had significantly heavier thrombus weights when compared to younger animals (2 months), and decreased thrombus inflammatory cell populations. Inflammatory cells, especially monocytes, have been noted to promote thrombolysis due to their secretion of proteolytic enzymes [44, 47, 98]. Consistently, older animals had significantly higher concentrations of MPs with TF activity than those of younger animals [131].

Moving Toward the Clinic: Novel Deep Venous Thrombosis Prophylaxis and Treatment in a Primate Model

The importance of P-selectin and its receptor PSGL-1 has been demonstrated in DVT using a primate model of stasis-induced IVC thrombosis. In this model, antibody inhibition of P-selectin or rPSGL-1-Ig inhibited inflammation and thrombosis when given prophylactically [53, 132]. Further study has demonstrated a significant dose–response relationship between rPSGL-1-Ig and thrombosis and rPSGL-1-Ig and spontaneous recanalization [62]. The peri-thrombus vein wall had decreased gadolinium enhancement (marker of inflammation) in all rPSGL-1-Ig groups compared to controls, despite no significant differences in inflammatory cell extravasation. In fact, the highest dosage produced the best inhibition of thrombosis, but was associated with the greatest inflammatory cell influx, suggesting that the prevention of thrombosis does not depend on inhibiting vein wall leukocyte influx. Importantly, these effects were observed with rPSGL-1-Ig with no systemic anticoagulation, bleeding time prolongation, thrombocytopenia, or wound healing complications.

Direct selectin inhibition also effectively treats established stasis iliac DVT in a primate model. Two days after thrombus development, baboons were treated with rPSGL-1-Ig, 4 mg/kg, LMWH or saline, and treatment continued once weekly (rPSGL-1-Ig) or daily (LMWH, saline) based on drug half-life assessment [133]. The animals were examined and killed 14 or 90 days after treatment initiation. Percent spontaneous vein reopening was increased significantly in the proximal iliac vein in rPSGL-1-Ig- and LMWH-treated animals compared to controls (Fig. 3.7). There were no differences in inflammation between groups. At 90 days after thrombosis, recanalization with iliac vein valve competence was found in the rPSGL-1-Ig- and LMWH-treated animals. Thus, rPSGL-1-Ig successfully treated established DVT as well as LMWH. The most recent studies with P-selectin inhibitory aptamers for the prophylaxis and treatment of DVT are consistent with these observations (unpublished data).

We have also documented the ability of an oral P-selectin inhibitor (called PSI-697) to inhibit thrombosis and inflammation. In a rodent model of stasis-induced venous thrombosis, PSI-697 preserved normal vein wall function after thrombosis compared to the LMWH [107]. In the primate iliac vein stasis occlusion model, the oral P-selectin inhibitor begun 3 days prethrombosis improved vein patency over a

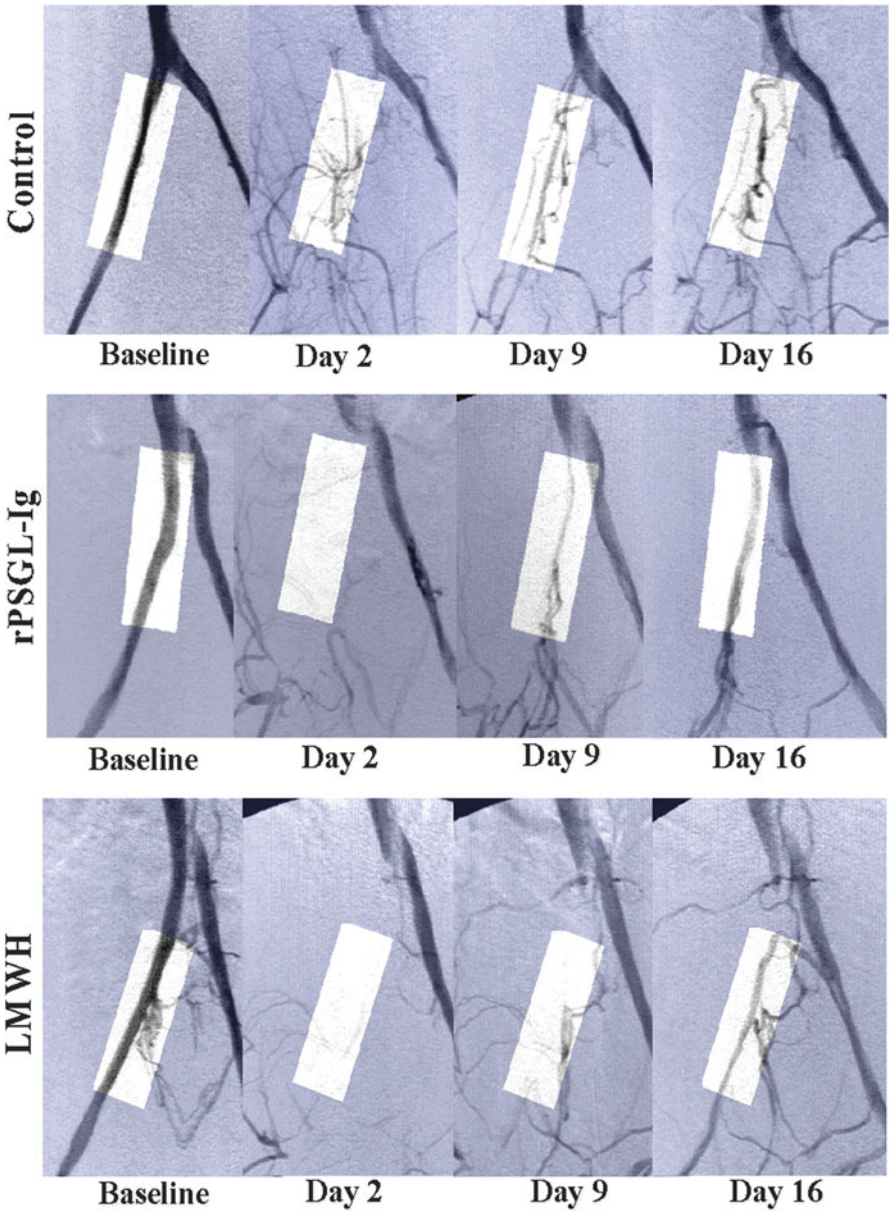

Fig. 3.7 Thrombus resolution in the primate following LMWH or recombinant PSGL-1 (rPSGL-1) treatment. Representative venographic examples at baseline, day 2, day 9, and day 16 from animals treated with saline (control), rPSGL-1-Ig, or low-molecular-weight heparin (LMWH). Note the improvement in recanalization in the rPSGL-1-Ig-treated animal, compared to the saline control and even the LMWH-treated animal. The *unshaded rectangle* indicates the evaluated area where the thrombus was created by temporary balloon occlusion. Reprinted from [133] with permission from John Wiley & Sons Inc

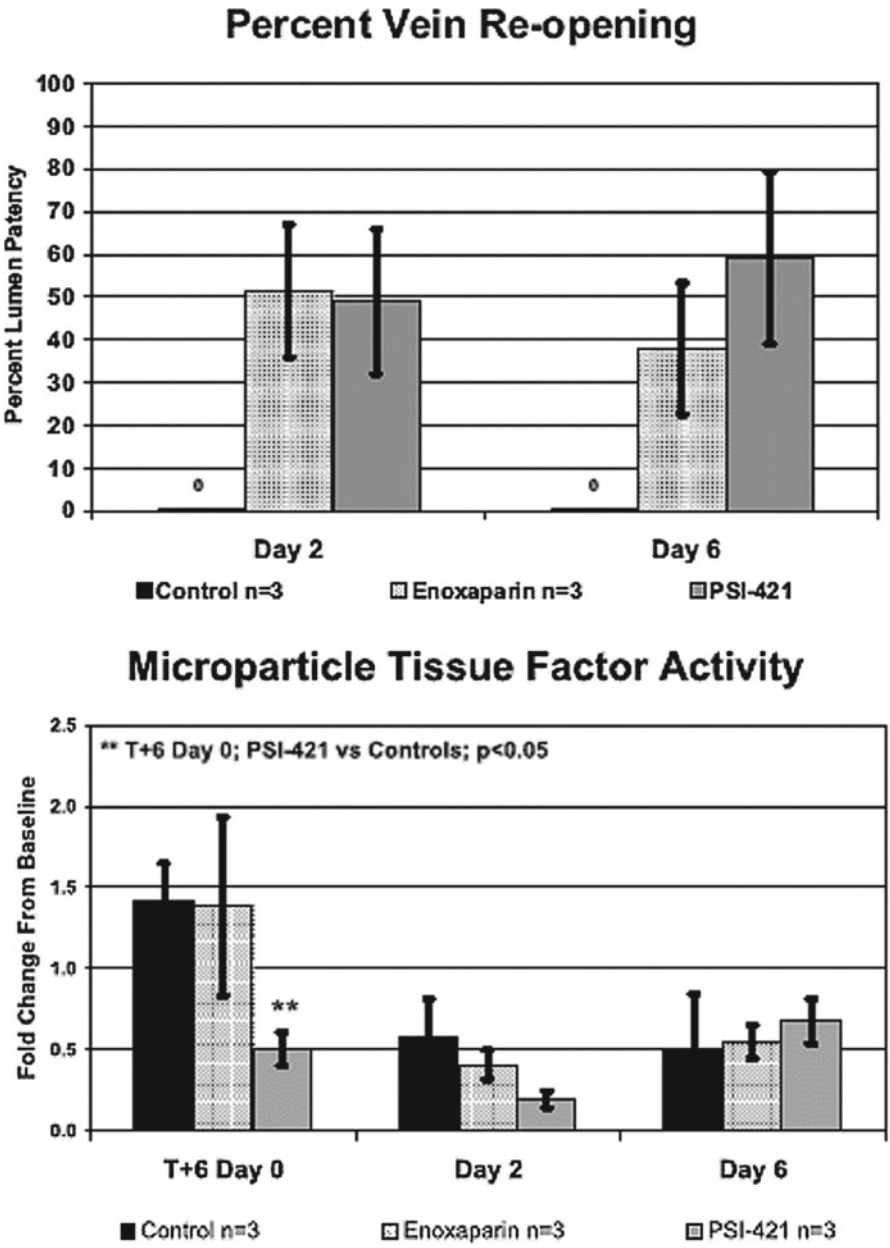

Fig. 3.8 An oral P-selectin inhibitor decreases microparticle tissue factor activity and enhances vein recanalization. Matched proximal and distal sections of thrombosed vein from a baboon model of DVT were evaluated for percent patency posttreatment with either low-molecular-weight heparin or a P-selectin inhibitor, PSI-421, by venography (*left graph*). Fold changes from baseline values of microparticle tissue factor activity normalized to total microparticle number. Animals receiving PSI-421 had a significant reduction in microparticle tissue factor activity compared to controls. T+6 day 0 = 6 h after thrombogenesis; $^{**}p < 0.05$. No difference was observed at the day 2 and day 6 time points (*right graph*). From [135] with permission from John Wiley & Sons Inc

6-day period better than LMWH, again with no change in coagulation function [134]. In this study, LMWH was given in a treatment dosage, although begun pre-thrombosis. Vein wall inflammation was decreased, as measured by MRI after gadolinium administration in response to LMWH.

Similar findings were demonstrated with another oral P-selectin inhibitor, PSI-421. Primates treated with PSI-421 had greater percent vein reopening and less vein wall inflammation than the low-molecular-weight heparin-treated and nontreated controls at day 6 (Fig. 3.8) [136]. Microparticle TF activity (MPTFA) was significantly lower in the animals receiving PSI-421 as measured immediately after thrombosis (6 h post thrombosis on the day of thrombus formation, day 0) suggesting lower potential for thrombogenesis in these animals. PSI-421 also reduced soluble P-selectin levels versus controls at 6 h post thrombosis on day 0, and on days 2 and 6. Experimental animals in all groups showed no coagulation defects, as suggested by normal bleeding time, normal prothrombin time, and normal activated partial thromboplastin time. This study was the first to demonstrate a reduction in MPTFA associated with vein reopening and reduced vein inflammation due to oral P-selectin inhibition in a baboon model of DVT.

Improved spontaneous thrombolysis in animals treated with rPSGL-1-Ig is similar to results found in primate, porcine, and rat models of arterial and venous thrombolysis using other methods of P-selectin inhibition [45, 133, 137, 138]. This protective effect is likely due to reductions in leukocyte–platelet interactions that lead to TF release and fibrin deposition, as these are P-selectin dependent [139]. Thus, P-selectin blockade inhibits leukocyte–platelet, leukocyte–endothelial cell, leukocyte–leukocyte, and even platelet–endothelial cell interactions; all actions that potentially decrease thrombus amplification after its initiation.

In the Clinic: P-Selectin and MPs in Patients with Deep Vein Thrombosis

Endothelial- and platelet-derived MP are elevated in patients with DVT [140]. However, MPs have been found to be present in healthy individuals and may have an anticoagulant function by promoting the generation of low amounts of thrombin, which activates protein C, supporting protein C's anticoagulant function [141]. These findings suggest that MPs have a steady-state physiologic level and that they become elevated with thrombosis.

Elevated levels of soluble P-selectin have also been associated with DVT [139, 142–144]. Most recently, a study of soluble P-selectin levels in patients with many different thromboembolic conditions found that the level of soluble P-selectin in DVT patients before beginning heparin treatment was 88.7 ± 41 ng/ml; 7 days after heparin therapy it was 54.5 ± 28.9 ng/ml; and in 30 control patients it was 22.1 ± 8.0 ng/ml [144]. Such data support the association of both MPs and soluble P-selectin with the formation of DVT.

We have also investigated the use of a combination of biomarkers to improve the ability to predict DVT. Currently, the serum marker D-dimer can reliably exclude DVT in the presence of a low clinical probability [145, 146]. However, no marker or combinations of markers improve upon the specificity of D-dimer (approximately 50%) for the diagnosis of VTE. Thus, diagnostic duplex imaging is the only practical means to diagnose DVT today and likewise spiral CT imaging to diagnose PE. Unfortunately, such imaging is not always immediately available, is costly, and is labor intensive. Recently, 62 positive and 116 negative DVT patients prospectively received a duplex scan, were evaluated for sP-selectin, D-dimer, CRP, and MP (total, leukocyte and platelet-derived and TF-MPs) concentrations, and assigned a clinical Wells score [147]. The biomarkers, or clinical scores, that differentiated DVT positives from negatives were sP-selectin, CRP, and Wells' score. With respect to the MP analysis, only platelet-derived MP differentiated DVT positive from negative patients. Using multivariable logistic regression, a combination of sP-selectin and Wells score could establish the diagnosis of DVT (cut-point ≥90 ng/ml + Wells ≥2), with a specificity of 96% and positive predictive value (PPV) of 100%, and could exclude DVT diagnosis (cut-point ≤60 ng/ml and Wells <2) with a sensitivity of 90%, a specificity of 33%, and a negative predictive value (NPV) of 96%. This study established a biomarker and clinical profile combination that can both confirm and exclude the diagnosis of DVT.

Finally, two human studies have solidified the importance of P-selectin in venous thrombogenesis and have suggested that elevated levels of sP-selectin are a marker for VTE. In the first of these studies, a case–control study of 116 consecutive patients and 129 matched controls underwent measurement of sP-selectin. These were patients with recurrent idiopathic VTE. The mean time between the sP-selectin measurement and the first episode of VTE was 11.5 years, and between the last VTE and the selectin measurement was 2.6 years. Using a cutoff of 55.1 ng/ml, the odds ratio = 8.5 for patients vs. controls, and the odds ratio = 11 after adjusting for factor V Leiden, prothrombin 20210A gene variant, factor VIII, homocysteine, and body mass index. In those patients with a P-selectin variant that results in lower levels of sP-selectin, the mean level of sP-selectin was 31.3 ng/ml [148]. In a second study of 544 consecutive patients with VTE, 63 patients demonstrated recurrence. Those with recurrence had sP-selectin levels of 45.8 ± 16.4 mg/dl vs. 40.1 ± 13.3 mg/dl, $p = 0.006$. When utilizing a sP-selectin level >75th percentile, the probability of VTE recurrence was increased by 1.7-fold [149]. Both studies suggest that high circulating levels of sP-selectin are a risk factor for recurrent VTE.

Summary

Venous thrombosis is a common and significant public health issue for which the true triggering events are not well defined in humans. Recent work has shown that links between coagulation and the inflammatory system play an important role in DVT. Venous thrombosis is both triggered by inflammation and causes localized

inflammation in the thrombus and vein wall. The selectin-type leukocyte adhesion molecules such as P-selectin and their ligands bridge the interface of thrombosis and inflammation by mediating leukocyte, platelet, and endothelial interactions and processes that contribute to both thrombus formation and thrombus resolution.

The role of coagulation factors, especially TF and thrombin, are well established in venous thrombus formation. Physiologic anticoagulants and thrombolytics counteract the prothrombotic process, and the endothelial cell plays a major role in modulating the local prothrombotic milieu by the production of both pro- and antithrombotic factors. In contrast to arterial thrombosis, the contribution of the platelet to venous thrombosis is not as clear. Endothelial- and monocyte-derived procoagulant MPs contribute to venous thrombus formation.

Venous thrombus resolution is a complex process that involves plasma-derived thrombolytics, but that is also partly dependent on leukocytes. Thrombus resolution is a sterile inflammatory process that involves leukocyte processing of thrombus components including coagulation proteins and necrotic cells, a process that is mediated in part by Toll-like receptor signaling. Leukocyte-derived uPA may be central to thrombus resolution, but other leukocyte factors such as certain cytokines and MMPs contribute significantly. Monocytes are particularly important to the later stages of the resolution process. In experimental models, the postthrombotic vein wall remodeling is primarily dependent on the mechanism of thrombosis instead of the absolute thrombus size. Vein remodeling and healing following DVT require contributions from both vascular smooth muscle and endothelial cells.

Recent studies have begun to translate some of these new basic science findings in DVT into the clinical setting. These studies have demonstrated that novel biomarkers for DVT such as P-selectin may be useful to increase the specificity of DVT diagnosis, and that P-selectin inhibitors may be a potential therapy for a nonanticoagulant means of DVT prophylaxis and treatment.

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Chapter 4
The Ubiquitin Proteasome System in Endothelial Cell Dysfunction and Vascular Disease

Najeeb A. Shirwany and Ming-Hui Zou

Introduction

It is estimated that the human genome encodes for approximately 30,000 discrete proteins [1]. Distinct cell types (endothelial cells, neuronal cells, hepatocytes, etc.) express a subset of these proteins relevant to their specialized function. This cell-specific fraction, while smaller than the total possible, can still represent a sufficiently large number of protein molecules for a given cell type. Regulating the turnover of these proteins, depending on cell type, makes the task of understanding homeostasis challenging. These proteins differ not only in structure and function, but also in individual half-lives reflecting rate of turnover. The longevity of a protein is related to its role in the cellular machinery and its protein quality. Some structural proteins remain unchanged for years, while regulatory proteins may have half-lives measured in a few brief minutes. Thus, the turnover of proteins can vary by several orders of magnitude. Nevertheless, over time, cells tend to accumulate a large amount of expended, sometimes aberrantly folded and oxidized proteins that must be eliminated, degraded, and/or recycled. This process of protein quality control is not only fascinating because of its unresolved detail despite being studied for nearly half a century, it has also become critical for our understanding of human physiology and pathology. The majority of eukaryotic protein degradation is executed by the ubiquitin proteasome system (UPS), and therefore this system has attracted the greatest attention in the context of protein homeostasis [2]. As such, the UPS is part of the posttranslational modification of proteins that includes processes such as acylation, alkylation, glycosylation, hydroxylation, and nitrosylation. These post-translational modifications, while essential for protein homeostasis, if perturbed

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sufficiently, can also lead to pathological change. Indeed, anomalies in the UPS have been implicated in the pathogenesis of cancer, neurodegenerative diseases, myopathies, viral infections, and cardiovascular diseases. In the cardiovascular system, the role of posttranslational protein modifications has been the subject of intense study in the last few years. Accumulating evidence has strongly implicated the UPS in cardiovascular system physiology and in the pathophysiology of a number of cardiovascular diseases. This chapter will cover a comprehensive overview of the role of the UPS in the vascular system with its complex and highly dynamic pathophysiology.

Overview of the UPS

The UPS is the major nonlysosomal intracellular system responsible for degrading most proteins in cells, particularly those that are short-lived and tightly regulated [3]. The UPS degrades proteins in a multistep process: the first step involves the targeting of proteins and the second is their successive degradation by the 26S proteasome [4]. Attachment of ubiquitin is the main mechanism which tags proteins for degradation by the proteasome. The ubiquitin-activating enzyme (E1) activates the 76 amino acid protein ubiquitin in an ATP-dependent manner and transfers it to one of several ubiquitin-conjugating enzymes (E2). These ubiquitin-conjugating enzymes then deliver the activated ubiquitin to a ubiquitin ligase enzyme (E3), which specifically binds to the substrate protein to catalyze the covalent attachment of ubiquitin to a specific lysine residue in the protein. This process of adding the first ubiquitin to a substrate is called monoubiquitination. Subsequently, polyubiquitin chains are synthesized by repeating this last step, which transfers activated ubiquitin to the lysine residues of previously placed ubiquitin. Depending on the exact binding sites between consecutive ubiquitin moieties, the polyubiquitin chains allow differential recognition by ubiquitin-binding domains (DUBs) as well as deubiquitinating enzymes. The ubiquitin system has a strict hierarchical structure [5]. Only one ubiquitin-activating enzyme (E1) has been identified, which activates ubiquitin for all modifications. The human genome is estimated to encode more than 20 ubiquitin-conjugating enzymes (E2s), and for more than 1,000 ubiquitin ligases (E3s).

Proteins are marked for degradation by the proteasome when they are covalently linked with a chain of ubiquitin proteins. The 26S proteasome complex is a multimeric protease, which consists of a catalytic core called 20S, and to regulatory particles called 19S. The 19S particles allow binding of the substrate to the proteasome complex. These particles consist of a ring of ATPases that are essential for the unfolding of the substrate and for the opening of the entry pore of the 20S complex. The barrel-like 20S complex has twofold symmetry and is composed of four stacked rings (2α and 2β rings) of seven subunits [6] (Fig. 4.1). Polyubiquitylated proteins bind to specific DUBs that are located in shuttling factors and the proteasome itself [7]. Once the unfolded protein enters the 20S complex of the proteasome, it is

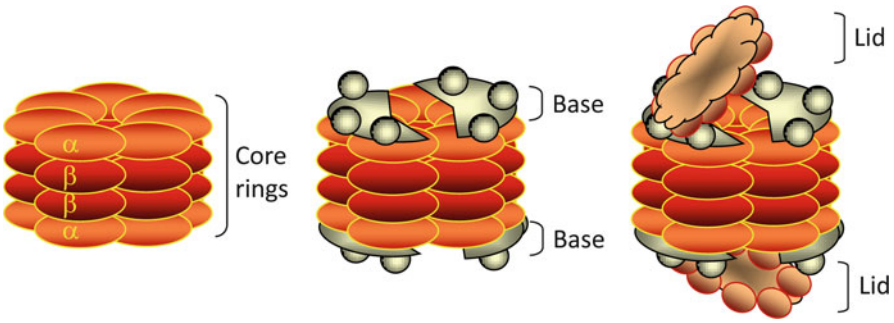

Fig. 4.1 Schematic representation of the structure of 26S proteasome. The 26S proteasome is a 2,000 kDa multiprotein complex comprised of a proteolytic 20S core particle that is capped by one or two 19S regulatory particles (RPs). The 20S–19S complex is termed the 26S proteasome. The 19S regulatory units recognize ubiquitinated proteins and control access to the proteolytic core

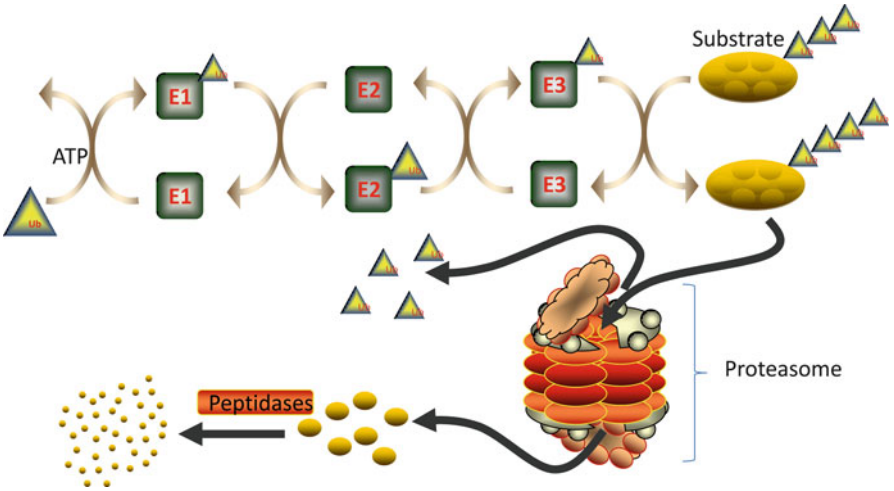

Fig. 4.2 Proteasome-mediated protein degradation. This schematic represents stages of polyubiquitination of substrate protein. Ubiquitin (Ub) is activated by enzyme E1 and translocated to enzyme E2. In the last stage, E3 ligase conjugates Ub to the substrate protein. The protein must be polyubiquitinated for Ub-dependent protein degradation by the proteasome

degraded into fragments varying in length from 3 to 25 amino acids and the ubiquitin is released and recycled by the deubiquitinating enzymes (DUBs). Approximately 100 DUBs have been identified with varying functions including regeneration and recycling of ubiquitin, reverse ubiquitination of proteins, and cotranslational activation of precursor ubiquitin proteins [4] (Fig. 4.2).

The UPS in Vascular Endothelial Cell Dysfunction

By specifically posttranslationally modifying proteins with ubiquitin and targeting them for degradation, the ubiquitin proteasome machinery acts as a central regulator and gatekeeper of protein turnover. Predictably, therefore, anomalies and disturbances in this machinery can and have been discovered to give rise to pathological states. Thus, the UPS has been directly or indirectly implicated in the pathogenesis of human disease. Examples of the UPS involvement in disease include Angelman's syndrome characterized by severe mental retardation, which is caused by mutations in the ubiquitin ligase, E6–AP. Another example is Liddle's syndrome, which is characterized by severe hypertension due to a mutation in the PPxY motif of the kidney epithelial Na^+ channel (ENaC). The ubiquitin ligase Nedd4 is unable to interact with the mutant ENaC channel, leading to its destabilization and increased reabsorption of Na^+ and water, resulting in severe hypertension. Alterations in ubiquitination are observed in most if not all malignant diseases as well [8]. These are manifest as destabilization of tumor suppressors such as p53, and overexpression of oncogenes like c-Myc and c-Jun. In human uterine cervical cancer, for instance, the tumor suppressor protein p53 is thought to be degraded by the human papilloma virus oncoprotein E6 and the ubiquitin ligase, E6–AP [9–12]. In addition, UPS dysregulation has been associated with colorectal and breast cancer underscoring the fact that disturbed protein homeostasis involving this critical system can trigger wide ranging and potentially serious pathologies. The UPS plays a role in the diseases in the cardiovascular system as well. Emerging evidence supports the idea that the UPS is critical to endothelial function in particular and the cardiovascular system operation, in general. We next discuss the role of the UPS in the endothelial cell function and dysfunction in disease.

The Ubiquitin Proteasome and Notch Signaling

The UPS is involved in the regulation of the vascular network by regulating notch signaling. In an important study by Nie et al., LNX was shown to be an important member of RING-finger-containing proteins that function as E3 ubiquitin ligases [13]. In these experiments ectopic expression of LNX enhanced TGFβ1 induced E-cadherin suppression, vimentin expression, accumulation of extracellular matrix, and promoted cell migration of HK-2 cells. The E3 LNX also ubiquitinates Numb, targeting it for degradation, which then enables Notch signaling to continue unabated [14]. It was also discovered that Numb PTB domain-binding motif and a PDZ domain of LNX are required for efficient substrate recognition and ubiquitylation [14]. The authors concluded that the LNX PDZ domains may be involved in the recognition of transmembrane receptors, which may in turn be substrates for ubiquitination by LNX [14]. The ubiquitin ligase FBW7/Sel-10 also targets Notch and targets it for proteasomal degradation. Tsenematsu et al. performed in vitro

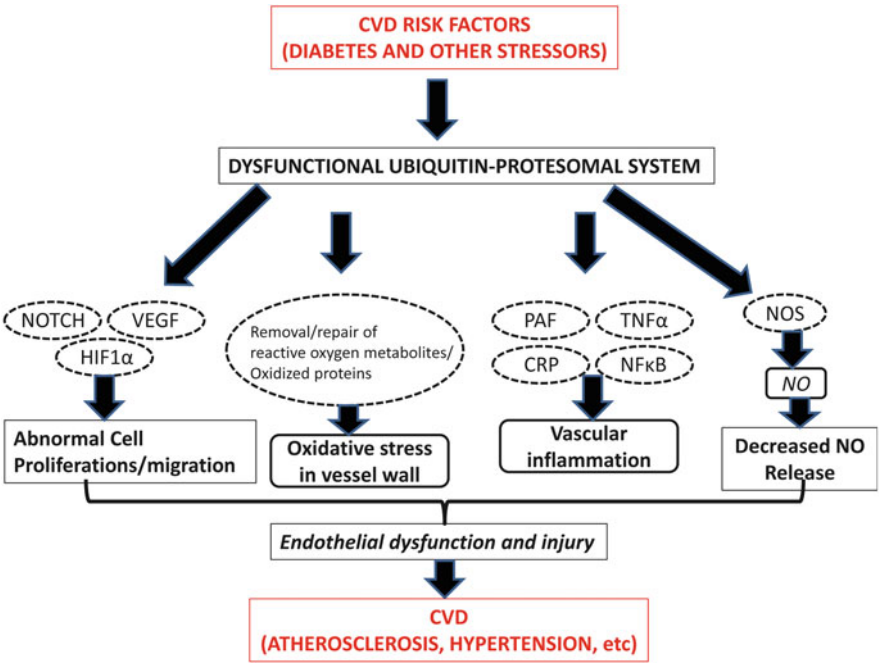

Fig. 4.3 The UPS appears to be positioned squarely in the center of processes that lead from metabolic stress on the endothelium to overt vasculopathy. In this hypothetical and idealized scheme, UPS derangement promotes heightened vascular tone leading to hypertension, manifestation of inflammatory change in the endothelium, oxidative stress in this cellular lining, and anomalies in vessel development. These cascading events are shown to lead to endothelial dysfunction, and through this disturbance, to the final outcomes of atherosclerosis, ischemia, infarction, etc

experiments on para-aortic splanchnopleural explant cultures from Fbw7$^{-/-}$ embryos and demonstrated an impairment of vascular network formation [15]. Thus, UPS regulation of Notch can occur in at least two independent ways providing for tight regulatory control of this signaling system (Fig. 4.3).

UPS Regulation of NO Through NOS

Nitric oxide (NO) and the enzyme that controls its synthesis, nitric oxide synthase (NOS), are fundamental to endothelial cell (EC) function. NO has a short biologic half-life, and its bioavailability is regulated by three isotypes of NOS: eNOS, iNOS, and nNOS. The endothelial cell (EC)-derived eNOS is constitutively expressed and generates NO at low levels in a Ca^{2+}-dependent manner [16]. eNOS is regulated by at least five different mechanisms in endothelial cells. These include (1) transcriptional regulation, (2) posttranslational modification, (3) interactions with proteins

such as Hsp90 and caveolin, (4) cofactor availability (like tetrahydrobiopterin 4; BH4), and (5) phosphorylation/dephosphorylation [17]. Details of posttranslational regulation of NO by the UPS are only beginning to emerge (Fig. 4.3).

UPS Regulation of eNOS

Recent studies have detailed mechanisms by which the ubiquitin proteasome regulates NO and eNOS. Recently it has been reported that the Hsp90 inhibitor geldenamycin can induce eNOS ubiquitination and subsequent degradation [18]. These studies also demonstrated that the ubiquitin ligase CHIP (C terminus of heat shock protein 70 interacting protein) assists in chaperoning eNOS in a ubiquitin-independent manner [18]; however, the study did not adequately preclude the possibility that the observed data were largely due to CHIPs role as a cochaperone. It has also been reported that the proteolytic degradation of eNOS may play a role in the endothelial dysfunction observed in postischemic processes, specifically the angiogenesis following ischemic injury in skeletal muscle, postischemic recovery in the aging myocardium, and postischemic reperfusion injury in the liver [4, 19–21]. Strangl and colleagues have reported that inhibition of the proteasome can modulate eNOS expression in a dose-dependent manner in a biphasic pattern: high doses of an inhibitor downregulated eNOS while lower concentrations had the opposite effect in ECs in vitro [4]. Therefore, the relationship between eNOS and UPS is dependent upon proteasome activity. Proteasome inhibition also causes the upregulation of eNOS expression in endothelial cells, thereby enhancing NO production and endothelial cell-dependent vasorelaxation in rat aortic rings [22]. When the tetrahydrobiopterin (BH4) levels in the endothelium are low, enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation. This results in generation of superoxide radical rather than the vasorelaxative NO, a phenomenon referred to as "eNOS uncoupling." eNOS uncoupling in turn is thought to be responsible for vascular oxidative stress and endothelial dysfunction. In this light, the regulatory role of the UPS in NO synthesis is critical in vascular function and pathology [23].

eNOS Phosphorylation Enhances Proteasome Activity

It has been demonstrated that increased NO-dependent phosphorylation of the transcription factor cAMP-response element-binding protein (CREP) elevates the activity of its promoter and induces the expression of the proteasome subunits LMP2 and LMP7. NO-dependent increase in proteasome activity also has been shown to attenuate protein kinase A (PKA) and protein kinase G (PKG) inhibitors, which can be reversed with transfection with antisense LMP2 and LMP7 oligonucleotides.

UPS Regulation of iNOS

iNOS is expressed in most vascular cells after induction with external stimuli such as lipopolysaccharide (LPS), proinflammatory cytokines, or shear stress [24, 25]. Enhanced expression of iNOS has been implicated in endothelial cell dysfunction, a process that contributes to vascular diseases. Principally this has been attributed to the induction of iNOS occurring in an oxidative environment, which leads to high levels of NO interacting with superoxide which forms peroxynitrite and induces cell toxicity [4]. iNOS activity can be controlled by the regulation of its synthesis, catalytic activity, and degradation. Degradation of iNOS by the UPS has been reported to be dependent upon the ubiquitin ligase/cochaperone CHIP (C-terminus of heat shock protein 70-interacting protein) [26]. Additionally, Osawa and colleagues have recently reviewed the evidence behind the induction by proteasome inhibitors (PSIs) and accumulation of iNOS, through inhibited degradation of IκBα, which prevents activation of NFκB [27]. Their key evidence came from cultured rat endothelial cells. The investigators subjected these cells to laminar shear stress in a parallel plate flow chamber [27]. When the cells were thus perturbed, the mRNA expression of iNOS was significantly elevated [27]. This increase could be completely attenuated when cells were pretreated with PDTC (an NFκB inhibitor) or MG132 (a potent inhibitor of the proteasome [27], indicating the role of the UPS in laminar shear stress-induced iNOS.

Proteasome Activity Regulates Vascular Inflammation

Several reports provide insight into how the UPS may affect specific components of the inflammatory response in the vasculature, thereby affecting the atherosclerotic process. Here we discuss how proteasome inhibition regulates regulatory T-cell responses, macrophage.

Regulatory T Cells

The UPS plays a role in modifying the function of regulatory T cells in attenuating the inflammatory and immune component of atherogenesis [3]. Regulatory T cells along with oxidized LDL, or uremic serum, cause a reduction in proteasomal activity, which ultimately leads to cell-cycle arrest and apoptosis [28]. In this study, the investigators demonstrated that patients with end-stage renal disease undergoing hemodialysis exhibited a reduction in the number of peripheral $CD4^+/CD25^+$ T_{reg} cells, which showed a reduced suppressor function. Considering the oxLDL effects, the relative percentage of $CD4^+/CD25^+$ T_{reg} of the total CD4 population was significantly reduced by incubation with oxLDL compared with a depleting effect

on $CD4^+/CD25^-$ T cells. The authors speculate that oxLDLs have a specific role on $CD4^+/CD25^+$ T_{regs}. Furthermore, $CD4^+/CD25^+$ T_{regs} from hemodialyzed patients exhibited early cell-cycle arrest and apoptosis. Mechanistically, these findings were linked with the oxLDL-inhibited proteasome proteolytic activity of p27Kip1 and Bax proteins, both of which accumulated in PHA-stimulated $CD4^+/CD25^+$ T_{regs} in vitro [28].

UPS Regulation of Macrophage

PSIs also reduce endotoxin-induced gene expression, including the Toll-like receptor 2, and can prevent LPS-induced inflammatory responses. When Qureshi and colleagues pretreated macrophage with the PSI, lactacystin, they found that in the RAW264.7 macrophage-like cell line, a dose-dependent inhibition of LPS-induced TNFα occurred. In addition, further pretreatment of LPS-treated macrophages with this inhibitor attenuated essentially all LPS-inducible genes measured including TNFα, IL-6, IL-12, p40, p35, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). Finally, lactacystin blocked the LPS-induced ERK phosphorylation but failed to inhibit interleukin-1 receptor-associated kinase 1 (IRAK-1) kinase activity. Lactacystin by itself triggered an increase in phosphorylation of p38 kinase and c-Jun N-terminal kinases (JNK), indicating that the proteasome's proteolytic activity may negatively regulate basal levels of activation of these two important signaling molecules [29].

Proteasome Inhibition Can Also Modify Expression of Inflammatory Mediators

TNFα stimulates inflammation and immune responses in part through the activation of NFκB. In plaques found in carotid arteries from human subjects, protein ubiquitination and 20S proteasome activity are correlated with the presence of TNFα and NFκB [30]. Suppression of the proteasome leads to increased vascular inflammation as well as negative outcomes in terms of severity of plaque formation. Plaques isolated from patients with an early morning blood pressure surge (a characteristic associated with atherosclerotic plaque instability and rupture) are reported to have increased protein ubiquitination and 20S proteasome activity, both of which are associated with increased NFκB [30]. These plaques also had increases in macrophages, T-lymphocytes, and TNFα, NFκB, and matrix metalloproteinase-9 (MMP-9), amongst others, as measured by immunohistochemistry and ELISA assay methods [30]. A recent study investigating the role of the UPS in atherosclerosis in rabbits reported that when the animals were treated with aspirin (acetylsalicylic acid), a common inhibitor of the 20S proteasome, not only were the atherosclerotic lesions reduced in size but the concentration of ubiquitinated

proteins were also enhanced in the atherosclerotic lesions [31]. In this study, New Zealand rabbits were placed on a normal diet or on a normal diet with aspirin or on an atherogenic diet, with or without aspirin for 12 weeks. Proteasome activity, plasma lipid concentrations, and peroxidation levels were measured [31]. In addition, ubiquitin, ubiquitin conjugates, IκBα, phosphorylated IκBα, and p65 expression levels were determined [31]. These investigators found that the concentrations of plasma lipids and peroxidation levels were higher in animals that were on an atherogenic diet or on an atherogenic diet with aspirin versus normal diet, with or without aspirin [31]. Severity of formation of atheromas was also increased in animals maintained on an atherogenic diet. In addition, ubiquitin and ubiquitin conjugates as well as IκBα were mainly localized in the subendothelium and the media of the aortic vasculature [31]. In addition, ubiquitin conjugates, IκBα, and phosphorylated IκBα were also increased in animals on an atherogenic diet. In this study, the activity of the 20S proteasome was robustly observed why that of the 26S proteasome did not appear to be affected across the groups. The overall conclusion by these authors was that the therapeutic effect of aspirin may manifest via inhibition of the proteasome [31]. While these are intriguing possibilities, further work in different species and/or models of mammalian atherosclerosis will have to be studied to validate the role of salicylic acid for its protective role as described here. Unfortunately, too many promising strategies fail to show benefits when more comprehensive empirical clues are sought or their benefits do not appear to hold up across the phylogenetic scale (Fig. 4.3).

Platelet-activating factor (PAF) is a potent mediator of inflammation and has been shown to be important in atherogenesis. It has been proposed by several investigators that oxidation of LDL in the circulation is dependent on several factors, which include diet and lifestyle. Although the synthesis of PAF is under tight regulation in the vasculature, dysregulated PAF synthesis has been observed following LDL oxidation. When PAF levels increase in an unregulated manner under the influence of oxidized LDL, then this molecule can initiate a rapid, local inflammatory response in the vasculature, which leads to damage of the endothelium and endothelial dysfunction leading to increased permeability-enhanced entry of circulating cells into the subendothelial space, formation of foam cells, and proliferation of smooth muscle cells, all being critical initiators in atherogenesis [32]. Its action is characterized by rapid desensitization which has been attributed it to downregulation of its receptor. PAF is a potent phospholipid mediator involved in atherosclerosis [33]. Following PAF stimulation, cells become rapidly desensitized via PAF receptor (PAFR) phosphorylation, internalization, and downregulation. PAFR downregulation has been reported to be dependent on proteasome-mediated receptor degradation [33]. Dupre and colleagues demonstrated that PAF-induced receptor endocytosis is a prerequisite for PAFR downregulation. They identified that PAFR receptor trafficking was independent of its ubiquitination. In their studies, ubiquitination of the PAF receptor was not ligand dependent. They examined the effects of the PSI and a lysosomal enzyme inhibitor (EST) on methylcarbamyl-PAF-induced PAFR degradation using COS-7 cells. When cells were pretreated with the PSI, the level of degradation was diminished by half and similar results were obtained with

clastolactacystin, another PSI. Overall, the study suggested that ubiquitination of the PAF receptor, in itself, was not sufficient for downregulation of the receptor. The authors conjectured that ligand-induced conformational change may expose the ubiquitin molecule and make it available for binding with a protein involved in intracellular sorting of the receptor [33]. In addition to PAF, C-reactive protein (CRP), which is also an acute phase respondent in inflammation, has been postulated to contribute to the formation of atherosclerotic plaques. In a recent study of LDL receptor-deficient mice, transgenic expression of human CRP was observed to reduce the size of atherosclerotic lesions along with increased expression of the 26S proteasome in aortic plaques [34]. The slower development of atherosclerosis in these mice was hypothetically, at least in part, linked with persistently high levels of proteasome activity (Fig. 4.3).

Several distinct pathways promote the activation of NFκB in response to inflammatory signals and other stimuli. With regard to UPS, ubiquitination plays several different roles in this process. These include targeting the NFκB inhibitor IκB for degradation by the proteasome [35–37] and the proteasome-dependent control of NFκB precursor proteins [38]. These mechanisms can be blocked or downregulated by specific DUBs [39]. In this manner, UPS can control NFκB activation at multiple levels. The NFκB dimer, which consists of the subunits p50 and p65, are present in the cytosol in an inactive state bound to inhibitory proteins collectively termed IκB. Activation of NFκB involves phosphorylation of the adjoined IκB, which is then recognized by a ubiquitin ligase which poly-ubiquitinates it, targeting it for degradation by the proteasome. This releases the inhibitory IκB, allowing nuclear translocation of the NFκB heterodimer which then induces the expression of NFκB-related genes. This process is induced by a variety of agents, including inflammatory or lymphoproliferative cytokines, microbial pathogens, and viruses [40]. Stimulus-induced proteolysis of IκB can then lead to activation of the NFκB transcription factor. In this way, the proteasome is intimately involved in the regulation of NFκB [40]. These findings are significant in the larger context of atherogenesis, despite the fact that the role of NFκB in this process is neither fully resolved nor widely accepted. However, NFκB and its target genes are involved in monocyte recruitment into subendothelial space. This event is a crucial milestone, because it is nearly impossible to reverse the progression of the lesion after this point [41] (Fig. 4.3).

The Proteasome Regulates Other ET-1, AngII, COX, and GPCR

Disturbances in endothelial function are also regulated by a variety of vasoactive factors, including endothelium-dependent relaxing factors (EDRFs) and endothelium-dependent contracting factors (EDCFs). In endothelial dysfunction, EDRFs levels decline, while EDCF expression increases, a phenomenon that increases basal vascular tone and increases blood pressure. The activity of the proteasome has been shown to be critical in these responses, particularly with respect to its regulation of endothelin-1 (ET-1).

In rats, it has been shown that treatment with PSIs decreases the level of ET-1 in the aortic wall. When Sprague Dawley rats were made hypertensive by using the deoxycorticosterone acetate-salt technique, a marked increase in aortic ET-1 concentration was observed in the hypertensive animals [42]. However, when these animals were administered a PSI [*N*-benzyloxycarbobyl-lle-Glu(*O*-*t*-Bu)-Ala-leucinal (PS-I)], the hypertensive phenotype was markedly attenuated [42]. The effect was accompanied by a decrease in aortic endothelin-1 content. The authors concluded that this indicated a proteasome-dependent proteolytic pathway that appears to play an important role in the enhanced production of endothelin-1 in blood vessels and the consequent increase in blood pressure in this model of hypertension.

Additional studies have found that proteasome inhibition regulates ET-1 in endothelial cells. When human umbilical vein endothelial cells (HUVECs) were treated with MG132, a potent and specific inhibitor of the 26S proteasome, expression of ET-1 was markedly reduced [43]. In this investigation, proteasome inhibition at nontoxic and toxic levels revealed a specific transcriptional response depending on the degree of proteasome inhibition in primary endothelial cells [43]. Furthermore, this study also demonstrated for the first time that partial inhibition of the proteasome at nontoxic doses of PSIs markedly attenuated the transcriptional response of ET-1 compared to toxic doses, with far fewer genes being regulated [43]. Proteasome inhibition resulted in the downregulation of ET-1 and its receptor ET_A [43]. Increased levels of vasorelaxing NO and decreased levels of ET-1 shifted the balance between vasoconstriction and vasorelaxation in favor of endothelium-dependent vasodilatation, which might contribute to improved vasorelaxation of aortic rings upon nontoxic proteasome inhibition [43], described in the previous paragraph.

The regulation of ET-1 by proteasome inhibition may be mediated by inhibiting TNFα. When rat aortic rings were treated with TNFα, the mRNA level of the ET-1 prepro-peptide was increased, indicating that TNFα regulates ET-1 expression [44]. When PSIs were given along with TNFα, the increase in ET-1 expression was completely blunted [44]. Specifically, Lorenz et al. demonstrated that incubation of rat aortic rings with TNFα resulted in dose-dependent reduction of acetylcholine-induced vasorelaxation [44]. Coincubation with TNFα and MG132 resulted in marked improvement in endothelium-dependent vasorelaxation compared to controls with TNFα alone [44]. Levels of eNOS mRNA and protein were attenuated despite improved vascular function following MG132 treatment [44]. The significance of these findings is that proteasome inhibition appears to prevent TNFα-induced vascular dysfunction. Lorenz and colleagues were also able to show that this protection from vascular dysfunction manifests via a reduction of superoxide production as well as the suppression of ET-1 levels [44].

Proteasome activity regulates the expression of angiotensin II (Ang II). Angiotensin II expression is enhanced in hypertension and has been implicated in endothelial dysfunction, through its induction ROS. Ang II has two native receptors, angiotensin I receptor and angiotensin II receptor (AT1 and AT2). When AT1 is stimulated, vasoconstriction occurs, via phospholipase-mediated second messenger

generation and also via coupling of AT1 to the prooxidant enzyme NAD(P)H oxidase [45, 46]. This pathway generates ROS and results in the formation of bradykinin as well as NO [47]. Intriguingly, at least in neurons, it has been shown that both AT1 and AT2 receptors undergo ubiquitination-dependent proteolysis. In these experiments, primary neuronal cultures established from the hypothalami and brainstems of Wistar–Kyoto rats exhibited angiotensin-induced downregulation of tyrosine hydroxylase (TH) activity under the influence of proteasomal inhibitors. The context of these experiments is that hypothalamic norepinephrine (NE) release regulates arterial pressure by altering sympathetic nervous system activity and angiotensin (Ang) decreases hypothalamic NE release. Ang diminishes TH enzymatic activity (the rate-limiting step in catecholamine biosynthesis) by reducing its phosphorylation. Angiotensin also downregulates TH expression by increasing its degradation through AT2 receptor activation by a proteasome-dependent pathway [48]. However, whether this phenomenon is also pertinent to vascular tissue is not certain. Xu et al. recently reported that when mice are infused with AngII, they found not only decreases the level of tetrahydrobiopterin (BH4; a critical cofactor for the biosynthesis of NO) and GTP cyclohydrolase I (GTPCH1; its rate-limiting enzyme), its effect can be abrogated by the administration of MG132 [49]. These studies indicate a number of mechanisms by which the proteasome regulates critical systems involved in endothelial cell function through its regulation of AngII signaling in endothelial cells.

Two other vasoactive compounds, endothelium-derived relaxing factor (EDRF) and EDCF = endothelium-derived contracting factor, contribute toward short-term, instantaneous changes in vascular tone and both of these depend on the activity of endothelial cyclooxygenase (COX). The mechanism of COX degradation is unclear; however, it has been demonstrated that COX is a substrate for the 20S proteasome [50]. In this interesting study, NIH/3T3 fibroblasts were employed and recombinant ovine COX and human COX were subcloned into a tetracycline inducible vector. Finally, fibroblasts that stably and inducibly expressed wild-type or mutant COX constructs were grown to confluence and used for the final data. The investigators showed that there may be two independent degradation pathways for COX-2, one involving the 26S proteasome and the other is triggered by fatty substrate oxygenation. These findings point, in part, to the fascinating complexity of protein homeostasis and the fact that UPS is only a part, albeit an important one, of the protein regulatory machinery of the cell.

Most of these vasoactive compounds such as AngII and ET-1 act by binding to specific G-protein-coupled receptors (GPCRs) on the surface of endothelial cells. It is possible that the proteasome-mediated regulation of these proteins occurs at the level of the receptor. The proteasome has been shown to play a role in GPCR turnover and regulation as well as signaling [51]. Overall, multiple loci in GPCR-initiated signaling pathways are targeted for regulated ubiquitination. GPCR-stimulated Ins(1, 4, 5)P3 receptor ubiquitination mediates its rapid proteolysis via the endoplasmic reticulum-associated degradation (ERAD) pathway. The effect of GPCR ubiquitination appears to be the modulation of GPCR endocytosis and trafficking to lysosomes for degradation. The first reported role for ubiquitination in endosomal

trafficking of GPCRs was the demonstration that this process facilitated the efficient endocytosis of the Ste2p in the budding yeast *Saccharomyces cerevisiae*. Hicke and Riezman showed that Ste2p undergoes significant ubiquitination following pretreatment with its physiological agonist, α-mating factor and further showed that ubiquitination of a single lysine residue within the C-terminal internalization signal was required for endocytosis of Ste2p [52, 53]. The physiological relevance of this ubiquitination process appears to be homeostatic in that it suppresses signaling in the face of persistent GPCR stimulation [54, 55].

The UPS and Vascular Oxidative Stress

There is a large body of evidence that implicates ROS in the pathogenesis of endothelial dysfunction. One of the underpinnings of endothelial dysfunction is excessive generation of ROS on one hand, and failure of antioxidant mechanisms in the endothelial cell on the other hand. Such an imbalance is also observed in cell types that make up the vascular wall, including smooth muscle cells. This ROS stress results from the impact on cells by free radicals that include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, hypochlorous acid (HClO), NO, and the peroxynitrite ($ONOO^-$). The generation of these free radicals is a consequence of oxygen metabolism. Under physiological conditions, these ROS serve some essential functions like growth stimulation and proliferation. However, when antioxidant defenses have been breached, these molecules collaborate in the oxidative modification of proteins and lipid membranes and can damage DNA. The UPS serves to control the rate of repair of oxidative damage and removal of reactive oxygen metabolites [3]. Approximately 90% of oxidized proteins are degraded by the 26S proteasome [56]. Therefore, the proteasome plays a critical role in the response to increased ROS, a common endpoint created in vascular disease, such as atherosclerosis (Fig. 4.3).

There is some evidence supporting the role of the proteasome in protecting against vascular stress. In a study of human endarterectomy specimens, taken to clear patients of severe atherosclerosis, the degree of oxidative stress correlated with proteasome activity in unstable plaques [57]. In this study, the content of ubiquitin conjugates in plaques from patients with symptomatic atherosclerosis in the carotid vasculature was found to be significantly higher when compared with asymptomatic patients and is correlated with the expression of 3-nitrotyrosine, and NADPH-oxidase p67 expression and with the percentage of cells undergoing apoptosis [57]. In this study, symptomatic patients had more unstable plaques and displayed an imbalance between the accumulation of ubiquitin conjugates and proteasome proteolytic activity, which was found to be reduced [57]. Similarly, when pigs were placed on a high cholesterol diet, an increase in oxidative stress parameters was found in the coronary arteries [4]. Thus a central role can be assigned to the interaction of the UPS and oxidative stress in the vasculature. Treatment with proteasomal inhibitors induces a stress response in the endothelium-characterized

upregulation of antioxidant defenses. However, it has also been noted that longer term treatment with these inhibitors may have deleterious effects and may even exacerbate oxidative stress in the endothelium [4].

The UPS in Vascular Development and Vascular Pathologies

Vascular Development

Vascular development begins when angioblasts derived from the mesoderm differentiating and forming vessel-like structures in a process called vasculogenesis [3]. Vasculogenesis provides an endothelial cell framework over which smooth muscle cells can proliferate and form the tunica media. Angiogenesis, or the process of forming new vessels from existing ones, parallels many of the mechanisms that are found in vasculogenesis. Both vasculogenesis and angiogenesis are guided by many signaling systems and pathways and include NOTCH signaling, activity of vascular endothelial growth factor (VEGF), and hypoxia-inducible factor 1α (HIF1α), among many others [58, 59]. The UPS plays an intimate role in all of these signaling systems. For example, the canonical Delta/Serrate/LAG-2 (DSL) ligands are responsible for the majority of NOTCH signaling. These DSL ligands are potential substrates for ubiquitination by E3 ligases. Several investigators have demonstrated that two distinct RING-containing E3 ligase families (neuralized 1 and neuralized 2, as well as Mind bomb 1 and 2) directly promote DSL ligand monoubiquitination and are required for DSL ligand endocytosis [60]. With regard to VEGF, a characteristic feature of one of its receptors, VEGFR2, is activation by sequential tyrosine phosphorylation and ubiquitination followed by proteolysis [61]. Analysis of VEGFR2 in primary human endothelial cells immortalized COS (monkey kidney fibroblasts), and human embryonic kidney (HEK) cells, has clearly shown that VEGF is regulated closely by ubiquitination and degradation [61] (Fig. 4.3).

Atherosclerosis

The UPS has also been reported to be involved in the progression of various stages of atherosclerotic disease. In the initial phases of atherosclerosis, lipid loaded cells in atherosclerotic lesions originate from blood-born monocytes and subsequently differentiate into macrophages. These macrophages engulf a large amount of lipids deposited in the subendothelium and take on the appearance of enlarged “foam cells.” Intense investigation has demonstrated that the number of scavenger receptors on the cellular surface of these cells are involved in this lipid influx [62]. This subendothelial migration is induced by the presence of bioreactive mediators called chemoattractants, in the tunica intima (inner most endothelial cell lining in the vasculature) [62]. Subsequent to this, smooth muscle cells invade this area from the media and transform it into an atherosclerotic plaque [62].

Proteasome activity can contribute to the activity of the foam cells through its regulation of adipose differentiation-related protein (ADRP). This protein is associated with lipid droplets inside the foam cells and a normally functioning proteasome is critical for the incorporation of this protein into foam cells. ADRP is induced during lipid accumulation in a variety of cell types, and its overexpression has been reported to inhibit the efflux of lipids and their accumulation in macrophages, a fact that has direct implications in atherogenesis [63]. It has been shown that expression of ADRP increases following lipid loading of human monocytes or macrophage-monocytic cell lines [64]. Additionally, ADRP mRNA has been detected in macrophage/lipid-rich areas of endarterectomy specimens [65, 66].

Proteasome inhibition inhibits the formation of neointima in the atherosclerotic process. When local balloon injury was induced in the rat coronary vasculature, the PSI lactacystin caused a marked reduction of neointimal formation [67]. This was accompanied by an upregulation of the p21 cyclin-dependent kinase inhibitor [67]. Treatment of vascular smooth muscle cells with lactacystin resulted in up to an 80% decrease in cell number compared with controls within 5 days of treatment [67]. In parallel with this change, there was an 86% decrease in S-phase entry of VSMCs and an increased level of the cyclin-dependent kinase inhibitor, p21 [67]. After 14 days of treatment post balloon injury, lactacystin significantly inhibited smooth muscle cell migration and resulted in a 59% reduction of the formation of neointima [67]. Barringhaus and Matsumara concluded that proteasome inhibition attenuated smooth muscle cell growth both in cultured cells and in an animal model of vascular injury likely via the upregulation of the p21. The study offered new insight in the role of the UPS in the vascular response to injury.

One of the consequences of atherosclerosis is vascular remodeling, a process that involves the turnover of the extracellular matrix and neovascularization. One important mediator of the turnover of the extracellular matrix is the cytokine transforming growth factor β (TGF-β). When TGF-β interacts with its receptor, it undergoes degradation by the proteasome [68]. Since the proteasome degrades the receptors, it would be hypothesized that proteasome inhibition would result in enhanced TGF-β signaling. However, experimental proteasome inhibition in animal models of atherosclerosis has actually found that proteasome inhibition reduces matrix metalloproteinase and collagen production in rat fibroblasts [69]. In addition to remodeling of the extracellular matrix, plaque neovascularization is another aspect of vascular remodeling in atherosclerosis. One of the central mediators of neovascularization is VEGF. VEGF expression is controlled by hypoxia-inducible factor I alpha (HIF1α), whose activity is regulated by the UPS [70]. HIF1α is a transcription factor composed of an α subunit that is stabilized under hypoxic conditions. Under normoxic conditions, HIF1α is hydroxylated by HIF prolyl-hydroxylases. When HIF1α is hydroxylated, it is recognized by the ubiquitin ligase von Hippel–Lindau (VHL), which poly-ubiquitinates it, targeting HIF1α for degradation by the proteasome [71]. Under hypoxic conditions, hydroxylation cannot occur, so HIF1α is not degraded and stabilized. When the HIF1α heterodimer binds to hypoxia response elements (HREs) in the nucleus, transcription of many genes involved in angiogenesis functions are initiated, such as erythropoiesis, iron metabolism, angiogenesis, and energy metabolism [72]. While the role of the UPS in regulating angiogenesis in

atherosclerosis is indirect, it likely plays a role in regulating oxygen sensing in tissues, a property that is particularly critical for the vasculature in response to atherosclerosis (Fig. 4.3).

The Therapeutic Potential of Manipulating the UPS in Human Vascular Pathologies

In view of the far-reaching impact of the UPS on diverse human vascular pathologies such as hypertension and atherosclerosis, it is provocative to consider that targeting the UPS in these diseases might be an extremely fruitful strategy. Although the exact relationships between UPS and human pathology are only just beginning to be understood, it is becoming clearer that the UPS must be considered a critical modulator of endothelial function as well as dysfunction, in view of its intricate interaction with several essential regulatory pathways. In a general sense, the bulk of evidence suggests that the UPS is functionally involved to varying degrees in the initiation, progression, and advanced (or complicated) stages of atherosclerosis. Inhibition of the system has shown some presumable advantageous effects in vascular smooth muscle function as well (as exemplified for example by the upregulation of eNOS and improvement in eNOS uncoupling; Stangl et al. [22]) which may have implications in hypertensive disease. However, bearing in mind that other studies have either produced ambivalent or even contradictory findings, some caution is warranted in the use of PSIs in cardiovascular disease and certainly more research is urgently needed to close this gap in knowledge (Fig. 4.4).

Recently, a number of substances have become available which can readily penetrate the plasma membrane and are targeted to inhibit the proteolytic function of the proteasome complex [73–76]. These compounds interfere with the nucleophilic attack of the regular proteasome substrates either by forming a transition state or by binding to the N-terminal active sites of the β subunits of the 20S proteasome. Either way, these inhibitors inhibit the chymotrypsin-like function of the proteasome [77, 78]. These compounds include both synthetic and naturally occurring drugs. Synthetic inhibitors that are widely used in experimental studies are reversible peptide aldehyde inhibitors (e.g., MG132), irreversible moronic acid inhibitors (e.g., PS-341), and vinyl sulfone inhibitors (e.g., NLVS) [78]. Naturally occurring molecules include lactacystin, epoxomicin, and PR-39 [78]. Given that the proteasome is involved in many critical cellular processes as well as implicated in a large number of human pathologies, PSIs are of great interest as potential therapeutic agents. Several PSIs have been used in clinical trials and are accepted treatments for some diseases. For example, a boronate inhibitor and a lactacystin inhibitor are currently in clinical trials for cancer and for stroke-associated ischemia reperfusion injury [79]. Several phase I trials have tested the use of PSIs in malignant disorders as well. Bortezomib (an N-protected dipeptide) for example has been used in trials with indications of activity in nonsmall cell carcinoma of the lung, androgen-dependent carcinoma of the prostate, mantle-cell and follicular-cell non-Hodgkin's lymphoma [80–83]. Bortezomib is also the standard therapy for multiple myeloma [84, 85].

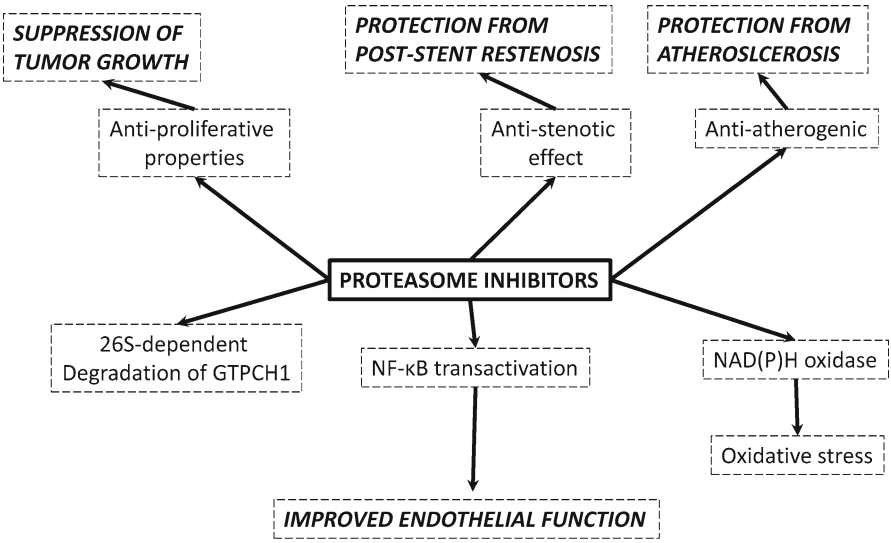

Fig. 4.4 Drugs (some of which are used clinically; e.g., metformin which might normalize UPS function via activation of AMPK) or compounds that inhibit the proteasome in a specifically targeted manner could theoretically exhibit therapeutic benefits in pathologies ranging from malignant disease to hypertension, and atherosclerosis. These pharmacological mechanisms are partly supported by recent empirical data discussed in the text

Thus far, most of the data relating to PSIs have been reported from cancer studies. In these studies, these compounds have been shown to exert a substantial antiproliferative effect in cellular systems, and this is generally been attributed to an increase in the activity of proapoptotic factors such as p53, p21, and Bax [86]. The impact of such drugs on the cardiovascular system is just beginning to be realized. One caveat has to be borne in mind and that is the significance of contrary reports that proteasome inhibition may lead to unwanted consequences and may even exacerbate the pathology that is being targeted. For example, reports from the Mayo clinic have shown that chronic inhibition of the proteasomal system may actually contribute to coronary atherosclerosis. In this study, female pigs were randomized into a group receiving a normal diet and one that received a proatherogenic high-fat diet. They were then treated with the PSI MLN-273. The data featured histological and immunohistochemical examination of the animal's coronary vasculature which exhibited accentuated oxidative stress and early appearance of atherosclerotic lesions [87]. Initial reports on the effects of PSIs in cardiovascular disease do indicate that proteasome inhibition might be an effective strategy to attenuate specific stages of atherogenesis such as the "proliferative phase" or the "progression stage" [78]. For example, a dose-dependent inhibition of proliferation and induction of apoptosis of vascular smooth muscle cells following treatment with PSIs and linked with decreased NFκB activation and increased p53 and p21 levels has been observed [88].

Studies also show that these drugs have the ability to attenuate restenosis after balloon angioplasty and implantation of endovascular stents for coronary artery disease [88, 89]. Animal models have also demonstrated that proteasome inhibition is a beneficial property in terms of the vascular disease severity. For example, in hypertensive rats, inhibiting the proteasome reduces ET-1 expression and improves endothelium-dependent vasorelaxation in vitro, in addition to increasing eNOS expression [22, 42]. It is also compelling to note that two of the most successful and widely used drugs that are the mainstay of cardiovascular medicine, aspirin and the statins, have both been shown to possess properties that inhibit the proteasome [90]. In order to improve the safety and efficacy of PSIs if they are to be used as drugs in the treatment of human pathology, more specific inhibition of UPS signaling processes have to be designed, and targeting earlier steps of the ubiquitin proteasome pathway will be more advantageous (Fig. 4.4). Such compounds are not yet available but with the growing amount of information about cellular substrates and components of the UPS, they may well become available in the near future.

Recent studies have demonstrated that increased AMPK activity inhibits proteasome activity in models of endothelial cell dysfunction to improve their function. AMPK is a very critical energy sensor in cellular systems and this is particularly true of the endothelial lining of blood vessels. For example, both the α1 and α2 isoforms of this kinase have been shown to be important in maintaining endothelial function [91]. Both AMPKα1 and AMPKα2 increase NO release by phosphorylating endothelial NO synthase at serine 1,177 and serine 635 in endothelial cells [92–95]. Recent studies have demonstrated that AMPK activation normalizes vascular endothelial function by suppressing 26S proteasome-mediated GTPCHI (which is the rate-limiting enzyme in biopterin biosynthesis) degradation in diabetes [96]. Consistent with these findings, Zou et al. have demonstrated that genetic deletion of AMPKα2 in low-density lipoprotein receptor knockout ($LDLr^{-/-}$) murine strain markedly increased 26S proteasome activity, IκB degradation, NFκB transactivation, NAD(P)H oxidase subunit overexpression, oxidative stress, and endothelial dysfunction, all of which were largely suppressed by chronic administration of MG132, a potent cell permeable PSI [91]. These studies demonstrate that AMPK-regulated proteasome activity is critical to endothelial cell function in animal models of atherosclerosis and may represent a novel mechanism by which proteasome activity is regulated in disease.

Pitfalls of Therapeutic Targeting of the Proteasome

There are several caveats that need to underscore any discussion of the therapeutic potential of targeting the UPS. First, proteasome inhibition can be proinflammatory (such as a proinflammatory response in neurons via COX-2 [97]), antiapoptotic, as well as anti-inflammatory (anti-inflammatory activity in HeLa cells [98]), depending on the cell system being studied. Second, differences in the response to

these drugs with regard to the stage and/or severity of the disease needs to be considered (such as the variable effect of Bortezomib in multiple myeloma [99]). Third, most if not all of these compounds lack pathway specificity in inhibition of the proteasome (e.g., the peptide aldehydes, MG 132, MG 115, and PSI, inhibit the proteasome complex's chymotrypsin-like activity in a potent but reversible manner but with low selectivity). However, this is not to preclude the fact that more specific and targeted molecules can be synthesized and developed (Fig. 4.4).

Knowledge Gaps and Future of Research on the UPS in Vascular Health and Disease

In recent years, the term "Degron" has been used to describe the very large variety of degradation signals that mark protein substrates for degradation by the proteasome. These recent studies have identified ubiquitin ligases that recognize structural motifs or degradation signals ("degron") within target proteins present within the substrate structure. A degron is a small segment of a protein sufficient for recognition and degradation. There are three components of the N-degron signal in eukaryotic proteins: (1) a destabilizing N-terminal residue; (2) its internal lysine residue where the polyubiquitin chain forms; and (3) the conformational areas around these components [3]. The identification of these degrons has been the subject of intense study. Though the general principles of substrate recognition have been investigated with great intensity, several fundamental questions remain. For example, there is limited knowledge about the structure of substrate-protein-bound E3 and E2 complexes in the context of ubiquitin transfer. Furthermore, the mechanisms of ubiquitin chain assembly on substrates are also not well understood. The current models of ubiquitin chain assembly remain to be tested with rigor. It is also not known if the attachment of ubiquitin chains of different linkages can result in differences in proteasome degradation and efficiency. The question of why different substrates are targeted directly to the proteasome while others are targeted to mobile adapter proteins remains an unresolved mystery. Research into answering these questions will allow a deeper understanding and a full comprehension of how the UPS is deployed and is linked with the myriad physiological pathways implicated in normal function as well as pathology. These components of the N-end rule pathway are essential for proper cardiac development as demonstrated by the severe cardiovascular abnormalities seen in UBR1- and UBR2-deficient mice [3] (a gene that binds to a destabilizing N-terminal residue of a substrate protein and participates in the formation of a substrate-linked multiubiquitin chain). This leads to the eventual degradation of the substrate protein. The protein described in this record has a RING-type zinc finger and a UBR-type zinc finger).

Table 4.1 Summary of the regulation of the vascular cells and vascular disease by the ubiquitin proteasome system

Signaling/process	Substrate	E3	Function	Vascular effects/associated diseases
NOTCH	NumB	LNX	LNX degrades NumB leading to dysregulated NOTCH signaling	Endothelial dysfunction/ atherosclerosis
Nitric oxide (NO) signaling	eNOS/iNOS	Specifics uncertain	Posttranslational modification of eNOS by UPS/GTP cylohydrolase I degradation by UPS	Endothelial dysfunction/ atherosclerosis
Vascular inflammation	Proinflammatory molecules such as TNFα and NFκB	Specifics uncertain	Enhanced inflammation in vascular wall	Promoting atheroma formation, plaque formation, dysregulated vasocontractility leading to hypertension
Vascular tone	Vasoactive molecules such as Ang-II, ET-1, EDRF, EDCF	Specifics uncertain	Increased vascular tone	Hypertension
ROS signaling in the endothelium	3-Nitrotyrosine/NADPH-oxidase	Specifics uncertain	Increased oxidative stress	Atherosclerotic plaque instability
Vasculogenesis	NOTCH/VEGF/HIF1α	Neuralized 1 and 2/Mind bomb 1 and 2	Disrupted angiogenic response or unregulated response	Ischemia/malignant solid tumors
Endothelial function	Adipose differentiation-related protein (ADRP)/NRF2/ SOD1/HO1	Uncertain	Foam cell formation	Atherosclerotic plaque
Neuronal apoptosis	Heat shock proteins/caspases/ cytochrome C/NFκB/Tau (τ)	CHIP/Mdm2	Ubiquitin-like immunoreactivity in hippocampus	Neuronal loss/Alzheimer's disease

This table identifies the known substrates, the type of ligase, the physiological role, and the pathological relevance of the specific example

Summary

Several decades of exciting and innovative research has revealed that the UPS is critically involved in a large number of cellular processes (summarized in Table 4.1). This system is not only central in the cellular machinery involved in protein homeostasis, but also plays a pivotal role in a large variety of human pathologies. Empirical support for these ideas comes from the entire spectrum of biomedical science, including animal models of human pathology, epidemiological studies focusing on many different kinds of human disorders, and pharmacological research, cell biology research, and genetics and epigenetics work, to name a few. In this discussion, we have provided an overview of the importance of the UPS and protein homeostasis in the context of vascular pathology. In this regard, we have discussed the role of the system in endothelial dysfunction, atherosclerosis, diabetic vascular disease, hypertension, as well as other diverse pathologies which are thought to have a vascular basis. We have specifically described the involvement of the UPS in NOTCH signaling, NO signaling (with related details regarding NOS and its isoforms), and in regulating a variety of vasoactive substances (such as EDRF, EDCF, and endothelin). We also describe the role of this protein homeostasis system in oxidative stress in so far as this important biological phenomena affects the vascular system. We have also discussed UPS in terms of ischemia, ischemia reperfusion, and ischemic preconditioning phenomena. Thus, it could be concluded that inhibition of the proteasome may have very useful effects in preventing the initiation and progression of vascular pathologies such as atherosclerosis. We have touched upon the fairly significant gaps in knowledge about the system and its significant potential to be a future therapeutic target to combat important and costly human pathologies such as diabetes, atherosclerosis, and hypertension.

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Chapter 5
Molecular Imaging of Vascular Inflammation, Atherosclerosis, and Thrombosis

Dan Jane-Wit and Mehran M. Sadeghi

Introduction

Traditional Cardiovascular Imaging

Pathological changes in coronary artery anatomy are commonly used to guide management of patients with coronary artery disease (CAD) and other vascular diseases. To this end, anatomical imaging platforms like invasive angiography, contrast-enhanced computed tomography (CT) angiography, or magnetic resonance angiography (MRA) provide images of the vascular tree, allowing visualization of individual arteries. These techniques rely on detection of luminal stenosis (or dilatation). The severity of each lesion is estimated on the basis of anatomic size of the lumen compared to nondiseased artery segments. This information is used to guide therapy which may consist of medical management or invasive percutaneous or surgical revascularization. Despite providing highly informative data regarding arterial anatomy, angiography yields little information about the cellular constituents or tissue characteristics of the vessel wall. The presence of calcification is a notable exception which may be detected by invasive angiography or CT and provides information on the extent of atherosclerosis and potentially the likelihood of complications. High resolution anatomical images of

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the vessel wall may be obtained using intravascular ultrasound (IVUS) and more recently optical coherence tomography (OCT). Both techniques are invasive, i.e., require catheterization of arteries through small surgical procedures. Using IVUS, the "total vessel" and luminal areas as well as intimal thickness may be readily measured and aspects of vessel wall structure, including lipid core and calcification, may be identified [1]. OCT provides very high resolution (~10 μm) images of the vessel wall and can be used to define other aspects of atherosclerotic plaque structure, including the thickness of fibrous cap which is an important determinant of plaque stability. A major shortcoming of OCT is that its depth of imaging is limited to 1–2 mm [2].

Myocardial perfusion imaging (MPI) with platforms such as single photon emission computed tomography (SPECT), positron emission tomography (PET), magnetic resonance imaging (MRI), CT, or echocardiography assesses myocardial blood flow and defines the functional significance of coronary artery stenosis. Through detection of relative or absolute reduction in myocardial perfusion, ischemic territories (or those at risk for ischemia) can be qualified in terms of overall size, distribution, and severity. The size and severity of territories at risk for ischemia are functional indices that, similar to coronary anatomy, may be used to guide therapy. Importantly, the prognostic information on CAD obtained through physiological imaging with nuclear-based MPI is additive to the data provided by coronary angiography. Despite its strengths, MPI yields scant information regarding subcritical coronary stenoses or normal-appearing segments of the artery. Further, the stability of established atherosclerotic lesions cannot be assessed using functional indices provided in perfusion studies; hemodynamically significant but stable coronary lesions cannot be segregated from those which are imminently prone to rupture.

Molecular Imaging

By providing anatomic and functional data, traditional imaging provides valuable diagnostic and prognostic information, often in the setting of established and symptomatic vascular disease. Traditional imaging approaches are routinely used in clinical cardiovascular medicine (and basic research) and have contributed to the decline in cardiovascular mortality observed in recent decades. However, it is now well recognized that beyond the presence of flow-limiting stenosis, other aspects of vessel wall biology (e.g., vessel wall inflammation and matrix remodeling) are key determinants of propensity to vascular complications. Molecular imaging is an emerging field developed to address such limitations of traditional imaging. It refers to imaging techniques used to monitor and detect the spatial and temporal distribution of molecular or cellular processes in vivo [3]. Characterization, visualization, and quantification of the target process are inherent components of molecular imaging. Unlike traditional imaging which may rely on contrast agents with nonspecific (vascular or otherwise) distribution, in molecular imaging a

systemically administered probe specifically localizes to an area with a biologically relevant molecular or cellular process. The probe signal may be detected using one of the existing imaging modalities (platforms), including PET, SPECT, MRI, ultrasound, CT, or optical imaging. As described above, aberrancies in anatomy and physiology detected by classical anatomical and functional imaging are often late (and rather incomplete) manifestations of cardiovascular diseases. This limits the diagnostic and prognostic value of traditional imaging and restricts its use for biological studies. Molecular imaging can potentially overcome these constraints, thereby broadening the applications of noninvasive (or invasive) imaging studies. For example, rather than imaging the late consequences of atherosclerosis, various molecular and cellular events involved in the initiation and progression of atherosclerosis and development of its complications may be detected using specific probes. In aortic aneurysm, molecular imaging may identify small aneurysms at high risk for rupture. In cardiac transplantation, where late detection of graft arteriosclerosis is a major challenge to patient management, molecular imaging may identify the disease at an early stage where preventive and therapeutic approaches may be more effective. As such, widespread adoption of molecular imaging could lead to a paradigm shift in the management of many cardiovascular diseases. Molecular imaging may also serve as a powerful investigational tool for basic biological studies of cardiovascular diseases. Finally, by tracking the effect of therapeutic interventions in vivo, molecular imaging may facilitate cardiovascular drug development.

Challenges in Vascular Molecular Imaging

Molecular imaging is based on the premise that systemically administered probes (in trace amounts without detectable biological activity) can be selectively targeted to regions of interest to produce a specific signal strong enough for detection using an imaging platform. The abundance and accessibility of the target, properties of the ligand, and inherent capabilities of the imaging platform are key determinants of the feasibility and success of molecular imaging. In general, molecular imaging probes consist of conjugated ligands or substrates with high affinity, specificity, and selectivity for the target. The target should be abundant enough to generate a detectable signal. This is especially a challenge in vascular molecular imaging where the size of the target organ is a major limiting factor. Nevertheless, as described in the following sections, adhesion molecules, proteases, and scavenger receptors are examples of the classes of molecules which are highly induced in vascular pathology and have been successfully targeted for molecular imaging. A variety of ligands, including antibodies, peptides, and small molecules, have been used as the targeting moiety to generate highly specific and selective probes. Amplification of the signal has been achieved through a number of strategies, including intracellular sequestration of probe and the use of multivalent probes.

Enhanced endothelial cell permeability, such as that found in atherosclerosis, promotes access to subendothelial structures, and also can lead to nonspecific uptake of the probe which is a confounding factor in many imaging studies of the vessel wall biology. Due to close proximity of the vessel wall with circulating blood, the residual blood pool activity can mask a specific signal from adjacent structures. Alternatively, this can lead to false positive results secondary to changes in blood volume, for example, in the case of arterial aneurysm. These confounding factors underscore the importance of appropriate controls and careful analysis of the data in evaluating novel tracers for imaging vessel wall biology. Favorable probe pharmacokinetics are instrumental in vascular molecular imaging as high target-to-background ratios can only be achieved with relatively durable high-affinity binding to target molecules in concert with rapid clearance of nonbinding probes. Finally, cardiac motion is a hitherto unresolved major problem which has limited the application of molecular imaging to coronary pathologies. As shown in the following sections, this myriad of challenges has been met with varying degrees of success by investigators. Advances in technology and ongoing basic research combined with considerable clinical and pharmaceutical interest should facilitate the resolution of these challenges and ensure clinical translation of molecular imaging.

Molecular Imaging Platforms

In addition to their application in traditional anatomic and physiologic imaging, a growing number of imaging platforms are used for molecular imaging of the cardiovascular system. The strengths and limitations of each modality are reviewed in this section. A good understanding of these issues helps investigators and clinicians in selecting the most appropriate modality (or their combination) for each specific application.

Nuclear Imaging

Single Photon Emission Computed Tomography

Nuclear imaging techniques including SPECT and PET (discussed below) have functioned as the mainstay imaging platforms for molecular imaging for several decades. Both techniques use trace concentrations of radiolabeled probes (pM to nM) to provide a detectable signal. Similar to planar imaging, SPECT cameras detect the γ-rays emitted by radioisotopes such as ^{111}In, ^{123}I, and ^{99m}Tc. In SPECT imaging, true volumetric three-dimensional images are obtained which can be cross-sectioned in any direction. Because each radioisotope has a distinct energy profile, multiple tracers may be imaged simultaneously. This is especially important for imaging highly complex processes such as arthrosclerosis where a single tracer may not provide a full picture of the underlying biology. Compared to

other modalities discussed below, SPECT and PET are highly sensitive and widely available. A disadvantage of SPECT imaging is spatial resolution which is ~1 cm for traditional clinical cameras. For small animal imaging, microSPECT cameras equipped with pinhole collimators offer a resolution of ~1 mm for ^{99m}Tc [4, 5]. Like PET, SPECT exposes subjects to ionizing radiation.

Positron Emission Tomography

PET cameras detect high energy γ-rays generated after annihilation of positrons emitted by certain radioisotopes [6]. Clinical PET cameras intrinsically offer higher spatial resolution (4–5 mm) than SPECT cameras. However, for small animal imaging the free path of positrons before decay (~2 mm for ^{18}F) adversely affects microPET resolution relative to microSPECT imaging. Compared to SPECT, PET is an even more sensitive technique. In part, because of its high count statistics and good temporal resolution, PET imaging can provide absolute quantification of biological activity expressed as units of radioactivity per unit of volume. Unlike classical SPECT, PET may be used for dynamic imaging to delineate regional tracer flow or kinetics [7]. A disadvantage of PET imaging is the short half-life of PET radioisotopes (e.g., 2 h for ^{18}F), which necessitates close proximity to a cyclotron to generate imaging probes. The availability of positron emitting isotopes such as ^{11}C which may be incorporated into a probe without altering its physical and chemical properties is a major advantage of PET imaging.

Magnetic Resonance Imaging

In MRI an external magnetic field is applied to a subject, eliciting alignment with the magnetic field of certain nuclei (e.g., hydrogen) which normally spin in different directions. This alignment may be perturbed with a weak rotating radiofrequency pulse. The recovery of magnetization (relaxation) can subsequently be measured in either a plane parallel or longitudinal to the external magnetic field (T_1 relaxation time), or in a transverse plane (T_2 relaxation time). Inherent differences in relaxation times are exploited to generate contrast between different tissues. In the presence of contrast agents, e.g., gadolinium and iron oxide particles, the magnetic properties of the environment are altered, and a signal (whether positive or negative) is generated.

A major advantage of MRI compared to nuclear imaging is its high spatial resolution (25–100 μm) [8], which in combination with its excellent soft tissue contrast allows for combined anatomic and biologic imaging in one setting. This is especially important for imaging atherosclerosis where a combination of anatomical and biological features determine plaque vulnerability. The main limitation of MRI is low sensitivity [8]. As such, targeted imaging of the vessel wall often requires large micromolar concentrations of contrast agents which may potentially lead to untoward biological effects. Technical advances in probe development and alternative

imaging sequences (such as ^{19}F MRI) can potentially increase sensitivity, reduce background signal, and improve quantification capabilities of MRI [9].

Several probe platforms have been used for molecular MRI. Gadolinium shortens water proton T_1 relaxation times and generates a positive signal enhancement (white) on T_1-weighted images. Nanoparticle liposomes [10], lipid-encapsulated perfluorocarbon emulsions [11], and micelles [12] containing gadolinium chelates have been designed and tested in vivo for imaging of angiogenesis, thrombus, and atherosclerosis. Superparamagnetic iron oxide-based agents may be classified into different categories based on the diameter of the iron oxide core [13]. Iron oxide agents alter T_2 relaxation times, creating hypointense areas that appear black on T_2-weighted images. The negative enhancing signal associated with iron oxide uptake may be difficult to discriminate from negative enhancement associated with motion or flow artifacts. Signal loss is also heavily dependent on image resolution and regional contrast agent concentration. To overcome these limitations, several groups have developed "positive" MRI sequences (which generate white, hyperintense signals) to specifically detect sites of iron oxide uptake [14].

Ultrasound Imaging

Ultrasound imaging is based on the use of gas-filled microbubbles which resonate when exposed to ultrasound. The microbubbles are injected systemically and upon exposure to ultrasound waves become acoustically active, resonating at a frequency that can be detected using high-frequency sonographic probes. Selective accumulation of targeted microbubbles subsequently generates a bright (echogenic) signal at given regions of interest. Using antibodies and other ligands, these microbubbles are engineered to selectively bind to their targets [15]. A major advantage of ultrasound imaging is real time, inexpensive, and noninvasive imaging with relatively high spatial and temporal resolution. This facilitates serial imaging to monitor biological processes in vivo. The ability of ultrasound imaging to delineate anatomy further facilitates image acquisition and interpretation. A main limitation of ultrasound-based imaging is that the size of microbubbles limits its use in molecular imaging to endothelial or intravascular targets. Furthermore, high ultrasound frequencies required for high resolution vascular imaging are not optimal for microbubble detection [15].

Optical Imaging

Optical imaging, with its multitude of forms, is a highly versatile imaging modality that can be readily adapted for molecular imaging applications [16, 17]. The most common optical imaging technique for cardiovascular molecular imaging is fluorescence. Fluorescent imaging relies on fluorescent probes that upon excitation with external light emit light at higher wavelengths. These probes may fit into one of three categories: nontargeted (e.g., indocyanine green for angiography), targeted

(ligands conjugated with fluorochromes), or activatable. The fluorescence in an activatable probe is in a quenched state at baseline, and can be dequenched upon cleavage of a specific substrate by an enzyme. This approach is associated with less background than other categories of probes and can provide highly sensitive information about enzymatic activity [18]. Distinct excitation and emission spectra of different fluorophores allow for simultaneous imaging of multiple targets in vivo. Given the exquisite sensitivity of optical imaging, fluorescent probes may be used at low concentrations and detect targets in the picomolar range. Besides versatility and sensitivity, a major strength of fluorescent imaging is spatial resolution which, depending on the detection system, can be in the μm to mm range. Limitations of in vivo fluorescent imaging include tissue autofluorescence, light scatter (refraction of signal traversing through body tissue), and depth of penetration (millimeter to low centimeter range). In addition, the chemical environment in tissue may affect fluorescence, contributing to inaccuracies and difficulties in quantification of the fluorescent probe concentration in deep tissues. Some of these limitations are in part overcome with imaging in the near infrared (NIR) window (excitation between the wavelengths of 650–900 nm). NIR fluorescent imaging is associated with reduced autofluorescence and markedly less photon absorption by hemoglobin and other endogenous molecules. As such, deeper tissues may be imaged. A new imaging platform, fluorescence molecular tomography (FMT), aims at producing mathematically derived three-dimensional images and quantitative information on fluorescent probe concentration in deep tissues [18, 19].

Computed Tomography

Differential attenuation of X-rays by various tissues is the basis for CT imaging which is classically performed for high contrast-high resolution anatomical imaging. This, in combination with newer techniques, including multislice and dual energy imaging, can provide information on the extent of atherosclerosis, presence of calcified or low attenuation plaques, and remodeling in coronary arteries. As such, while not molecular imaging in its purest definition, CT imaging may identify aspects of plaque biology that determine its propensity for complications. Hybrid imaging with CT (PET/CT, SPECT/CT) is used for signal localization, attenuation correction, and partial volume correction for molecular nuclear imaging. A recent application of CT involves contrast containing nanoparticles for detection of macrophage phagocytic activity in atherosclerosis [20].

Vascular Biology Processes as Targets for Molecular Imaging

Vascular diseases share a set of critical overlapping basic biological processes. These processes, whether alone or in combination, may serve as targets for molecular imaging, and their imaging can provide important and often unique information on the initiation, progression, and response to therapy of vascular diseases.

Endothelial cell activation, inflammation, angiogenesis, matrix remodeling, apoptosis, and thrombosis are especially important in the pathogenesis of atherosclerosis. In parallel with advances in technology, considerable progress has been made in recent years in imaging these processes, and some of the approaches initially validated in animal models are at the cusp of entering clinical practice. The following section, albeit by no means exhaustive, reviews some of the key recent developments in molecular imaging of atherosclerosis, inflammation, and thrombosis with a focus on vascular imaging.

Inflammation is critical to initiation, progression, and instability of the atherosclerotic plaque [21]. Retention of low-density lipoprotein (LDL) in the vessel wall and endothelial cell activation promote the recruitment, activation, and accumulation of inflammatory cells in early atherosclerotic lesions. Lipid phagocytosis by monocyte-derived macrophages, release of free radicals, and the resultant generation of modified lipids in conjunction with local production of chemokines and cytokines sustain a proinflammatory milieu, which further promotes the development of atherosclerosis. Through outward remodeling, the lumen area remains preserved in even fairly advanced atherosclerotic lesions. However, with the progression of plaque, the lumen eventually narrows down to the degree that the lesion may become symptomatic. Symptoms of ischemia may also occur when plaques rupture or erode which then exposes blood to the procoagulant subendothelial structures, promoting focal thrombus formation and sudden reduction in blood flow [22–24]. Plaque rupture (and healing) also contributes to rapid increases in plaque size. The mechanisms of plaque erosion are poorly understood. However, it is now well established that plaque rupture is linked with the presence of high densities of inflammatory cells that through production and activation of various proteases weaken a thin fibrous cap. In addition to its key role in atherosclerotic plaque progression and rupture, vessel wall inflammation plays an important role in other vascular diseases. Aortic aneurysm and transplant vasculopathy are examples of other vascular diseases where the presence of inflammatory cells (including macrophages, T lymphocytes, and dendritic cells) is linked with pathogenesis of the disease and its complications.

While the presence of edema and certain patterns of light scatter (in the case of OCT [2]) may detect aspects of inflammation, targeted molecular imaging stands out as a uniquely powerful tool for imaging vessel wall inflammation. Endothelial cell activation, inflammatory cell recruitment, activation and death, protease activation, vascular smooth muscle cell (VSMC) proliferation, and angiogenesis are intertwined processes that contribute to or are the result of vessel wall inflammation. To facilitate the discussion, approaches to imaging each process are reviewed under a separate heading.

Endothelial Activation

The endothelium serves as an active barrier between blood and subendothelial cell structures. In response to injury or proinflammatory cytokines, endothelial cells

convert to a procoagulant, proadhesive phenotype and this barrier is disrupted. The disruption of endothelial cell barrier function may be detected based on passive diffusion of labeled macromolecules [e.g., ^{99m}Tc-polyclonal immunoglobulin (~150 kDa), Gd-DTPA-albumin (~70 kDa)] [25]. In atherosclerosis imaging, this passive diffusion promotes the delivery of both targeted and nonspecific probes beyond the endothelium.

Endothelial activation is an early step in the initiation of atherosclerosis. Vascular cell adhesion molecule-1 (VCAM-1) is inducibly expressed on the adluminal surface of activated endothelial cells during the acute, early stages of vascular inflammation [26]. Upregulation of VCAM-1 mediates leukocyte adhesion and their subsequent transmigration through the vessel wall. In addition to endothelial cells covering the lumen of arteries prone to atherosclerosis, a large number of endothelial cells are present within the neovessels (vasa vasorum) of atherosclerotic plaque and its vicinity. As such, selective expression of VCAM-1 on activated and dysfunctional endothelial cells has rendered VCAM-1 a natural candidate for visualization of inflammation associated with atherogenesis [27].

Several probes have been developed for targeted visualization of VCAM-1 expression using MRI or optical imaging [28, 29]. In one approach, microparticles of iron oxide (MPIO) which contain large amounts iron oxide were decorated with an anti-VCAM-1 antibody and selectively localized to sites of IL-1β-induced cerebral vascular inflammation in murine hosts. These sites could be conspicuously visualized noninvasively with MRI [28]. In another approach, peptide sequences with homology to $\alpha_4 \beta_1$ integrin, a native ligand for VCAM-1, were selected through phage display screening [30, 31]. These were incorporated into multivalent nanoparticles to generate probes detectable by MRI and optical imaging. One such probe, VINP-28, localized to aortic root atherosclerotic lesions in murine hosts and was able to discriminate response to statin therapy by MRI [31]. More recently, a VCAM-1-specific tetramer peptide labeled with ^{18}F was used to assess atherosclerosis and response to therapy in apoE$^{-/-}$ mice by PET [32]. Of note, these peptides have been selected against murine VCAM-1 and their binding to human VCAM-1 has not been demonstrated. In addition, a previous report has questioned whether there are sufficient numbers of VCAM-1 molecules for imaging on human endothelial cells [27].

Endothelial cell adhesion molecules are ideal targets for ligand-coated microbubbles, which can be detected by ultrasound imaging. As such, intercellular adhesion molecule-1 (ICAM-1) targeted microbubbles were shown to bind to activated endothelial cells [33]. Microbubbles coated with antibodies against VCAM-1, P-selectin, or their combination have been successfully used to detect endothelial activation in murine models of atherosclerosis (Fig. 5.1) [34, 35]. Other probes targeting adhesion molecules such as E-selectin, P-selectin, or ICAM-1 [36–38] have similarly been investigated as noninvasive means to detect acute cerebrovascular inflammation by MRI.

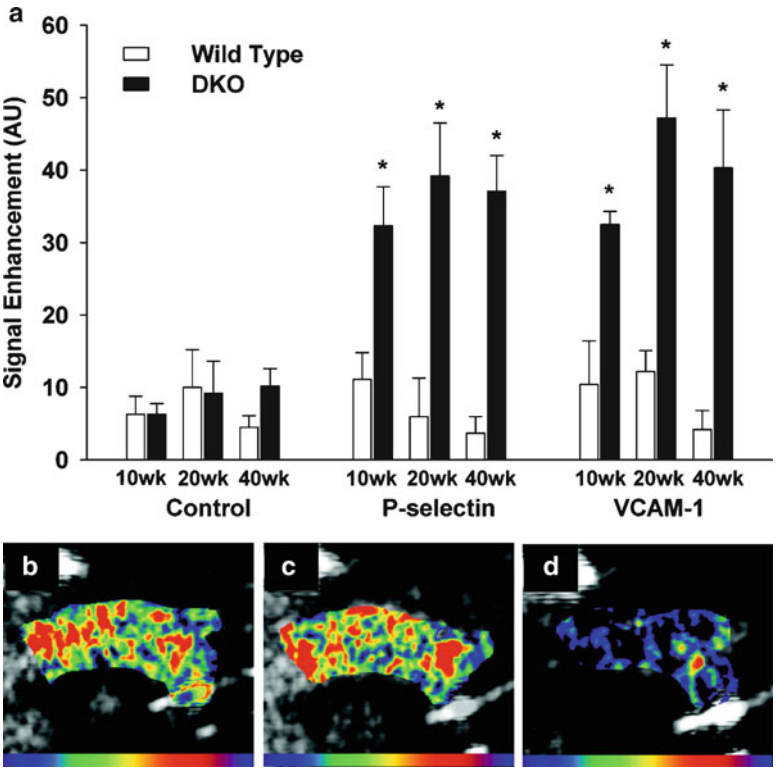

Fig. 5.1 Contrast-enhanced ultrasound images of aortic arch using VCAM-1-targeted (**a** and **b**), P-selectin-targeted (**a** and **c**) or control (**a** and **d**) microbubbles in wild-type (WT) or LDL receptor and apobec-1 double knock out (DKO) mice at 10, 20, or 40 weeks of age. (**a**) Background-corrected signal intensity in the proximal aorta, $^{*}p < 0.05$. (**b**–**d**) Examples of targeted ultrasound imaging in 40-week-old double knockout mice, demonstrating the presence of a high signal (in *red* and *yellow*) in the aortic arch with VCAM-1- (**b**) and P-selectin (**c**)-targeted probes. Reprinted from [35] with permission from Lippincott Williams & Wilkins

Lipid Accumulation

LDL and its modified derivatives play an important role in the initiation and progression of atherosclerosis, and plaque vulnerability has been linked with the presence of a large lipid-laden necrotic core [39, 40]. IVUS is an invasive tool used for assessing lipid core which appears as an area of echolucency on IVUS and its size has been linked with clinical events [41, 42]. However, echolucency is not specific for lipid core and may also be seen with intraplaque hemorrhage. MRI can characterize plaque components, including its lipid content with accuracy [39, 43]. More recently, CT angiography has been proposed for assessing plaque volume (in part related to the size of necrotic core) with relative accuracy [44].

Molecular imaging can complement these data by providing important additional information, including the presence of lipid modification and specific receptors in atherosclerosis. Radiolabeled LDL (e.g., with ^{125}I, or ^{99m}Tc) was one of the first agents used for plaque characterization in animal models of arthrosclerosis [45–47]. Due to slow clearance of the labeled probes, this approach was found to be ineffective for in vivo imaging and was abandoned. Other early approaches to imaging lipids in atherosclerosis included the use of labeled lipoprotein derivatives and oxidized LDL [48, 49]. Eventually, all these were abandoned for a targeted approach aimed at detecting lipid modification and receptors involved in lipid uptake in atherosclerosis. MDA2, an antibody raised against a neoepitope found on oxidized LDL, and other similar antibodies have been incorporated into various imaging probes. Labeled MDA2 localizes in atherosclerotic lesions in murine and rabbit models of atherosclerosis, and its imaging can track the effect of dietary intervention on plaque development [50–52]. Interestingly, in rabbit atherosclerosis, macrophage immunostaining is prominent in plaque areas with high MDA2 uptake, while areas with less uptake show stronger staining for collagen and VSMCs [52]. Targeted micelles incorporating MDA2 and other similar antibodies localize within macrophages of atherosclerotic plaques in apoE$^{-/-}$ mice [53, 54]. It is reported that membranes of cells undergoing apoptosis express higher levels of oxidized phospholipids, which may be targeted by oxidation-specific antibodies [55]. As such, micelles incorporating these antibodies may be used for in vivo assessment of complementary aspects of plaque biology by MRI.

Macrophage Biology

Radiolabeled leukocytes are traditionally used to identify sites of inflammation by nuclear imaging in humans. A similar approach was used to assess monocyte trafficking to atherosclerotic lesions by autoradiography and microSPECT imaging [56, 57]. Purified syngeneic monocytes labeled with ^{111}In-oxine were injected to apoE$^{-/-}$ mice and were detected in atherosclerotic lesions within 5 days after intravenous administration. Monocyte accumulation in the vessel wall increased with age of the mice and correlated with plaque area in animals of different ages and on different diets [56]. Importantly, microSPECT imaging demonstrated an approximately fivefold reduction in monocyte trafficking in animals treated with various statins [57]. Radiolabeling of leukocytes is clearly a powerful experimental tool for quantifying the trafficking of leukocyte subpopulations in atherosclerosis. However, changes in focal leukocyte numbers due to cell proliferation or apoptosis cannot be differentiated by this approach.

Activated macrophages display surface proteins that confer sensitivity to chemokines. One such surface protein, CCR-2, the receptor for macrophage chemotactic protein-1 (MCP-1), was exploited as a target to noninvasively visualize macrophages in atherosclerotic plaques. In a rabbit model of atherosclerosis, ^{99m}Tc labeled MCP-1 uptake (presumably by macrophages) in the balloon-injured

aorta could be visualized using SPECT imaging. Furthermore, tracer uptake quantified ex vivo correlated well with macrophage immunostaining in atherosclerotic lesions [58].

Exploiting the phagocytic phenotype of activated macrophages, investigators have used iron oxide particles as a selective probe for imaging macrophages using MRI [59]. Microcrystalline iron oxide particles coated with dextrans or siloxanes can be efficiently delivered to resident macrophages in atherosclerotic plaques at sufficient density as to generate negative MRI contrast on T_2- or T_2^*-weighted images. Initially tested in rabbit models of atherosclerosis [60, 61], the observation on negative plaque enhancement using superparamagnetic iron oxide particles has been extended to humans [62–64]. Importantly, in a study in symptomatic human carotid disease, ultrasmall particles of iron oxide (USPIO) accumulation in carotid arteries was detectable by MRI as early as 24 h and colocalized with areas of high macrophage content on endarterectomy samples [64]. The exact mechanism of ultrasmall particles of iron oxide uptake in plaque macrophages is not clear, and may involve transmigration of monocytes that have endocytosed the particles in the blood, or diffusion of ultrasmall particles of iron oxide into subendothelial space due to enhanced vascular permeability and their subsequent in situ uptake [59]. A number of confounding factors should be considered when using iron oxide particles for imaging macrophages in atherosclerosis. Superparamagnetic iron oxide uptake is not specific to macrophages or reticuloendothelial cells and can be seen by other cell types [59]. The size of the particles, their coating and charge and the cell type determine the mechanisms through which the particles are taken up by target cells. The extent of signal drop and concentration of iron oxide particles in the vessel wall may not be linear under every experimental condition. Finally, there are major differences in iron particle clearance between different species which should be considered in extrapolating observations made in rodents to humans [59]. The development of multimodal magnetofluorescent nanoparticles (MFNPs) provided an opportunity to directly investigate the localization of iron oxide particles. When injected to atherosclerotic apoE$^{-/-}$ mice, plaque macrophages represented ~65% of MFNP-positive cells with the rest consisting mostly of endothelial and VSMCs [65]. Similarly, a ^{64}Cu-labeled multimodal nanoparticle has been developed and validated for in vivo imaging of inflammation in murine atherosclerosis [66]. More recently, an iodinated nanoparticle contrast agent that is phagocytosed and accumulates in macrophages, N1177, has been developed and tested for CT imaging of atherosclerosis in rabbits [20]. CT enhancement using this agent correlates well with ^{18}F-FDG uptake and macrophage immunostaining [67]. It remains to be determined whether this enhancement is of sufficient magnitude for imaging human atherosclerosis.

Scavenger receptors are pattern recognition receptors that were initially identified based on their ability to mediate cellular uptake of modified (e.g., by oxidation or acetylation) LDL. They now include a diverse group of proteins categorized into eight classes based on their structural homology [68]. Over the years it has become clear that in addition to modified LDL, these receptors bind to many other ligands. Macrophages express several scavenger receptors, including scavenger receptor A (SR-A), CD36, CD68, and lectin-like oxidized LDL receptor 1 (LOX-1) [69].

Scavenger receptor AI (SR-AI), one of the first modified LDL receptors identified, is upregulated in the course of macrophage differentiation [70]. Based on this observation and low levels of SR-A expression in normal arteries, gadolinium-containing SR-A targeting immunomicelles were used for detecting macrophages in aortic wall of apoE$^{-/-}$ mice by MRI [12]. Consistent with SR-A expression pattern, much (but not all) of the immunomicelle uptake colocalized with CD68 positive areas and there was a strong correlation between gadolinium signal enhancement and CD68 staining.

LOX-1 is an inducible scavenger receptor that was originally discovered in endothelial cells [71, 72], but is also expressed on the surface of macrophages and VSMCs [73]. Using a ^{99m}Tc-labeled anti-LOX-1 antibody, atherosclerotic aortas could be visualized in Watanabe heritable hyperlipidemic rabbits by planar imaging [74]. In Watanabe heritable hyperlipidemic rabbit aortas, ^{99m}Tc-labeled anti-LOX-1 antibody uptake was twofold higher than the uptake of a control antibody. The difference was tenfold between ^{99m}Tc-labeled anti-LOX-1 antibody uptake in the aortas of Watanabe heritable hyperlipidemic compared to control rabbits, underscoring the magnitude of nonspecific tracer uptake in atherosclerosis. Tracer uptake in atherosclerosis correlated well with a vulnerability index defined as the ratio of lipid component area (macrophages and extracellular lipid deposits) to the fibromuscular component area (smooth muscle cells and collagen fibers) [74]. Similarly, a second probe targeted against LOX-1 has been validated for detection of atherosclerotic lesions in vivo [75]. This probe was designed for use across multiple imaging platforms and consists of a liposomal shell coated with anti-LOX-1 antibody, Dil (1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) fluorescent molecules and either gadolinium or ^{111}In. In vivo, this probe was shown to preferentially bind to macrophages and localize in the shoulder region of plaques.

Enhanced cellular metabolic activity mainly attributed to macrophages in atherosclerosis may be detected by ^{18}F-2-deoxy-D-glucose (FDG) PET imaging [76]. Cellular uptake of ^{18}F-FDG, a glucose analog, is mediated by glucose transporters. Once inside the cell ^{18}F-FDG is phosphorylated by hexokinase to ^{18}F-FDG-6-phosphate which cannot be further metabolized and is trapped inside the cell. Retained ^{18}FDG can be detected by PET imaging and ^{18}F-FDG-6-phosphate eventually decays to glucose-6-phosphate. ^{18}F-FDG uptake is seen in several leukocyte populations, including human monocyte-derived macrophages in culture, where uptake level is comparable to several cancer cell lines [77]. Hexokinase activity is considerably enhanced in activated macrophages [78] and macrophage activation leads to enhanced ^{18}F-FDG uptake [77]. Interestingly, ^{18}F-FDG uptake by human endothelial cells in culture is reported to be several folds higher than macrophages [79].

^{18}F-FDG uptake can be detected in rabbit atherosclerosis and the uptake correlates with macrophage density [80–82]. ^{18}F-FDG PET imaging following administration of Russell's viper venom to induce aortic thrombosis (presumably due to plaque rupture) in atherosclerotic rabbits showed slightly (albeit nonsignificantly) higher ^{18}F-FDG uptake in thrombosed areas [83]. Importantly, treatment with probucol (an antihyperlipidemic drug with antioxidant properties) led to a significant reduction in ^{18}F-FDG uptake in parallel with the reduction in macrophage infiltration

in WHHLMI rabbit aortas [84]. There are conflicting data on ^{18}F-FDG uptake in atherosclerotic mice, some of which may be related to the timing of imaging [85, 86]. It is reported that ^{18}F-FDG binds to sites of vessel wall calcification, presumably through nonspecific entrapment by hydroxyapatite [87].

Expression of integrins by inflammatory cells has raised the possibility that integrin imaging may be used to detect vascular inflammation. Arginine–glycine–aspartate (RGD)-based peptides bind to integrins with the flanking sequences determining selectivity for specific members of the integrin family. Integrin $\alpha_v\beta_3$ (discussed more in detail later in this section) is expressed at high level on monocytes and macrophages. ^{18}F-galacto-RGD, a probe targeting α_v integrins, localizes in areas rich in nuclei (presumably consisting mostly of macrophages) in murine aortic atherosclerosis [88]. Similarly, an optical probe RGD-Cy 5.5 was used to image lesions induced by carotid ligation in high fat-fed apoE$^{-/-}$ mice [89]. Histological analysis demonstrated colocalization of the tracer with macrophages in carotid lesions.

Matrix Remodeling

Extracellular matrix serves as an anchoring scaffold for cellular constituents of tissues. Matrix remodeling through synthesis, reorganization, and degradation of macromolecules plays a central role in the pathogenesis of vascular diseases. Matrix remodeling is an integral part of vascular remodeling, a process that involves persistent changes in vessel wall composition and geometry. The two components of vascular remodeling, geometrical remodeling, and changes in vessel wall composition play a more or less prominent role in specific vascular diseases. For example, in aortic aneurysm, expansive (outward) remodeling of the artery is most prominent, while in transplant vasculopathy, intimal hyperplasia may be considered the key pathological feature. In atherosclerosis both components of vascular remodeling play major and complementary roles in the development of an atherosclerotic plaque and its complications. Compared to those plaques with inward (negative, constrictive) remodeling, atherosclerotic plaques with outward (positive, expansive) remodeling have higher lipid and macrophage content, features that are associated with plaque vulnerability [90]. This association of remodeling with plaque composition (and propensity to rupture) has been repeatedly observed in other studies, including a study in patients with renal artery stenosis where the necrotic core was found to be larger in lesions with positive remodeling [91, 92].

In addition to its role in geometrical remodeling and intimal hyperplasia, matrix remodeling plays a direct role in plaque rupture. A recent prospective study in patients undergoing percutaneous coronary interventions for acute coronary syndromes (which excluded patients with any remaining lesion with diameter stenosis >50%) did not find any association between remodeling index (dichotomized around the median value of 0.94) and major adverse cardiovascular events (MACEs) over a 3-year follow-up period [1]. However, relevant to the discussion on matrix

remodeling, compared to plaques not associated with MACEs, those associated with MACEs were more likely to have been classified as thin-cap fibroatheromas (defined by radiofrequency-IVUS) in the initial analysis [1]. This is presumably because the thin-cap fibroatheromas are more prone to rupture. It is believed that reduced matrix protein synthesis secondary to VSMC paucity and enhanced matrix degradation due to high density of inflammatory cells weaken the fibrous cap and predispose it to rupture.

Matrix turnover is mediated by several families of proteases including adamalysins [93], matrix metalloproteinases (MMPs) [94], cathepsin cysteine proteases [95], and serine proteases [96]. Of these, MMPs and cathepsins have been used as targets for molecular vascular imaging. The MMP family consists of at least 23 secreted and membrane-bound zinc and calcium-dependent endopeptidases which may be classified into different classes based on their structure or preferred substrates [94, 97, 98]. Expression of collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), matrilysins (MMP-7), and membrane-bound MMPs (MMP-14) and other MMPs (MMP-12) has been reported in atherosclerosis and related diseases [98]. Collectively, these proteins are potent proteases that catalyze the degradation of connective tissue and extracellular matrix components including fibrillar and nonfibrillar collagens, elastin, and basement membrane glycoproteins.

Matrix Metalloproteinases

In addition to their direct effect on matrix proteins, MMPs regulate vessel wall biology through other mechanisms, including proteolytic cleavage and release of cytokines, chemokines, and growth factors. ECs, VSMCs, and inflammatory cells are the main sources of MMP activity in the vessel wall [94, 98]. A large body of evidence exists on the role of MMPs in the pathogenesis of atherosclerosis, and its complications, as well as other vascular diseases [98]. As discussed above, MMPs modulate plaque stability through regulation of extracellular matrix protein deposition and degradation, and also VSMC proliferation, and inflammation [94, 99, 100]. Different members of the MMP family have distinct effects on plaque biology. As such, atherosclerosis in apoE/MMP-12 double knockout mice shows features associated with plaque stability, i.e., increased VSMC and reduced macrophage content compared to mice deficient in apoE alone [101]. Similarly, VSMC content, a feature of plaque stability, is increased in apoE/MMP-7 double knockout mice. This is in contrast to the effect of MMP-3 or MMP-9 deletion which confer features of plaque instability to atherosclerotic lesions [101].

MMP activity is regulated at transcriptional and posttranscriptional levels. With a few notable exceptions, MMPs are in general produced as inactive secreted or membrane-bound proenzymes. MMP activation involves delocalization of the so-called MMP prodomain through enzymatic cleavage or allosteric displacement to expose the zinc-dependent catalytic domain [97]. Substrate specificity is in part determined by binding to sites other than the catalytic site (so-called exosites) which

may serve as targets for tracer development in the future. Interaction with other molecules including *t*issue *i*nhibitors of *M*M*Ps* (TIMPS) and compartmentalization are additional mechanisms of regulating MMP activity [97].

Two strategies have been developed for imaging MMP expression and activity in vascular diseases. The first strategy exploits the enzymatic activity of MMPs. In one approach, following enzymatic cleavage of a specific substrate, quenching fluorochromes are removed from an optical probe, generating a fluorescent signal detectable by optical imaging [102]. In theory, this approach allows for high specificity based on the substrate structure, low background activity, and considerable signal amplification in the presence of MMP activity. One such probe that incorporates a peptide sequence recognized by MMP-2 and MMP-9 was used to detect MMP activity in murine atherosclerosis. Ex vivo images demonstrated localization of the probe in macrophage-rich areas of atherosclerotic aorta [103]. A similar probe that can be activated in vitro in the presence of MMP-13, MMP-12, MMP-9, MMP-7, MMP-2, and MMP-1 was used to detect MMP activation in aortic aneurysm in fibulin-4 mutant mice (a model of Marfan's disease) [104]. Interestingly, in this model, tracer uptake in the aorta preceded the development of aneurysm. An alternative approach based on enzymatic activity involves the use of activatable cell-penetrating peptides. In this approach, cleavage of the substrate allows a cell-penetrating peptide to enter the cells [105]. While promising, this approach has not been tested for imaging vascular pathologies, and a recent report suggested that rather than occurring in the vicinity of target cells, activation of the peptide occurs remotely in the vascular compartment, indicating that it might not be optimal for vascular imaging [106].

An alternative strategy for imaging MMPs employs ligands, including MMP inhibitors, which selectively bind to MMPs. Based on such ligands, several radiolabeled probes, including ^{123}I-CGS 27023A, ^{111}In-RP782, and ^{99m}Tc-RP805, have been developed and used to image MMP expression in the vessel wall. When administered intraorbitally, ^{123}I-HO-CGS 27023A localized in ligated carotid arteries in apoE$^{-/-}$ mice yielding a signal that was detected by planar imaging [107]. A subsequent study using ^{111}In-labeled RP782, a tracer that specifically binds to the activation epitope of multiple MMPs, demonstrated considerable uptake of the tracer in murine carotid arteries following wire injury [108]. In this model, carotid injury leads to considerable neointimal hyperplasia over a period of 4 weeks, which predominantly consists of VSMCs. Tracer uptake was detectable in vivo by microSPECT/CT imaging by 2 weeks (and by autoradiography, a more sensitive technique, as early as 1 week) after injury. Consistent with the role of MMPs in vascular remodeling, RP782 uptake paralleled weekly changes in the cross-sectional vessel wall, but not total vessel or luminal, area [108]. In a follow-up study, serial microSPECT/CT imaging at 2 and 4 weeks after carotid wire injury with RP805, a ^{99m}Tc-labeled homologue of RP782, was used to assess the effect of dietary intervention on vascular remodeling. Tracer uptake (reflecting MMP activation) in injured arteries was reduced as early as 1 week after withdrawal of a high fat diet. Furthermore, MMP activation in the vessel wall detected by noninvasive imaging at 2 weeks after injury correlated well with neointimal area at 4 weeks in the same

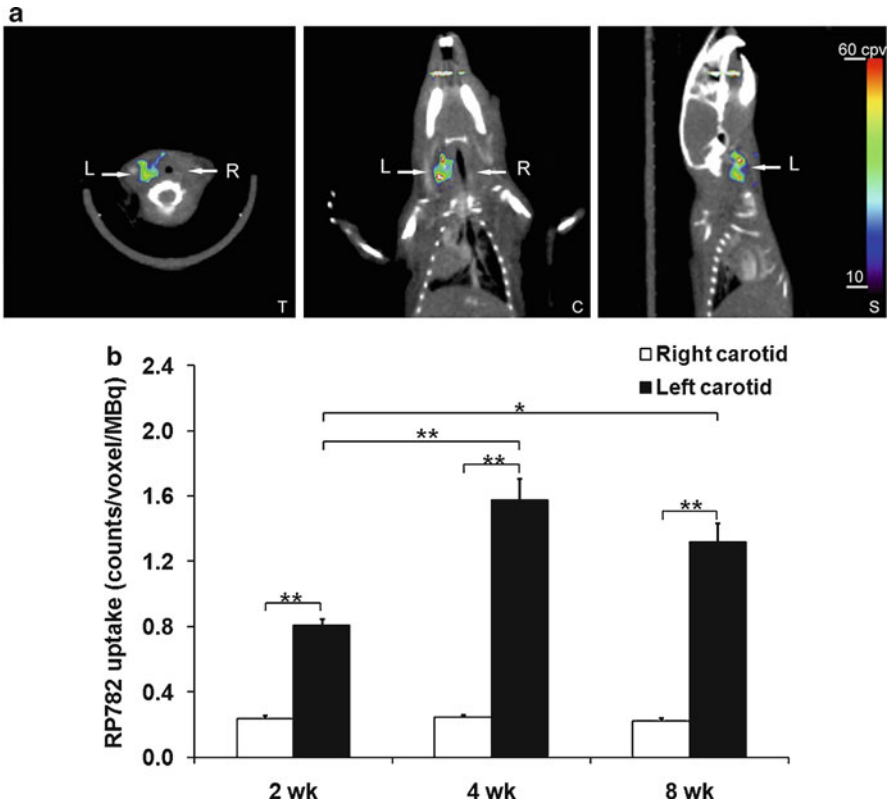

Fig. 5.2 MMP-targeted imaging of carotid aneurysm. (**a**) RP782 microSPECT/CT images of an apoE$^{-/-}$ mouse at 4 weeks after surgery to induce left common carotid artery aneurysm, demonstrating higher tracer uptake in aneurismal left (L) than control right (R) carotid artery. (**b**) Quantification of tracer uptake in carotid arteries at 2, 4, and 8 weeks (wk) after surgery. $^{*}P = 0.01$; $^{**}P < 0.001$. *C* coronal slice, *S* sagittal slice, *T* transverse slice. Reprinted by permission of the Society of Nuclear Medicine from [110]

animal, indicating that MMP-targeted imaging may be used to predict the effect of therapeutic interventions on intimal hyperplasia [109]. Similarly, in a model of $CaCl_2$-induced carotid aneurysm in apoE$^{-/-}$ mice, MMP-targeted imaging was able to predict the propensity of an aneurysm to expansion in vivo [110] (Fig. 5.2). Similar tracers were used to detect MMP activation in rabbit [111, 112] and murine [113] models of atherosclerosis. In rabbit atherosclerosis, MMP tracer uptake in atherosclerotic aortas was detectable by planar imaging [111]. Ex vivo quantification of tracer uptake in the aorta showed a significant reduction in MMP activation in animals treated with a statin, minocycline (an MMP inhibitor), or high fat withdrawal [112, 113]. There was a good correlation between tracer uptake and macrophage immunostaining. Similarly, a correlation existed between aortic macrophage immunostaining and ex vivo quantification of tracer uptake in the mouse models of atherosclerosis [113].

P947 is a novel MR contrast agent with μM affinities for various MMPs obtained by coupling gadolinium to an MMP inhibitor [114]. Ex vivo, this agent can bind to MMP-rich human carotid endarterectomy samples. When injected to atherosclerotic mice, considerable signal enhancement may be detected in the aorta by in vivo MRI [114]. Using a fluorescent homologue, it was shown that P947 localizes near the fibrous cap of murine atherosclerotic plaques in areas positive for MMP-2, MMP-3, and MMP-9 immunostaining [115]. A similar enhancement of the aortic wall was observed in rabbits fed a high fat diet. Repeated MRI after 4 months in animals that continued the high fat diet showed persistence of the signal. However, in animals switched to normal chow, in conjunction with the reduction in MMP-2 activity, the signal was significantly reduced on repeat MRI [116]. MRI in a rat model of abdominal aortic aneurysm showed significantly higher normalized signal enhancement with P947 compared with an inactive homologue or Gd-DOTA [117]. P947 signal enhancement was also higher in aneurismal, as compared to sham operated, aortas. The predictive value of this approach for aneurysm expansion remains to be determined.

Cathepsins

Cathepsins are a group of 11 predominantly intracellular enzymes that belong to the family of cysteine proteases [95]. They are produced as preproenzymes with enzyme activation occurring in endoplasmic reticulum, endosomes, or lysosomes. Enzyme activity is regulated by a group of inhibitors including cystatin C. It is reported that human macrophages secrete cathepsins B, L, and S into the peri-cellular space. Several cathepsins including cathepsins B, K, and S are expressed in human and murine atherosclerosis where cathepsin immunostaining localizes to macrophages, endothelial cells, and VSMCs. Cathepsins modulate atherosclerosis through regulation of elastase and collagenase activity, lipid metabolism, and inflammation [118]. Deletion of cathepsin S in atherosclerosis-prone mice leads to reduction of plaque area [119]. Cathepsin K deletion similarly leads to smaller plaques with reduced frequency of rupture in brachiocephalic artery [120].

An activatable near infrared fluorescent (NIRF) probe with poly-L-lysine backbone was initially developed for imaging cathepsin enzymatic activity in tumors [121] and has been used to detect proteolytic activity in atherosclerotic lesions in the mouse [122]. The activity of the probe was validated in the presence of tumor cells capable of internalizing the probe [121]. Protease inhibitors such as E64 (which inhibits cathepsins B, H, L and a number of other proteases) and leupeptin (a serine, cysteine, and threonine protease inhibitor) completely inhibited NIRF signal generation. A similar inhibitory effect was seen for trypsin inhibitor (tosyl-L-lysyl chloromethyl ketone) and trypsin-like serine protease inhibitor (3,4-dichloroisocoumarin), but not pepstatin, a cathepsin D inhibitor [121]. In apoE/eNOS double knockout mice on a Western-type diet, it was shown that cathepsin B is upregulated in atherosclerotic lesions and following administration of the probe, the resultant fluorescent signal can be detected in vivo by fluorescence-mediated tomography. On ex vivo analysis, fluorescent activity localized in the aortic arch

and abdominal aorta. Fluorescent activity on microscopy was detected in the endothelial and subendothelial areas which were also positive for cathepsin B immunostaining [122]. The same cathepsin B probe was used to assess protease activity ex vivo in human endarterectomy samples [123]. Protease-related fluorescent signal was present both in the plaque and in emboli obtained at the time of intervention, and subtle differences in signal distribution was observed between plaques from symptomatic and asymptomatic patients. An activatable MMP probe generated a similar signal which localized in the vicinity of macrophage-rich areas of the plaque [123]. Cathepsin K is expressed by macrophages and VSMCs and shows considerable elastinolytic and gelatinolytic activity. A probe, incorporating a cathepsin K sensitive peptide, was used for imaging proteolytic activity in mouse aortic atherosclerosis and in human carotid endarterectomy samples [124]. This probe demonstrates twofold higher sensitivity for cathepsin B compared to other cathepsins and a ~10-fold higher sensitivity compared to MMPs. In both models, considerable fluorescent signal was generated by the probe. Interestingly, while both VSMCs and macrophages express cathepsin B, the signal localized mostly with macrophages, but not VSMCs, indicating differences in cathepsin activation or secretion or probe uptake between the two cells [124]. Novel derivatives of protease sensors with different pharmacokinetics and sensitivity have been developed and evaluated for their binding to atherosclerotic lesions in the mouse [125]. To overcome depth-related limitations of optical imaging, a catheter-based strategy for detection of NIRF has been developed and tested in the aorta of atherosclerotic rabbits [126]. Some of the limitations and technical difficulties associated with endovascular NIRF imaging may be addressed using novel algorithms developed for quantitative imaging through blood [127].

Fibronectin

Imaging neoepitopes on matrix proteins is a complementary approach to detection of matrix remodeling. One such epitope is the extra-domain B (ED-B) of fibronectin which is generated by alternative splicing during angiogenesis and remodeling [128]. Radiolabeled and fluorescent-labeled antibody against ED-B localizes in atherosclerotic, but not normal artery and the corresponding signal can be detected ex vivo. In combination with imaging protease activation or activity, these molecular imaging approaches have become powerful experimental tools for assessing vascular remodeling in vivo, and their imminent clinical application may have a transformational effect on diagnostic and therapeutic approach to vascular diseases.

Smooth Muscle Proliferation

VSMCs play a dual role in atherosclerosis. On one hand, proliferation and migration of VSMCs into the developing plaque is an early step in atherogenesis [129].

Activated VSMCs produce inflammatory mediators and matrix proteins that promote plaque development and its stability. On the other hand, plaque rupture is linked with a paucity of VSMCs and the resultant thinning and weakening of the fibrous cap. In other diseases, e.g., in-stent restenosis and transplant vasculopathy, VSMC proliferation is predominantly a pathogenic process. In these cases, imaging VSMC proliferation, by targeting epitopes expressed on proliferating cells, provides unique information on the neointimal hyperplastic process.

Z2D3, an antibody originally discovered by screening of hybridomas generated against human atherosclerosis, displays binding specificity for VSMCs with a proliferative phenotype [130]. Derivatives of this antibody were amongst the first molecular imaging probes studied for imaging vessel wall biology [131]. Although not optimal for imaging and large-scale production, radiolabeled Z2D3 fragments have been shown to detect VSMC proliferation in coronary in-stent restenosis and transplant rejection in vivo [130, 132].

Another putative target for molecular imaging of VSMC proliferation is the integrin family of adhesion molecules. Integrins are heterodimeric surface proteins involved in cell–cell and cell–matrix adhesion. Integrins $\alpha_v\beta_3$ is upregulated and undergoes conformational change on the adluminal surface of endothelial cells as well as medial VSMCs during angiogenesis and arterial remodeling [133]. Underscoring the importance of $\alpha_v\beta_3$ integrin in vascular remodeling are reports that $\alpha_v\beta_3$ inhibition ameliorates neointima formation induced by vascular injury [134–136]. $\alpha_v\beta_3$ integrin is upregulated in injury-induced vascular remodeling and transplant vasculopathy. RP748, an ^{111}In-labeled quinolone with specificity for α_v integrins' high affinity conformation, was evaluated for detection of vascular remodeling in murine models of mechanical or immune-mediated injury. In both models, RP748 uptake in the vessel wall detected by autoradiography increased after injury and its uptake paralleled cell proliferation at different time points after injury [137, 138]. It remains to be empirically determined whether integrin-targeted probes provide sufficient signal for imaging cell proliferation in vivo.

Angiogenesis

A key feature of atherosclerosis is the angiogenic expansion of the vasa vasorum in the adventitia. Mural neovascular development accelerates lesion progression and instability by providing conduits for immune cell invasion and by increasing the foci for intraplaque hemorrhage [139–143]. Nontargeted imaging may be used to evaluate the extent of plaque vascularization using traditional imaging techniques [144–146]. However, targeted molecular imaging has the potential of detecting the angiogenic *process*. The molecular events surrounding angiogenesis involve cell proliferation, migration, and adhesion as well as matrix remodeling. The integrin family of adhesion molecules expressed on endothelial cells mediates many of these processes and has been extensively investigated as targets for molecular imaging of

angiogenesis. Integrin $\alpha_v\beta_3$ is the most commonly used target for imaging angiogenesis, although many of the probes developed to date show a broader specificity for α_v integrin. In an early study using hyperlipidemic rabbits, targeted imaging of the $\alpha_v\beta_3$ integrin using paramagnetic perfluorocarbon nanoparticle coated with an RGD-mimetic small molecule allowed noninvasive detection of atherosclerotic lesions in the aorta with MRI [147]. The enhancement signal could be blocked with nonparamagnetic nanoparticles, establishing specificity of the signal. On histological analysis, $\alpha_v\beta_3$ integrin positive vessels (present at the adventitia–media interface) constituted a fraction of all of the total vasculature detected by CD31 staining. As discussed above, in addition to endothelial cells, $\alpha_v\beta_3$ integrin is expressed by VSMCs and macrophages. The inability of bulky nanoprobes to leave the vascular space assures selective targeting of angiogenic endothelial cells with this approach. In a subsequent study, fumagillin, an antiangiogenic agent, was intercalated within the surfactant layer of the $\alpha_v\beta_3$ integrin nanoparticle such that the $\alpha_v\beta_3$ integrin probe could be used for selective drug delivery to atherosclerotic sites [148]. Repeat imaging in atherosclerotic rabbits following drug delivery showed reduced signal enhancement in fumagillin-treated animals, and histological analysis showed fewer microvessels in these animals [148]. Interestingly, while statin therapy had no long-term antivascular effect, targeted delivery of fumagillin combined with statin therapy showed a synergistic effect in decreasing integrin-mediated angiogenesis in vivo (Fig. 5.3) [149]. It is worthwhile to note that a number of other probes based on other targets (VEGF, Robo4) and imaging platforms (ultrasound) have been developed for imaging angiogenesis, and their usefulness for imaging atherosclerosis remains to be determined [150–152].

Apoptosis

Apoptosis occurs in a number of cell types within atherosclerotic lesions, and may contribute to plaque instability by enhancing the size of the necrotic core and thinning of the overlying fibrous cap [153]. Apoptosis is a regulated and energy-dependent process triggered by either extrinsic (e.g., tumor necrosis factor-α, Fas ligand) or intrinsic (e.g., free radicals, calcium) signals. During apoptosis, alterations in the cell membrane and cytoplasmic proteins occur, which may be targeted for imaging [154]. As part of the apoptotic process, the organization of the plasma membrane is altered such that phosphatidylserine, a phospholipid typically confined to the inner bilayer, becomes exposed on the outer surface [155]. Once believed to be a hallmark of apoptosis, phosphatidylserine translocation is also observed in nonapoptotic cells [156, 157].

In atherosclerosis, there is extensive macrophage apoptosis (especially in the fibrous cap) in ruptured plaques, suggesting that imaging apoptosis may help identify high risk lesions [158]. Because annexin V has high affinity for phosphatidylserine, annexin V-based agents have been investigated as probes for imaging

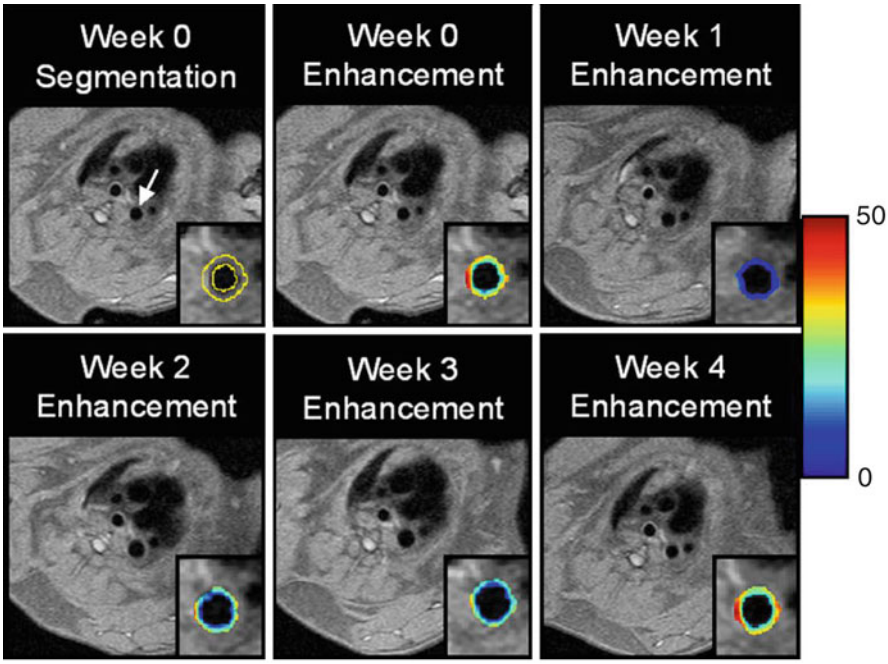

Fig. 5.3 Integrin $\alpha_v\beta_3$-targeted MRI of plaque angiogenesis and targeted fumagillin (a suppressor of angiogenesis) delivery in atherosclerotic rabbit aorta. Color-coded signal enhancement overlaid on T_1-weighted MR images demonstrating a reduction in signal enhancement 1 week after targeted fumagillin therapy. The signal enhancement of $\alpha_v\beta_3$-targeted nanoparticles subsequently gradually increases and returns to baseline level (week 0) after 4 weeks. Reprinted from [149] with permission from Elsevier

apoptosis in various pathologies [159]. In a rabbit model of atherosclerosis, planar imaging with ^{99m}Tc-annexin V demonstrated uptake of the tracer along the aorta. Tracer uptake was confirmed by autoradiography and histological analysis demonstrated enhanced tracer uptake in advanced lesions [160]. Similarly, in murine models of atherosclerosis, ^{99m}Tc-annexin V SPECT/CT imaging demonstrated areas of enhanced tracer uptake. Tracer uptake in the aorta was confirmed by autoradiography and was found to be highest in apoE$^{-/-}$ (or LDLR$^{-/-}$) mice on a high fat diet followed by apoE$^{-/-}$ (or LDLR$^{-/-}$) mice fed normal chow, and considerably less in wild-type control animals [161]. On histological analysis, tracer uptake correlated with macrophage and apoptosis immunostaining [161]. In a model of balloon injury to coronary arteries in high fat-fed swine, ^{99m}Tc-annexin V localized in a subset of injured arteries, with tracer uptake correlating with apoptosis rate quantified on histological samples [162].

Direct comparison of MMP-targeted and annexin-based imaging in rabbit [163] and murine [164] models of atherosclerosis has been performed. However, to draw

reliable conclusions, a number of technical challenges associated with dual tracer and serial imaging will need to be addressed. Ultimately, annexin imaging may not be an optimal approach as it suffers from a several major limitations for detection of apoptosis. As discussed above, phosphatidylserine translocation is not specific to apoptotic cells. Annexin V binds to several other membrane proteins unrelated to apoptosis (e.g., interferon-γ receptor [165]), and annexin V alone cannot differentiate between cell necrosis and apoptosis as loss of membrane integrity in necrosis exposes phosphatidylserine to annexin V. To overcome these limitations, alternative approaches to imaging apoptosis (e.g., targeting caspase activation) are under development [159].

Calcification

In clinical studies, arterial calcification is often used as surrogate for atherosclerosis burden. Nonetheless, vascular calcification is a complex process resembling embryonic bone formation and may take several forms. Intimal calcification is linked with atherosclerosis, while medial calcification is often observed in patients with type 2 diabetes mellitus and chronic kidney disease [166]. In atherosclerotic calcification, the presence of small calcific deposits in a spotty or speckled pattern is linked with plaque vulnerability and unstable angina, although there is considerable overlap between calcification pattern and different clinical presentations [23, 167, 168]. CT is the most commonly used imaging modality for detecting arterial calcification. However, small deposits are often missed by CT imaging. IVUS can differentiate different patterns of calcification and has been used in clinical studies to investigate potential associations with symptoms [167, 168].

Phosphonates which bind to hydroxyapatite, the major mineral product of osteoblasts, may be used for the detection of calcification. The classical example is ^{99m}Tc-methylene diphosphonate (MDP) which is used for imaging bone lesions. The development of a NIRF probe based on conjugated biphosphonates (e.g., OsteoSense750) has allowed in vivo imaging of osteoblastic activity by optical imaging [169]. When injected to atherosclerotic apoE$^{-/-}$ mice in conjunction with NIRF probes for detection of macrophages or protease activity, there was a strong correlation between OsteoSense750 and macrophage signals in different regions of the aorta, suggesting an association between the two processes. Interestingly, there was a discordance between the temporal pattern of signals from the protease and OsteoSense750 probes, with the signal from cathepsin K probe detectable in carotid arteries of 20- and 30-week-old mice, while the osteogenic signal (which did not colocalize with cathepsin K signal) was detected only at 30 weeks [170]. Importantly, NIRF imaging provided sufficient sensitivity and resolution for ex vivo detection of foci of microcalcifications in the aorta that were undetectable by microCT imaging.

Thrombosis

Plaque rupture or erosion generates a prothrombotic interface for platelet activation, adhesion, and aggregation. The platelet aggregate may progress into an occlusive thrombus, causing acute coronary syndromes or alternatively remain asymptomatic and contribute to the progression of atherosclerosis [171]. As such, imaging thrombosis (platelet-rich or organized) can be useful for assessing atherosclerosis and its consequences. Thrombus formation involves a cascade of events including platelet activation, activation of coagulation factors, conversion of fibrinogen to fibrin, and subsequent cross-linking of fibrin. The platelet-specific glycoprotein $\alpha_{IIb}\beta_3$ integrin plays an important role in platelet recruitment and aggregation at the sites of endothelial injury. Similar to $\alpha_v\beta_3$ integrin, activation of $\alpha_{IIb}\beta_3$ integrin involves conformational changes in its quaternary structure that generate high affinity binding sites for fibrinogen and von Willebrand factor [172]. These epitopes may be used as targets for imaging thrombosis. Apcitide is a ^{99m}Tc-labeled probe that binds to integrin $\alpha_{IIb}\beta_3$ on activated platelets and can be used for imaging acute deep venous thrombosis [173–175]. Similarly, MPIO conjugated to an anti-$\alpha_{IIb}\beta_3$ integrin single chain antibody can detect platelet-rich human thrombi by MRI ex vivo [176]. In a model of arachidonic acid-induced carotid thrombosis in the mouse, P975, a Gd chelate-conjugated cyclic RGD peptide targeting $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ was used to detect arterial thrombosis by MRI in vivo [177]. While this agent may potentially be useful for imaging plaque-associated thrombosis, the requirement for delayed (2 h) imaging to generate a specific signal excludes its use in acute settings.

Fibrin generation is another aspect of thrombosis which may be used as an imaging target. During acute thrombosis, fibrin's circulating precursor, fibrinogen, becomes selectively cleaved by activated thrombin [178]. Consequently fibrin is selectively deposited in abundant quantities over plaque-associated thrombi, making it an attractive target for molecular imaging. A gadolinium-based fibrin-specific contrast agent, termed EP2104R, has been validated in human subjects for noninvasive detection of coronary, pulmonary, and peripheral arterial thromboses by MRI [179–182]. An alternative approach to targeted imaging of thrombosis involves the activated form of coagulation factor XIII (FXIIIa) generated in the course of thrombogenesis. FXIIIa covalently cross-links α_2-antiplasmin (α_2-AP, the main inactivator of plasmin) to fibrin [183], and α_2-AP-based optical and MR probes have been used to detect arterial thrombosis in vivo [184, 185]. To date, none of the methods developed for targeted imaging of thrombosis has been shown to detect atherosclerotic plaque-associated thrombosis, and it remains to be determined if this approach is able to compete with nontargeted approaches to detect thrombosis.

Vascular Molecular Imaging in Humans

^{18}F-FDG Imaging of Vessel Wall Inflammation

As discussed earlier, ^{18}F-FDG PET imaging may be used to detect vessel wall inflammation in animal models of atherosclerosis. ^{18}F-FDG uptake in human arteries was first noted on PET studies performed for cancer staging [186]. Correlative studies demonstrated ^{18}F-FDG uptake to be associated with age, hypertension, hyperlipidemia, and smoking [187, 188]. Interestingly, vascular calcification and ^{18}F-FDG uptake rarely overlapped, suggesting the presence of distinct processes [189]. To further investigate whether plaque inflammation could be visualized and quantified noninvasively using ^{18}F-FDG PET in humans, patients with symptomatic carotid stenosis underwent ^{18}F-FDG PET imaging before carotid endarterectomy. On PET images there was approximately fourfold higher ^{18}F-FDG uptake in symptomatic carotid arteries as compared with asymptomatic contra-lateral lesions [190]. Furthermore, autoradiographic examination of carotid endarterectomy samples from symptomatic patient exposed to ^{3}H-deoxyglucose (an ^{18}F-FDG analogue) ex vivo showed predominant uptake of the tracer at the lipid core-fibrous cap border which is rich in macrophages [190]. Subsequent data demonstrated a strong correlation between ^{18}F-FDG uptake in endarterectomy samples and macrophage (CD68) staining [191] and established reproducibility of ^{18}F-FDG PET imaging in carotid arteries and aorta [192]. In patients with ^{18}F-FDG uptake in carotid arteries or aorta, repeated imaging after 3 months demonstrated a reduction in ^{18}F-FDG uptake in those treated with simvastatin compared to those who underwent dietary modification, establishing the ability of PET imaging to track the effect of therapeutic interventions on vessel wall biology [193]. Similarly, life style modification for an average of 17 months in a group of asymptomatic patients with risk factors for atherosclerosis led to a significant reduction in the number of ^{18}F-FDG-positive lesions along the great vessels. Interestingly, in this study, the changes in ^{18}F-FDG uptake best correlated with changes in high density lipoprotein (HDL) plasma levels [194].

Much of the work in this area has focused on imaging the aorta and great vessels. Cardiac motion and ^{18}F-FDG uptake by normal myocardium are impediments to imaging coronary arteries. To overcome this limitation, a number of protocols have been developed to suppress myocardial ^{18}F-FDG uptake [195]. Using this approach for cardiac ^{18}F-FDG PET imaging, a difference in target-to-background ratio between lesions culprit for acute coronary syndromes and those corresponding to stable symptoms was reported (Fig. 5.4) [196]. Despite these advances, because of cardiac motion the left main and proximal left anterior descending arteries are probably the only coronaries which can be imaged with relative accuracy using this approach.

The correlation between ^{18}F-FDG uptake on PET images and anatomical features of plaque detected by CT and MRI was investigated in a group of patients

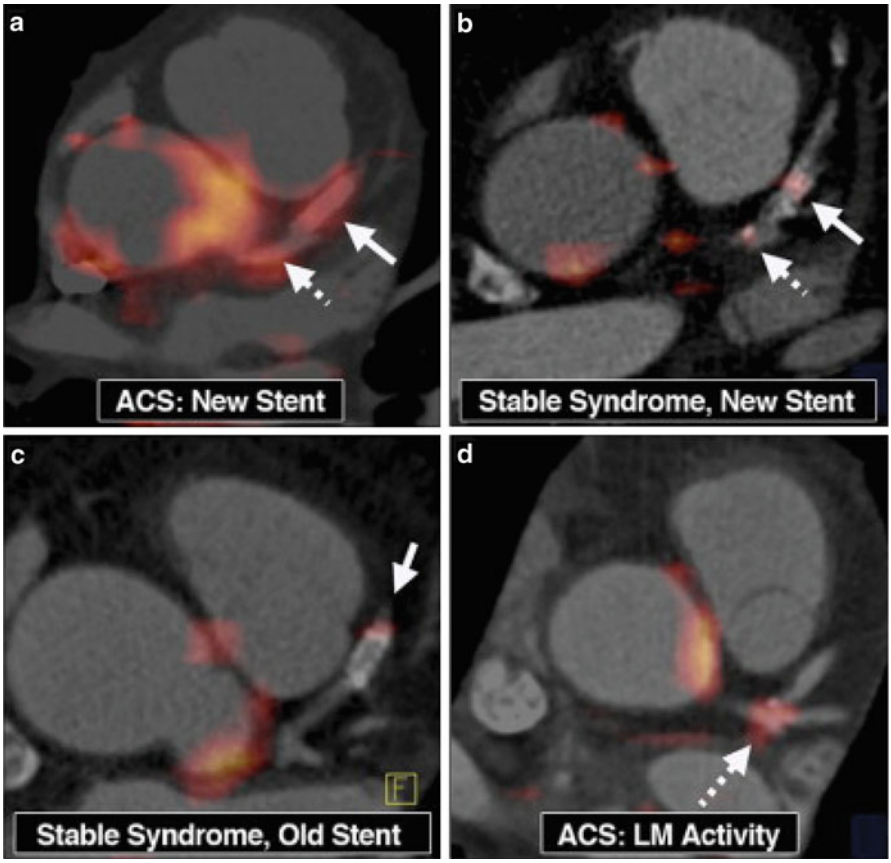

Fig. 5.4 ^{18}F-FDG PET/CT images demonstrating tracer uptake in the left main (LM) coronary artery in patients who presented with acute coronary syndrome (ACS) and uptake to a lesser degree in patients with stable syndrome. *Solid arrows* show stent locations. *Dashed arrows* show lesions within the LM. Reprinted from [196] with permission from Elsevier

with recent neurological symptoms and ipsilateral carotid stenosis. Surprisingly, there was no strong correlation between ^{18}F-FDG uptake and any CT or MRI measurement. ^{18}F-FDG uptake was higher in lesions with intraplaque hemorrhage but not in lesions with a thin or ruptured fibrous cap [197]. In another study, ^{18}F-FDG PET imaging in patients undergoing screening for carotid disease by ultrasound was positive in a subset of patients without detectable atherosclerosis. This raises questions pertaining to the source of ^{18}F-FDG uptake in carotid arteries in these patients [198]. Regarding the source of ^{18}F-FDG uptake in atherosclerosis, much of the existing literature points to macrophages. In most studies, macrophages are defined by their CD68 positive immunostaining. While the representativeness of immunostaining on random histological sections for the whole lesion may be

questioned, a study of ^{18}F-FDG PET imaging prior to endarterectomy in patients with symptomatic carotid stenosis found a similar correlation between CD68 mRNA from 3-mm-thick segments of the artery and ^{18}F-FDG uptake [199]. As a marker for macrophages, CD68 is somewhat nonspecific and may be expressed by other cells, including adipocytes [200] and lipid-loaded VSMCs [201]. Despite these limitations, ^{18}F-FDG is the most clinically advanced vascular molecular imaging probe available at the present time and is currently extensively employed to assess efficacy of antiatherosclerotic drugs in clinical trials [202].

Ultrasmall Superparamagnetic Iron Oxide Imaging of Carotid Inflammation

The relatively long blood half-life of ultrasmall superparamagnetic iron oxide (USPIO) in humans (~30 h) allows their accumulation in macrophages. This has led to their proposed use for detection of metastatic cancer in lymph nodes [203]. Preliminary studies in animals [60] and incidental uptake in atherosclerotic arteries noted on cancer staging studies [63] led to investigation of USPIO for imaging inflammation in carotid atherosclerosis. In a prospective study, patients with symptomatic carotid stenosis underwent MRI before and 24 or 72 h after USPIO (Sinerem®) administration [62]. USPIO was taken up primarily in macrophages but also in endothelial cells, myofibroblasts and VSMCs in 10 out of 11 endarterectomy samples, and MRI signal was detectable in half of these patients. USPIO uptake in carotid atherosclerosis in association with macrophages was confirmed in several follow-up studies [64, 204]. More recently, this agent was used in a prospective study to assess the effect of statin therapy on carotid inflammation. In asymptomatic patients with at least 40% carotid stenosis and USPIO signal loss on an initial MR study, treatment with high-dose atorvastatin (80 mg) for 12 weeks led to a modest, yet significant reduction in USPIO signal intensity on T_2^*-weighted MR images [205]. These changes were not observed in the low-dose treatment group receiving 20 mg daily of atorvastatin. While several major technical challenges associated with MR imaging of USPIO uptake in atherosclerosis persist [59, 206], this study provided evidence for possible use of USPIO-based MRI for assessing the effect of therapeutic interventions on atherosclerosis.

Fibrin-Specific MR Imaging of Thrombosis

In a proof of principle study, a fibrin-specific gadolinium-based contrast agent, EP-2104R, was used to detect cardiac, aortic, or carotid thrombosis by MRI [182, 207]. In addition to detecting the great majority of thrombi identified by other modalities, this approach identified additional loci of thrombus in two patients. Additional studies are needed to delineate optimal imaging parameters and the effectiveness of EP-2104R for imaging plaque-associated thrombosis.

Conclusion

Molecular imaging is critically dependent on identification of appropriate targets, development of specific and selective probes, and refinements in imaging technology. The small size of the vessel wall and cardiac motion considerably increase the challenges associated with vascular molecular imaging. A diverse range of molecules, including adhesion molecules, proteases, scavenger receptors, and clotting factors, are induced and somewhat selectively expressed in vascular pathologies and are thus putative targets for molecular imaging. While the direct exposure of endothelial cells to blood facilitate the delivery of targeted MRI and ultrasound probes to targets expressed on endothelial cells, imaging of subendothelial cell structures remain challenging for these imaging platforms. Nuclear and fluorescent imaging are particularly useful for this purpose. An important factor to keep in mind is the vastly different requirements and challenges associated with imaging in small animals (e.g., for vascular biology research) and in humans. For example, fluorescent imaging is a powerful tool for basic research studies in small animals. However, application of fluorescent imaging to imaging blood vessels in humans is currently only possible with invasive strategies. Nuclear imaging modalities (PET and SPECT) are highly quantitative in nature and appear as especially promising for clinical translation of molecular imaging. Ultimately, vascular molecular imaging will most likely be based on multimodality imaging (e.g., PET/CT) which combines the strengths and overcome the limitation of each imaging modality.

Translational studies applying molecular imaging to human disease are ongoing. Refinement of these techniques will broaden the theoretical limitations of noninvasive imaging, allowing for early identification and risk stratification of patients with coronary artery and other vascular diseases. Significant progress has already been made with regards to developing probes targeted at relevant molecular markers of vascular diseases. From an economic standpoint, ultimately those probes that target key biological processes common to several pathologies (e.g., inflammation, MMP activation) have the highest likelihood of being viable for clinical translation. Similarly, PET and SPECT are widely available and routinely used in clinical cardiovascular medicine. This increases the likelihood of their use in clinical vascular molecular imaging. Indeed, FDG PET imaging is currently under fairly advanced investigation as a tool for detecting inflammation in human carotid, coronary, and aortic disease.

When targeted imaging becomes clinically viable, its application to patient care will fundamentally alter the paradigm of management of a broad range of cardiovascular diseases including CAD, peripheral vascular disease, myocardial infarction, and aortic aneurysm. Molecular imaging will allow providers to identify at-risk patients, subsequently permitting individualization of therapies which is expected to confer efficiencies to health care delivery by identifying patients who will benefit most from costly or invasive therapies. An important application of molecular imaging is in providing early endpoints for clinical trials in vascular diseases, which can considerably shorten the time and reduce the associated costs.

In concert with established pharmacologic and invasive therapies, molecular imaging may in the future serve as an indispensable component in providers' armamentarium to diagnose and treat vascular diseases.

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Part II
The Therapeutic Potential of Angiogenesis, Nanomedicine, and Endothelial Progenitor Cells in the Treatment of Vascular Disease

Chapter 6
Molecular Regulation of Vasculogenesis and Angiogenesis: Recent Advances and Future Directions

George E. Davis

Introduction

The molecular control of vascularization responses during development, postnatal life, and following tissue injury is being elucidated using a variety of experimental approaches, including both in vitro and in vivo model systems [1–16]. Both positive and negative modulatory signals have been discovered that contribute to the considerable complexity of the responses that characterize typical biological systems. Much of the early work was focused on identifying specific factors and receptors for the vascular system, including vascular endothelial growth factors (VEGFs), angiopoietins, VEGFR2, Tie1, and Tie2 [11, 16]. Later studies revealed that the specificity for the vascular system is not absolute as other cell types, such as leukocytes and mural cells, can express some of these receptors, along with endothelial cells (ECs). In some cases, these other cell types and ECs can be derived from common progenitor cells, a possible reason for such overlapping receptor expression. Also, during vascular development a proportion of hematopoietic cells are derived from EC precursors from the dorsal aorta as well as other vascular beds [17, 18]. Much recent work has focused on identifying additional genes and signaling pathways that affect EC specification, EC development into specific lineages, including arterial, venous, and lymphatic lineages, and molecules that control EC functions such as vascular morphogenesis and maturation events [7, 11, 12, 14, 16, 19–25].

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Additional lines of investigation have addressed the morphologic and molecular characterization of vascular morphogenesis, including critical steps such as proliferation, survival, motility, invasion, tube morphogenesis, and sprouting events [2, 8, 10, 14, 20, 23, 26]. It is clear that all of these processes are necessary to establish vascular tube networks in three-dimensional (3D) tissue environments. Major regulators of ECs that control these processes include the extracellular matrix (ECM) [8, 14, 27–30], growth factors and cytokines [8, 11, 28–30], and interactions with other cell types, such as mural cells and macrophages [7, 30, 31]. Since blood vessels are forming along with other parenchymal cells to develop tissues, ECs interact with a variety of cell types to affect these processes. Furthermore, EC-lined tubes are critical conduits to locally deliver oxygen and nutrients that are necessary for these processes to occur. They also communicate with other cell types through growth factors, cytokines, peptides, and other small molecules such as nitric oxide and prostacyclin (PGI_2).

Finally, major efforts have been directed toward addressing how mural cells, including vascular smooth muscle cells (present around larger vessels) and pericytes (present around vessels in the microvasculature, particularly capillaries), affect vascular development, vascular function and maturation, blood flow, and blood pressure [1, 7, 30, 32, 33]. Importantly, it appears that vascular development is a gradual process extending into postnatal life [34]. It takes time to recruit mural cells, induce mural cell proliferation, establish appropriate cell–cell and cell–ECM contacts, and deposit, as well as appropriately cross-link, the vascular ECM in order to develop and regulate the maturation of both large and small blood vessels [35]. In this chapter, we highlight eight areas of recent advancement in our understanding of vasculogenesis and angiogenesis, and we address both our current understanding and the future potential of these new insights.

Morphologic and Molecular Control of Vasculogenesis and Angiogenesis

A major advance in our understanding vascular morphogenic events has occurred as a result of developing real-time imaging technologies that have allowed for direct visualization of these events in vitro and in vivo [20, 36–41]. The majority of the imaging thus far has occurred in vitro or using zebrafish, quail, or mouse embryos in which green fluorescent protein (GFP) or other fluorescent reporters are expressed in an EC-specific manner or antibodies are tagged to allow for visualization of developing ECs [36, 42–44]. An underappreciated aspect of vascular morphogenesis is the rapid speed by which blood vessels form and connect with the circulation through the heart pump. The time course of these events varies depending on the species, but it is clearly essential that the vascular tube networks rapidly assemble and connect with the heart to establish the circulatory system in order to deliver oxygen and nutrients to developing embryos. Despite the rapidity by which the tubes assemble, it takes blood vessels considerable time (extending into postnatal life) to fully develop and mature, particularly with respect to the assembly and

cross-linking of the vascular ECM [35]. In the latter case, it is critical that this vascular ECM is properly cross-linked (particularly the elastic interstitial matrix composed of elastin, fibrillin, etc.) so that large vessels such as the aorta can appropriately function in their elastic recoil ability to propagate blood and to prevent rupture in response to elevated blood pressure requirements [35]. In fact, deficiencies in either fibrillin or lysyl oxidases lead to aortic rupture in postnatal life. Lysyl oxidase enzyme activities control ECM cross-linking through collagens, elastin, and elastin-associated proteins within the developing vascular ECM [35].

Characteristic morphologic events that describe vasculogenic tube formation include the assembly of individual or small clusters of developing ECs into interconnecting networks of EC-lined tubes in 3D ECMs [1, 6, 7, 29, 36, 37, 40, 45–49]. In vitro systems developed to characterize the molecular and signaling requirements for this process have been described in great detail [37–39, 45, 50–62]. Detailed histologic and real-time video analysis reveal that ECs undergo dramatic intracellular vacuole formation during this process [45, 54, 55], representing a major mechanism underlying rapid creation of tube structures by ECs [7, 46, 49] (Fig. 6.1). In essence, lumen formation requires the creation of apical plasma membranes exposed to fluid and basal plasma membranes exposed to ECM in individual ECs or groups of ECs to assemble tubes. Importantly, intracellular vacuoles primarily represent pinocytic membranes that invaginate from the plasma membrane surface in contact with ECM [36, 45, 55] (Fig. 6.1). Inclusion of membrane-impermeable dyes in the extracellular space strongly labels these pinocytic vacuoles during the lumen formation process [6, 45, 46, 55]. This pinocytic process depends on EC integrin-dependent contact with ECM and requires Cdc42- and Rac1-mediated signaling, as well as both the microtubule and actin cytoskeletons [45, 46, 49, 58]. This process has been observed in real-time in vitro and in vivo movies where intracellular vacuoles were shown to play a role during both EC lumen formation and tubulogenic events [20, 36–38, 63, 64]. Key events appear to be the ability of vacuoles to form through pinocytosis, to fuse with each other and other intracellular compartments such as Weibel–Palade bodies and to traffic to the apical plasma membrane surface to create the lumenal compartment [45, 55]. Previous work suggests that the pinocytic vacuoles accumulate in a polarized fashion around centrosomes [6], a critical control structure for microtubule elongation. This accumulation may account for the ability of intracellular vacuoles to traffic along microtubules through motor proteins to fuse with the developing apical lumenal surface.

It is important to consider not only the creation of the apical membrane surface during lumen formation, but also its maintenance within stabilized vessels. Interestingly, maintenance of the apical membrane surface is critically dependent on cytoskeletal signaling as we previously demonstrated through experiments showing the fundamental role of the microtubule cytoskeleton in maintenance of EC lumen and tube networks [56]. Addition of microtubule-disrupting agents (i.e., colchicine, vinblastine, thrombin) causes rapid collapse of EC tubes (and loss of the apical membrane surface), while addition of actin cytoskeletal disassembly agents such as cytochalasin B on its own does not [56]. However, collapse of EC-lined tubes depends on microtubule disassembly followed by actin-based cell contractility, a process controlled by the GTPase, RhoA [56]. Interestingly, RhoA activates a

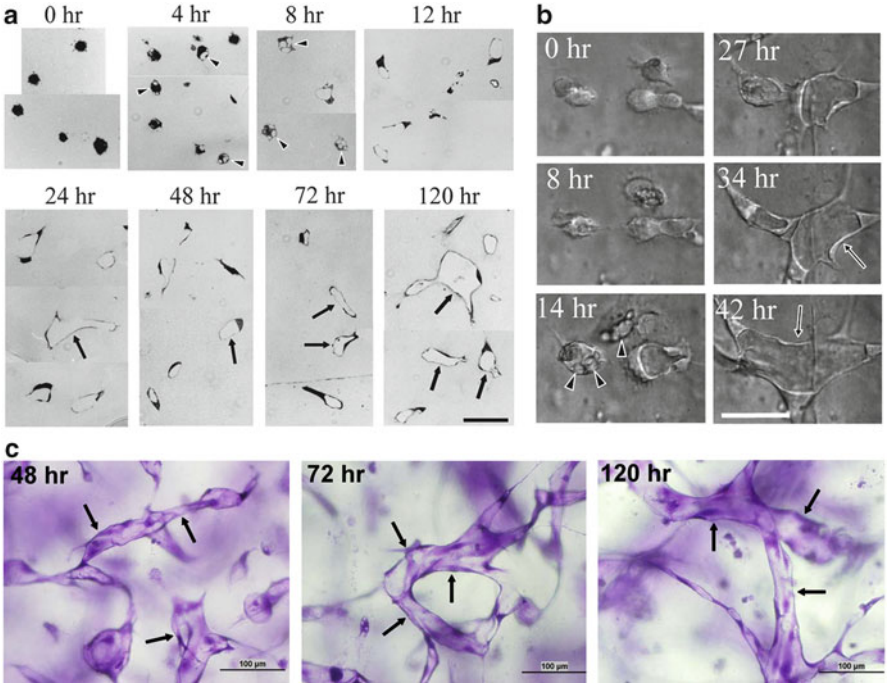

Fig. 6.1 Human endothelial tube morphogenesis in 3D collagen matrices under defined media culture conditions. (**a**) Human ECs were seeded in 3D collagen matrices under defined serum-free media culture conditions and at the indicated times, cultures were fixed, embedded in plastic, and thin cross sections were prepared. Sections were stained with toluidine blue and were photographed. *Arrowheads* indicate EC intracellular vacuoles, a critical way for ECs to rapidly generate apical membranes during lumen formation. *Arrows* indicate EC lumen structures in 3D matrices. Bar equals 50 μm. (**b**) Real-time video analysis of EC lumen and tube formation in 3D collagen matrices. Cultures were established and were photographed at 10 min intervals over 48 h. Images at the indicated times are shown revealing the dynamic process of EC vacuole, lumen, and multicellular tube formation. *Arrowheads* indicate EC intracellular vacuoles and *arrows* indicate the borders of EC lumens and developing tubes. Bar equals 50 μm. (**c**) EC were seeded in 3D collagen matrices and were fixed at the indicated times, were stained with toluidine and photographed to indicate multicellular EC tube assembly and remodeling over time. *Arrows* indicate the borders of multicellular EC tube structures in 3D collagen matrices. Bar equals 100 μm

number of effectors, including Rho kinases, which play a significant role in vascular wall dysfunction, including effects on EC tube stability as well as EC–EC contacts to control processes such as vascular permeability [7, 56, 65–68].

It is apparent during real-time movies that developing EC lumens and tubes expand from their lateral surfaces [37, 38, 40] (Fig. 6.1), a process termed cord hollowing [69]. Considerable data suggest that this lumen expansion mechanism in 3D matrices is dependent on ECM proteolysis by ECs [37, 38, 40]. These processes work in conjunction with the intracellular vacuole mechanism to control lumen and tube development [6, 7, 30]. Interestingly, blockade of membrane-type matrix

metalloproteinases (MMPs) using inhibitors fails to block intracellular vacuole formation, but does interfere with vacuole–vacuole fusion and lumen expansion mediated by ECM proteolysis [7]. Thus, the major blocking influence of MMP inhibitors appears to occur during lumen expansion events during which ECs have been shown to create physical spaces termed vascular guidance tunnels [38, 39] within the ECM.

ECs also elongate and connect with their neighbors through membrane protrusive activity (i.e., process extension), which occurs during tubulogenesis and sprouting. Signals that control cell shape changes through cytoskeletal rearrangements occur through ECM and growth factor-mediated events, which appear to be primarily controlled by Rho GTPases (and their downstream effectors) that represent master regulators of the cytoskeleton [6, 7, 40, 48, 49, 55, 58, 70–73]. Considerable work has been conducted regarding the influence of Rho GTPases on EC structure, function, and morphogenesis [7]. They affect the function of the actin, microtubule, and intermediate filament cytoskeletons to affect these cell behaviors. Important considerations include the role of these signals in EC specification, differentiation, morphogenesis, and the development of stability and quiescence. Another key point is the influence of EC behaviors on adjacent ECs and underlying cells, including mural cells, in the vascular wall. Flow forces are known to significantly affect cytoskeletal regulation, and these affect cell shape, junctional stability, and production of nitric oxide. Importantly, flow-induced signaling can be regulated in a number of ways, including upregulation of transcription factors such as KLF-2 [74], although considerable information suggests that a flow sensory system exists within EC–EC junctions and involves molecules such as VE-cadherin, PECAM, and VEGFR2 [5, 75–77]. Interestingly, ECs also reorient in a polarized fashion in the direction of flow through centrosomal rearrangement in a manner dependent on the Rho GTPase, Cdc42 [75]. Importantly, abnormalities in flow forces appear to occur in diseases such as atherosclerosis where EC dysfunctional changes lead to upregulation of adhesion molecules (e.g., VCAM-1), which in turn leads to monocyte recruitment and accumulation of these cells within the intimal wall where they phagocytose and retain oxidized lipids, a major pathogenic feature of atherosclerotic lesions [2, 77].

Molecular Control of Vascular Lumen Formation and Tube Morphogenesis

Major advances in our understanding of EC lumen and tube formation in 3D ECM environments have occurred over the past two decades [7, 8, 20, 30, 78] (Figs. 6.1 and 6.2). This understanding has resulted from the development of sophisticated in vitro and in vivo models of these events and accompanying technologies that have allowed for a dissection of the molecular and signaling requirements for the distinct stages of EC tubulogenesis. A number of recent comprehensive reviews address the details of these events [6–8, 20, 30, 79]. A key point is that EC lumen

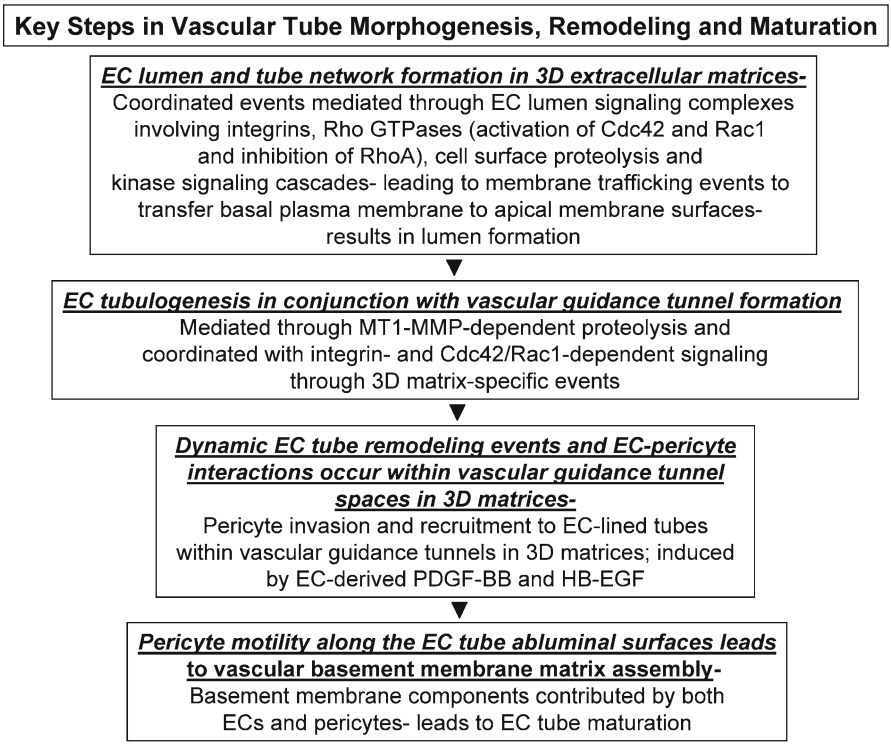

Fig. 6.2 Critical steps controlling EC tube formation, remodeling, pericyte recruitment, and pericyte-induced tube stabilization secondary to vascular basement membrane matrix assembly. Many of the fundamental steps of EC lumen and tube formation in 3D matrices have been elucidate using defined in vitro models. These are indicated in a stepwise fashion and reveal the major progress that has occurred in recent years to identify critical molecular and signaling regulators of EC lumen and tube formation in 3D matrix environments

and tube morphogenesis are events that occur selectively in three dimensions, and they do not occur on 2D surfaces [7, 40]. Importantly, EC tubulogenesis is strongly stimulated by exposure to particular ECM environments, in particular, interstitial collagens and the provisional ECM proteins fibrin and fibronectin, which are deposited following tissue injury responses [8, 29]. Interestingly, collagen and fibrin matrices have been utilized as important 3D matrices to establish models of vascular tube morphogenesis in vitro where tube networks have been shown to form [8, 29]. Many investigators have utilized Matrigel-based systems (laminin-rich matrices) to study EC morphogenesis in vitro, but there is little to no evidence for actual tube formation using such approaches. In fact, laminins appear to possess inhibitory activity for vascular morphogenesis [29, 60], and the lack of lumen formation when laminin matrices are utilized may be a reflection of this inhibitory signaling function of laminin. Unpublished work from our laboratory strongly supports such

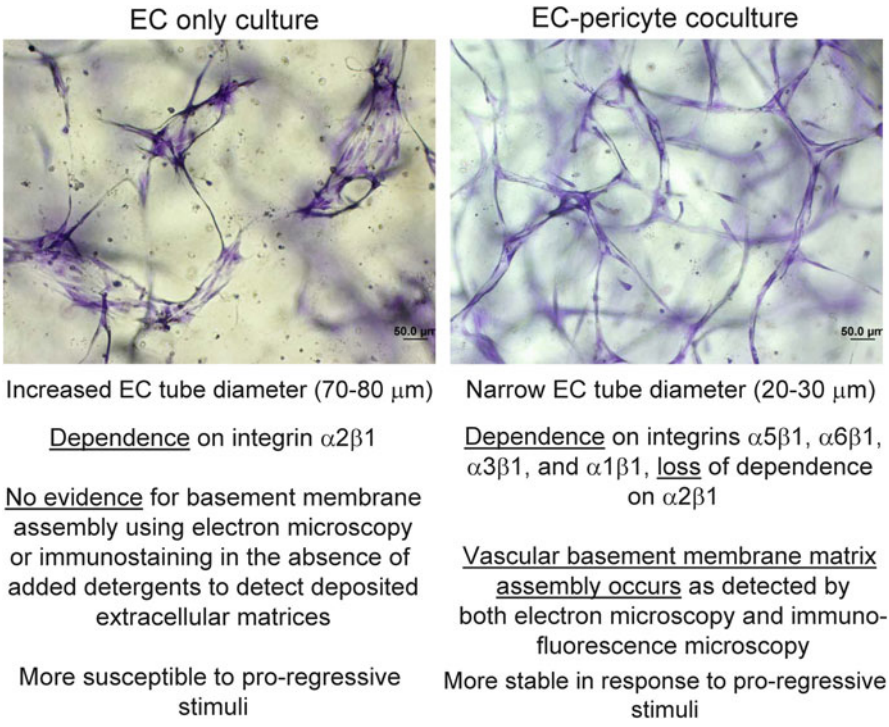

Fig. 6.3 Pericyte recruitment to EC-lined tubes leads to marked tube remodeling and vascular basement membrane matrix deposition events during EC–pericyte tube coassembly in 3D collagen matrices. EC-only cultures versus EC–pericyte cocultures under defined serum-free media conditions and utilizing stem cell factor, interleukin-3 and stromal-derived factor-1a were established for 5 days in 3D collagen matrices. At this point, cultures were fixed and stained with toluidine blue. As indicated in the representative panels, EC-only tubes are much wider and less interconnected while EC–pericyte coassembled tubes are much narrower tubes that show much greater interconnections. Thus, pericyte recruitment to EC tubes has a major remodeling influence accompanied by vascular basement membrane matrix assembly and results in tube stabilization with less susceptibility to proregressive stimuli. Furthermore, the ECM remodeling events that result from pericyte recruitment lead to new integrin requirements for ECs and pericytes during this process. While the collagen receptor α2β1 is solely responsible for the formation and maintenance of EC only cultures (due to continuous exposure to collagen matrices), this integrin loses its influence in EC–pericyte cocultures due to the vascular basement membrane assembly process (that leads to loss of collagen type I exposure for ECs) and then EC and pericyte integrins with affinity for the basement membrane components becomes important (i.e., α5β1 for fibronectin, α3β1 and α6β1 for laminin isoforms and α1β1 for collagen type IV) for tube maturation and stabilization events. Bar equals 50 µm

a conclusion since addition of distinct laminin isoforms within collagen matrices markedly inhibits the EC lumen formation process. Also, vascular basement membrane assembly restricts EC lumen diameter, as demonstrated when EC-only versus EC–pericyte cocultures are compared [39, 80] (Fig. 6.3). Genetic knockout of laminin in mice results in increased vessel diameter in embryoid body cultures, which

also supports this conclusion [81]. In addition, angiogenic sprouting can be initiated by dissolution of the vascular basement membrane [8, 82] (a laminin-rich matrix and a structure associated with EC tube stability and quiescence) and exposure of the new sprouts to interstitial collagens leads to stimulation of vascular tube morphogenesis [8].

Thus, vascular lumen formation is a process that depends on EC interactions with ECM, and a major controller of these events is integrin-based signaling by interactions with these matrices [39, 40, 45, 48, 54, 83–85]. Considerable data show that integrins control EC lumen formation both in vitro and in vivo, and this has been demonstrated using multiple models and different animals including human, mouse, avian, and zebrafish species. The particular integrins that are involved depend on the matrices that the ECs are exposed to, however, as previously discussed, collagen and fibrin matrices appear to have the strongest promorphogenic activity yet described. With human ECs, the α2β1 integrin plays a key role in collagen matrices [45, 84], while the integrins, α5β1 and αvβ3, play a major role in fibrin/fibronectin matrices [54, 84]. Similar results have been obtained using the mouse aortic ring assay system [86]. Both α2β1 and α1β1 have been implicated in vivo during mouse angiogenesis [87], and recently α2β1 was shown to control developmental vascularization in zebrafish embryos [88].

Integrins are known to initiate a variety of signaling cascades that likely play a role during vascular tube morphogenesis. Importantly, integrins can act in conjunction with other signaling receptors (e.g., receptor tyrosine kinases) and molecules to control vascular morphogenesis [23]. The Rho GTPases, including Cdc42, Rac1, and RhoA, are key controllers of the cytoskeletal rearrangements that occur downstream of integrin–ECM interactions. The marked morphologic changes that occur during EC tubulogenesis in 3D matrices have been clearly linked with activation and function of Rho GTPases (Figs. 6.2 and 6.4). Since Rho GTPases play a key role in controlling the major cytoskeletal systems, including actin, microtubule, and intermediate filaments, they represent fundamental control points for the vascular morphogenesis process [7]. Rho GTPases are controlled like other members of the Ras superfamily of proteins by guanine dissociation factors (to control availability of Rho GTPases in the GDP bound form), guanine exchange factors (GEFs) to activate Rho GTPases (to convert them from a GDP to GTP bound form), and GTPase-activating proteins (GAPs) to inactivate the Rho GTPases (to convert them from a GTP to GDP bound form) [70]. Although some progress has been made on both the upstream and downstream control of Rho GTPase activation and inactivation during vascular morphogenesis and stabilization, this is a critical area that has not been explored in sufficient detail (particularly the identity of relevant RhoGEFs and RhoGAPs that affect these events).

A major advance in the lumen and tube formation field was the demonstration that Cdc42 and Rac1 control EC lumen formation while RhoA does not [55, 58]. This original work utilized dominant negative (DN) mutants of these GTPases as well as constitutively active (CA) mutants [55]. Interestingly, CA RhoA markedly blocks lumen formation [55]. More recent work suggests that Cdc42 and Rac1 activation controls lumen formation while suppression of RhoA activation occurs

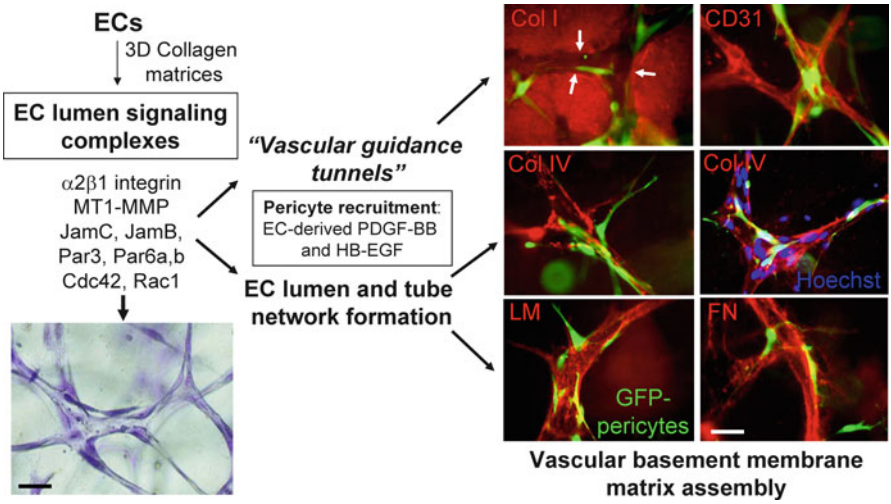

Fig. 6.4 Fundamental molecular and signaling requirements for human EC tubulogenesis and EC–pericyte tube coassembly events leading to tube maturation and stabilization in 3D collagen matrices. EC lumen formation in 3D collagen matrices requires a recently described lumen signaling complex of proteins that coordinate integrin signaling, cell surface proteolysis, and Rho GTPase activation, which are all required signals for EC tubulogenesis. It was demonstrated that the integrin, $\alpha_2\beta_1$, the cell surface MMP, MT1-MMP, the junction adhesion molecules, JamC and JamB, Par3, Par6b and Par6a, and Cdc42-GTP are present in a complex that controls the EC tubulogenesis process in a 3D matrix-specific manner. Importantly, this signaling complex is particularly operative in 3D matrices due to the fact that EC tube formation is a 3D-specific process that does not occur on 2D surfaces. Importantly, tube formation also generates proteolytically created vascular guidance tunnels, which are physical spaces that allow for EC tube remodeling as well as serve as matrix templates to allow for pericyte assembly and motility on the EC tube abluminal surface. These EC–pericyte interactions are highly dynamic (through migratory events which have been demonstrated with real-time videos) within vascular guidance tunnels and lead to continuous vascular basement membrane matrix assembly around EC tubes. The *lower left image* is representative of an EC–pericyte coculture fixed and stained with toluidine blue after 5 days of culture. Bar equals 25 μm. The *right fluorescence panels* were derived from EC–pericyte cocultures in which GFP-labeled pericytes were utilized to distinguish them from the unlabeled ECs. After 5 days, the cultures were fixed and immunostained for the indicated antigens (which are shown staining *red*). *Col I* collagen type I; CD31; *LM* laminin; *FN* fibronectin; *Col IV* collagen type IV. *Arrows* indicate the borders of vascular guidance tunnels that are characterized by a lack of staining with collagen type I antibodies secondary to MT1-MMP-dependent proteolysis of collagen type I, which creates the tunnel spaces during lumen and tube formation. Staining for basement membrane matrix antigens was performed in the absence of detergent to assess only extracellular deposition of these matrix proteins. ECs were stained with CD31; GFP-labeled pericytes are shown to recruit to the abluminal surface of the EC tubes within vascular guidance tunnel spaces. Pericytes have been shown to recruit to EC-lined tubes through EC-produced PDGF-BB and HB-EGF. Hoechst dye was added in one panel (*blue*) to indicate nuclei. Bar equals 25 μm

to prevent lumen collapse during this process [7, 56]. Also, RhoA activation occurs during collapse of vascular tubes following depolymerization of microtubules in response to treatment with colchicine or vinblastine [56]. Blockade of RhoA using the DN RhoA mutant prevents tube collapse following treatment with

these microtubule disrupting agents [56]. Interestingly, microtubule disrupting agents have been utilized in many studies as antiangiogenic as well as antitumor therapeutic drugs.

Both Cdc42 and Rac1 control EC lumen formation as well as EC intracellular vacuolation, a key step in this process [49, 55]. Both of these GTPases also control EC invasion into 3D collagen matrices during angiogenic sprouting behavior [58]. An important advance in the field is also the identification of downstream effectors and regulators of Cdc42 and Rac1 activation that control these morphogenic events [58, 59]. In addition, Cdc42 is known to control cell polarity by affecting centrosome repositioning during cell motility or flow-mediated events in ECs. Interestingly, Cdc42-GTP is known to interact with the polarity regulator, Par6, which, through its interaction with Par3, contributes to the establishment and control of polarization in cells [70, 89, 90] (Figs. 6.2 and 6.4). Rac1-GTP can also interact with Par6 isoforms. Par3 has many binding partners, but notable ones include the tight junction proteins, junction adhesion molecules (Jams), and the adherens junction protein, VE-cadherin [91, 92].

Recent studies demonstrate a critical role for the Cdc42 and Rac1 effectors, Par6 and Pak2, in EC lumen formation [58]. The Cdc42-selective effector, Pak4, also plays a critical functional role during these events [58]. Pak2 and Pak4 activation are controlled by both PKCε and Src kinases (Src and Yes) that are upstream of the Paks during this process [59]. Furthermore, both Src and Pak participate in activation of B-Raf/C-Raf which activates MEK-1, followed by Erk1/Erk2 activation. Disruption of any of these kinases has been demonstrated to abrogate EC lumen and tube formation [59]. Previous work also implicates this pathway in controlling pathologic angiogenesis [93]. Importantly, these critical kinases control tube morphogenesis (and not proliferation) by affecting cytoskeletal rearrangements, MT1-MMP-dependent proteolysis, EC survival, and transcriptional mechanisms (e.g., through Erk activation) [40, 58, 59]. Cdc42 activation is also associated with binding of Par6, which further interacts with Par3 to control EC lumen formation in vitro [40, 58]. A recent in vivo study implicating Par3 in arteriolar EC lumen formation [85] supports this finding. Also, both Pak2 and Pak4 have been shown to play a role in vascular morphogenesis during development [94, 95].

A new study in 3D collagen matrices identifies EC lumen signaling complexes that contain critical regulators of EC lumen formation, including Cdc42, Par6b, Par3, JamB, JamC, membrane type 1 matrix metalloproteinase (MT1-MMP), and the α2β1 integrin [40] (Fig. 6.4). JamB and JamC both bind Par3 through their cytoplasmic tails, and expression of JamB or JamC mutants without these tails markedly interferes with EC lumen formation [40]. JamA does not control lumen formation in this system [40] and was shown to be an inhibitor of this process perhaps through competitive interactions with Par3 which would disrupt interactions with JamB and JamC. Importantly, the function of the EC lumen signaling complex appears to be selective for the 3D matrix-specific EC lumen and tube formation process [40]. For example, disruption of this complex did not affect random motility of ECs on 2D collagen substrates and, furthermore, it affected Cdc42 activation selectively in 3D collagen matrices and not on 2D collagen surfaces. Expression of

the JamB and JamC constructs without their cytoplasmic tails also blocked Cdc42 activation in 3D collagen matrices, but not on 2D collagen surfaces [40]. In contrast, RhoA activation remained the same whether lumen formation was blocked by the Jam mutants or not. Of great interest is that the influence of JamB and JamC on Cdc42 activation in 3D matrices was recapitulated with siRNA suppression or chemical blockade of MT1-MMP, which completely interfered with the 3D matrix-specific Cdc42 activation events necessary for this process [40]. In addition, blockade of MT1-MMP interferes with EC motility and lumen formation in 3D matrices but not on 2D matrix surfaces. Overall, these data demonstrate a key interdependence between Cdc42 and MT1-MMP to coordinate the EC lumen and tube formation process in 3D matrix environments and further show that ECM proteolysis is a necessary step in EC tube morphogenesis during vascularization events [7, 30, 40].

A major regulator of EC lumen and tube formation in 3D ECM environments is the membrane tethered MMP, MT1-MMP, or MMP-14 [37, 96, 97]. MT1-MMP also plays a major role in controlling EC invasive events that characterize angiogenic sprouting [37]. Blockade of MT1-MMP with chemical inhibitors such as GM6001, protein inhibitors such as TIMP-2, TIMP-3, and TIMP-4, siRNA suppression of MT1-MMP, or expression of a DN mutant of MT1-MMP markedly abrogates EC lumen formation or sprouting events in 3D matrices [37, 38, 40, 84]. As discussed previously, MT1-MMP is a central component of EC lumen signaling complexes that control the EC tubulogenic process. It appears to have strong affinity for the collagen-binding integrin $\alpha 2\beta 1$, particularly when ECs interact with collagen type I [40]. Together $\alpha 2\beta 1$ and MT1-MMP control vascular tube formation in 3D collagen matrices and through coordinated signaling events regulate Cdc42 and Rac1 activation as well as protein kinase cascades that control the morphogenic process [7, 40].

An important functional role of MT1-MMP is to create physical spaces in the ECM, termed vascular guidance tunnels, in which EC-lined tubes reside [38–40]. In every case that we have examined, there is a direct correlation between lumen formation and concomitant creation of vascular guidance tunnels [38, 40]. Blockade of EC lumen formation using inhibitors of protein kinase C, Src, $\alpha 2\beta 1$ integrin, Cdc42, Rac1, or MT1-MMP leads to marked abrogation of vascular guidance tunnel formation [38]. Thus, the creation of tunnel spaces by ECs represents a fundamental aspect of their ability to form and remodel EC-lined tubes. Importantly, these tunnels (following tube formation) allow for EC motility events that regulate tube remodeling and maturation and also affect EC–mural cell interactions [38, 39]. Of great interest is that pericyte recruitment to EC-lined tubes occurs within vascular guidance tunnels spaces that were created during the tube formation process [39]. Furthermore, these spaces allow for both cells to readily migrate during subsequent remodeling and maturation events [39, 80]. It is clear that pericytes migrate within vascular guidance tunnels selectively on the EC abluminal tube surface, and these heterotypic cell–cell interactions lead to deposition of vascular basement membrane matrix on this abluminal surface, a fundamental step that characterizes vascular tube maturation and stabilization [39, 80]. Thus, the EC lumen and tube formation signaling cascade creates networks of tubes as well as tunnel spaces that

are necessary for mural cell recruitment along the abluminal surface of EC tubes. Also, the tunnels allow for motility of both ECs and mural cells and cell–cell interactions that are necessary to control vascular basement membrane matrix assembly around these tubes [39, 80].

In recent years, a number of very interesting regulators of EC lumen formation have been identified. In particular, cerebral cavernous malformation 2 (CCM2) (also called Osm), CCM1 (also called Krit1) and CCM3 (also called PDCD10) were observed to control lumen formation in vitro and in vivo [64, 67, 68, 92, 98, 99]. These genes are known to be mutated in humans with cerebral cavernous malformations. Knockout mice missing these genes have both cardiac and vascular abnormalities with deficiencies in vascular lumen defects in the developing aorta and its branches leading to a lethal phenotype. Interestingly, suppression of CCM2 or CCM1 expression leads to marked RhoA activation, a state which is incompatible with EC lumen formation [56, 67, 68, 100]. As stated previously, RhoA mediates EC lumen and tube collapse (i.e., regression); other lines of research suggest that RhoA must be actively inhibited during the lumen formation process [7, 48, 55]. Interestingly, Pak kinases such as Pak2 and Pak4, which are important in EC lumen formation, are capable of inhibiting RhoA GEFs through phosphorylation events to reduce RhoA activation [101]. Pak2 and Pak4 are activated downstream of Cdc42/Rac1 activation (although Pak4 shows more selectivity toward Cdc42) in the EC lumen formation cascade. Rasip1, a newly identified regulator of EC lumen formation is expressed predominantly in an EC-specific manner [48]. Knockout of Rasip1 in mice or siRNA suppression of Rasip1 leads to marked blockade of EC lumen formation in vitro [48]. Knockout mice shows markedly reduced lumen formation in all of the large and small vessels examined. Of great interest is that Rasip1 binds Arhgap29, a RhoGAP that is known to inactivate RhoA [48]. Knockdown of Arhgap29 also leads to blockade of EC lumen formation. In both cases, Rasip1 and Arhgap29 knockdown resulted in reduced Cdc42 and Rac1 activation with increased RhoA activation. Interestingly, their inhibitory phenotypes are rescued by expression of either DN RhoA or concomitant knockdown of RhoA [48]. These data indicate that Rasip1 controls EC lumen formation by stimulating Cdc42 and Rac1 activation and by inhibiting RhoA activation [7, 55, 56].

ECM Control of Vascular Morphogenesis and Stabilization

The ECM is a critical regulator of vascular morphogenesis, vessel functions such as vascular tone and contractility, and also vessel maturation and stability [8, 27–29, 102]. ECM-stimulated vascular cell signaling occurs through a variety of receptors including: (1) integrins; (2) nonintegrin receptors such as cell surface proteoglycans and other adhesion receptors; (3) pattern recognition receptors such as toll-like receptors and scavenger receptors; and (4) co-signaling receptors such as receptor tyrosine kinases that are activated by growth factors and cytokines [2, 8, 23, 27, 103]. In the latter case, considerable evidence shows how growth

factors and cytokines work in conjunction with ECM to signal various cellular behaviors including proliferation, survival, motility, invasion, and morphogenesis [8, 23]. Importantly, growth factors and cytokines anchor to the ECM and this facilitates the opportunity for co-signaling functions of growth factor and matrix receptors that lead to important cellular responses during vascular morphogenesis and following tissue injury. Also, unique signals to vascular cells appear to occur under these circumstances when particular ECM proteins anchor specific growth factors. This ECM binding can occur through glycosaminoglycans such as heparan sulfate or through protein–protein interactions with specific ECM components. For example, VEGF can bind fibronectin [104] and bone morphogenetic protein 4 (BMP4) can bind collagen type IV [105] through protein–protein interactions. Interestingly, matrix-bound VEGF was able to signal distinctly from soluble VEGF, and mutations in VEGF (or splice variants) that alter its matrix affinity markedly can affect vessel morphogenic responses in the context of developmental or tissue injury situations such as within the tumor microenvironment [106, 107]. More work is necessary to investigate the significance of these interactions during different vascular formative and functional events.

An important aspect of the interactions of vascular cells with ECM is that they are able to remodel ECM and deposit unique matrices that relate to their functional role [8]. In addition, vascular cells can degrade ECM in order to perform functions including 3D motility, tube morphogenesis, or tube regression [30]. ECM remodeling is necessary to facilitate vascular tube morphogenesis as well as tube maturation and stabilization, and such events appear to underlie the initiation of angiogenic sprouting during pathologic vascularization responses [8]. Degradation of basement membrane components such as laminins (which have inhibitory activity) and exposure to interstitial matrices such as collagen type I (which has stimulatory activity) to allow promorphogenic signaling appear to play important roles during such responses. More work is necessary to understand why collagen versus laminins matrices regulate such distinct promorphogenic, inhibitory, or stabilized EC behaviors.

A recent key observation is that ECs degrade ECM during vascular morphogenesis to create physical tunnel spaces in which EC-lined tubes reside [38]. Importantly, MT1-MMP is necessary to create the physical tunnel spaces during EC lumen and tube formation, and during this process MT1-MMP is necessary for EC motility within 3D matrices [38]. However, once the tunnels are present, MT1-MMP and other MMP activity are not required for EC motility within vascular guidance tunnels. Thus, tube remodeling within existing tunnel spaces can occur even following MMP inhibition [38]. Other experiments have revealed that EC tubes that have collapsed can regrow within vascular guidance tunnels [38], a phenomenon that has been implicated in the context of tumor angiogenesis, where tumor vessels can apparently regrow in tunnel-like spaces following vessel regression secondary to inhibition of VEGF signaling [108]. Experimentally, our laboratory showed that thrombin addition to existing vessel tube networks in vitro caused rapid collapse of the structures within vascular guidance tunnels. Following addition of the thrombin inhibitor, hirudin, the collapsed tubes could be shown to regrow within the vascular guidance tunnel matrix template [38].

Thus, vascular guidance tunnels, which are generated as a consequence of vascular tube morphogenesis and sprouting, can serve as matrix templates for EC motility and tube remodeling events in 3D matrices. Also, these matrix templates may be critical to facilitate the separation of arterial, capillary, and venous networks through cell–cell repulsion events mediated by ephrinB2 and EphB4 interactions [9, 22, 42, 109–112]. Thus, the cell–cell repulsive interactions necessary to create such arteriovenous separation likely occur on such a matrix template (mimicking a 2D matrix surface) in 3D matrices. Vascular guidance tunnels, like 2D matrix surfaces, should allow for cell sorting events so that ECs can actively migrate with their like neighbors and migrate away from cells that are distinct to create distinct arterial versus venous populations of ECs. Interestingly, there is clear evidence that ephrinB2 and EphB4 expressing cells are intermixed in early development and later sort out during the EC tubulogenic process that creates arteries, capillaries, and veins [42, 110, 112]. Importantly, vascular guidance tunnel spaces are fundamental matrix conduits that affect these critical developmental processes and are coupled to the molecular signaling cascade that creates vascular tubes in 3D ECMs.

Finally, vascular guidance tunnel spaces are also directly linked with the ability of EC–mural cell interactions to control vascular remodeling, maturation, and stabilization [39]. Our laboratory demonstrated that mural cells such as pericytes are recruited along EC tubes on their abluminal surface and furthermore migrate along this surface within vascular guidance tunnel spaces (Fig. 6.4) [39, 62, 80]. These dynamic EC–pericyte interactions lead to continuous vascular basement membrane matrix assembly, a key step necessary to stabilize and mature developing blood vessels (Fig. 6.4). Importantly, this basement membrane matrix assembly process requires both cells [39] and likely requires the active motility of both ECs and mural cells within vascular guidance tunnels. Thus, vascular guidance tunnels, which are generated as a result of MT1-MMP-mediated ECM remodeling [38], serve as a conduit by which EC–pericyte interactions stimulate a second ECM remodeling event—assembly of the vascular basement membrane matrix [39]. EC–pericyte tube coassembly induces marked extracellular deposition of fibronectin, collagen type IV, laminins, nidogens, and perlecan, a process that does not occur with ECs alone [39]. As discussed previously, deposition of this specialized ECM leads to vessel maturation events and interestingly, components within the basement membrane such as laminin isoforms and tissue inhibitor of metalloproteinase (TIMP)-3 can interfere with further tube morphogenesis [37]. Thus, basement membrane assembly can suppress morphogenesis while at the same time facilitate tube stabilization.

EC–Mural Cell Interactions in Vascular Morphogenesis and Maturation

In recent years, important advances have occurred in our understanding of the function of mural cells and their interactions with ECs during development as well as under various disease conditions such as cancer and diabetes [33, 113]. A major

regulator of mural cell function is platelet-derived growth factor (PDGF)-BB a PDGF isoform that is synthesized by ECs and presented to mural cells to affect EC–mural cell intercellular communication [31]. Potential roles for PDGF-BB in these events are affecting mural cell proliferation, mural cell motility, and invasive functions or controlling production of additional mural cell-derived factors that control EC–mural cell interactions. EC-specific knockout of PDGF-BB has been shown to interfere with mural cell association with the microvasculature, while larger vessels such as arteries and veins appear less affected [114]. Capillaries in these knockout animals are abnormal and show increased vascular permeability, dilation, and hemorrhage, which are all signs of decreased vascular stability [114]. Thus, pericyte association is fundamental to stabilization of the developing microvasculature and this is strongly linked with EC PDGF-BB and PDGFRβ expressed by mural cells. Interestingly, vascular abnormalities in the EC-specific PDGF-BB knockout mouse resemble changes in the microvasculature frequently observed in diabetes [113], suggesting that pericyte abnormalities are likely to be involved in the microvascular dysfunction in this disease. Recently pericyte interactions with ECs have been shown to play a key role in the development of the blood–brain barrier [115, 116]. Pericyte association with tumor vasculature occurs but is also abnormal compared to the normal microcirculatory vessel circuits [117]. Attempts to increase pericyte association with vessels in the context of the tumor vasculature appear to normalize this vasculature to a more functional microcirculation [118, 119]. Thus, alterations in pericyte association with the microvasculature in the context of disease lead to abnormal microvessel function, which strongly contributes to the abnormalities observed.

Interesting questions remain in our understanding of how vascular smooth muscle cells are recruited to EC-lined tubes and how they become circumferentially oriented around large vessels such as arteries and small vessels such as arterioles. Vascular smooth muscle cells around the developing aorta are derived primarily from the cranial neural crest [120]. Interesting lineage tracing studies reveal considerable heterogeneity among vascular smooth muscle cell populations around different developing vessels [120]. One interesting issue is the potential that pericytes could differentiate into vascular smooth muscle cells in some contexts, particularly in small vessel development such as arterioles.

A fundamental question that has recently been addressed is how pericytes are recruited to developing tubes. Also, what is the function of pericytes once they are recruited to developing tubes and how does this lead to vascular tube maturation and stabilization? Using a model of human pericyte recruitment to human EC-lined tubes in 3D matrices, it was observed that EC-derived PDGF-BB and heparin-binding epidermal growth factor (HB-EGF) are required for pericytes to recruit to EC tubes and also to migrate in 3D collagen matrices [80] (Fig. 6.3). Importantly, pericyte motility in 3D collagen matrices requires the copresence of ECs, and this appears to be due in part to the production of PDGF-BB and HB-EGF by ECs [80]. The presence of pericytes was shown to dramatically restrict EC tube diameter and thus reduce the width of vascular tubes compared to EC cultures in the absence of added pericytes [39, 80] (Fig. 6.3). This restriction of vessel diameter was shown to

correlate with the ability of pericytes to induce vascular basement membrane matrix deposition, an inhibitor of tube formation [39, 80]. Blockade of both PDGF-BB and HB-EGF derived from ECs disrupts pericyte motility and recruitment to developing tubes and concomitantly interferes with basement membrane formation, leading to increased vessel widths. Furthermore, blockade of PDGFRβ as well as EGFR (both receptors are highly expressed by pericytes) in vitro or in vivo during quail vascular development leads to blockade of pericyte recruitment, decreased basement membrane matrix formation, and increased vessel widths [80]. Interestingly, mouse knockouts of fibronectin, an important component of the vascular basement membrane, lead to embryonic lethality in large part due to severe cardiac and vascular abnormalities [121, 122]. One of the consequences of this knockout phenotype is that vessel widths are markedly increased, mimicking in vitro cultures with ECs by themselves or EC–pericyte cocultures in which pericyte recruitment has been inhibited (Fig. 6.3). Furthermore, disruption of fibronectin matrix assembly or blockade of the α5β1 integrin (which binds fibronectin) disrupts basement membrane assembly and significantly increases EC tube width [39]. Thus, EC–pericyte interactions regulate vascular basement membrane assembly, a critical process that regulates vessel diameters and subsequent maturation events [39, 80].

Although pericytes are known to be important in vascular stability, the reasons underlying this phenomenon were less clear. Our recent studies demonstrating that pericyte recruitment to developing EC-lined tubes during vascular development controls vascular basement membrane matrix assembly provide key evidence for this function. Another important potential function of pericytes is to support EC survival during tube assembly events. One growth factor that could play a role here may be angiopoietin-1, a factor that can be produced by mural cells [16]. Of great interest is that angiopoietin-2, a competitive inhibitor of angiopoietin-1/Tie2 signaling, is produced by ECs and is induced during EC tube morphogenesis [57]. Although angiopoietin-1 can facilitate EC survival functions, a specific role during such events in a 3D model system has not yet been convincingly demonstrated. Mouse knockout of angiopoietin-1 or its receptor, Tie-2, leads to embryonic lethality, which is consistent with defects in EC–pericyte interactions secondary to these deficiencies. It remains to be shown whether angiopoietin signaling is necessary during such EC–pericyte tube coassembly events under defined conditions. Because of the complexity of these types of questions, it is quite clear that the solutions to these issues will require the use of both in vitro and in vivo models. When delivered in vivo angiopoietins have very strong phenotypes, such as the marked inhibition of vascular permeability when expressed in a variety of pathological angiogenic conditions [16]. It is important to examine the influence of angiopoietins not only on individual ECs or pericytes, but also when they are combined in a physiological 3D matrix context. Since Tie-2 appears to be constitutively phosphorylated in adult ECs in vivo [123], one interesting possibility is that angiopoietin-Tie2 signaling occurs optimally in the presence of flow, but not in its absence. This type of question also needs to be addressed in molecular detail to be able to understand the important functional relevance of angiopoietins. There is much to be learned about EC–pericyte interactions as well as

EC–vascular smooth muscle cell interactions. More work is necessary to elucidate how these interactions function normally and are specifically altered in human disease.

Growth Factor/Cytokine Control of Vascular Morphogenesis and Stabilization

Growth factors and cytokines are known to play a fundamental role in vascular development as well as postnatal vascularization responses to tissue injury and disease [3, 11]. Among the growth factors, two appear to be particularly critical: the VEGF isoforms, such as VEGF-A 165, and the FGF isoforms, such as FGF-2. VEGF is known to be downstream of sonic hedgehog (SHH) in regulating vascular development and controlling artery formation and specification [124]. VEGF isoforms (VEGF-A and VEGF-C) are known to act through primarily the receptor tyrosine kinases, VEGFR2 and VEGFR3, which bind to overlapping sets of these isoforms [11]. FGF-2 and FGF-1, along with VEGF, have been demonstrated to be strongly proangiogenic, particularly in the context of pathologic angiogenesis. A series of studies have demonstrated the combined influence of VEGFs and FGFs in vascularization [11, 125]. Their influence involves EC survival, proliferation, permeability, motility, and morphogenesis in normal and pathologic vascular responses in multiple tissues (responses to tumors, ischemia, and other tissue injuries). Due to these studies, many therapeutic approaches have been based on VEGF-mediated or FGF-mediated signaling pathways to affect tissue vascularization in the context of disease. Another interesting finding was the discovery of secreted variants of VEGFR1 that can serve as so-called VEGF traps [126, 127] to sequester and inactivate VEGF isoforms preventing their interaction with either VEGFR2 or VEGFR3. These secreted VEGF traps can bind not only VEGF-A and VEGF-C (which induce phosphorylation of both VEGFR2 and VEGFR3) but also other related isoforms, including VEGF-B and placental growth factor (PlGF), which do not activate these receptors [11]. Interestingly, these latter factors may compete with VEGF-A/C to be blocked by the VEGF traps and, thus, may exert biologic effects in this manner. Also of great interest is that secreted VEGFR1 is found at high levels in the avascular cornea, and deletion of this protein in this site leads to abnormal vascularization of the cornea [128]. In addition, soluble VEGFR1 isoforms appear to be increased under pathologic conditions such as pre-eclampsia in which placental vascular insufficiency is observed [126]. Like other growth factors, VEGF and FGFs show affinity for ECM including heparan sulfate proteoglycans, as well as fibronectin in the case of VEGF. Interestingly, particular VEGF isoforms can interact with neuropilins, cell surface receptors for other proteins, including the cell repulsive factors, semaphorins [11].

VEGFs and FGFs activate their receptor tyrosine kinases in similar fashion to other growth factor-receptor tyrosine kinase activation pathways. VEGFR2 has interestingly been linked with a number of other cell surface receptors, including

VE-cadherin, which plays a major role in the mechanisms underlying why VEGF has such dramatic effects on inducing vascular permeability. In particular, VEGF treatment leads to increased Src-dependent phosphorylation of the VE-cadherin cytoplasmic tail, resulting in the dissociation of beta-catenin and p120 catenin from the tail and disruption of EC–EC junctions [129–131]. Interestingly, angiopoietin-1, through interactions with its EC receptor, Tie-2, antagonizes VEGF-induced permeability in part by antagonizing Src activity that prevents this signaling pathway [132]. Also, angiopoietin-2, through its antagonism of angiopoietin-1 binding to Tie-2, can synergize with VEGF to facilitate processes such as increasing vascular permeability. In tissue injury responses, angiopoietin-2 can be rapidly mobilized from EC Weibel–Palade bodies along with von Willebrand factor to facilitate such acute EC responses [16].

There are many growth factors, cytokines, and peptides that affect EC responses. Some of the most prominent include VEGF isoforms, FGF isoforms, SHH, Indian hedgehog (IHH), bone morphogenic protein (BMP) isoforms (BMP-4, BMP-2 and BMP-6), PlGF, hepatocyte growth factor (HGF), TGF-beta isoforms, connective tissue growth factor, SDF-1α, stem cell factor (SCF), interleukin-3 (IL-3), and the peptides, adrenomedullin and apelin [11, 28, 62, 133–135]. Inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), can also markedly affect vascularization. Important cytokines that affect mural cell responses include PDGF isoforms, particularly PDGF-BB and epidermal growth factor (EGF) isoforms such as HB-EGF and neuregulin isoforms. Sources of these growth factors include ECs, mural cells, fibroblasts, circulating stem cells, mast cells, macrophages, and neutrophils [2, 23, 136]. Growth factor and cytokine networks between ECs, mural cells, and their cellular neighbors such as tissue parenchymal cells (keratinocytes, other epithelial cells, etc.), leukocytes, and stem cell progenitors play critical roles in how ECs are affected by other cell types and how ECs affect adjacent cell types. Increasingly, blood vessels are known to affect tissue development not only by delivery oxygen and nutrients, but also by delivery of growth factors, cytokines, and peptides to the developing tissue microenvironment.

It is clear that a detailed understanding of the specific role of particular growth factors, cytokines, and peptides on ECs and mural cells is critical to our understanding of vascular development, the role of the vasculature in disease states and our ability to manipulate the vasculature in applications such as tissue engineering. One of the difficulties of this work is that many of the approaches to test the functional role of these factors utilize in vivo assays. In vitro strategies are also used, but in most of all cases, these assay systems have employed serum-containing media, making what the cell types are actually exposed to unclear. Thus, it is critical for investigators to perform EC and mural cell assays under defined media conditions so that the additives to a particular system that affect biological responses are known. This is one of the key strategies that my laboratory has employed to investigate EC tubulogenesis, EC–pericyte tube assembly, and maturation events under serum-free defined conditions [50, 62, 80].

In recent work, we defined serum-free conditions that would support human EC tubulogenesis as well as pericyte recruitment to EC-lined tubes with concomitant

vascular basement membrane matrix assembly in 3D collagen matrices [62]. Despite the presumed ability of VEGF and FGF to support such vascular morphogenic responses, in fact, under these defined conditions (without serum), this combination of factors failed to support tube morphogenesis or survival [62]. After years of screening known recombinant growth factors (as single, double, or triple combinations of factors), we identified a combination of three factors that dramatically induces the formation of EC-lined tubes in 3D collagen matrices in the absence or presence of added pericytes [62]. The factors that support human vascular tube morphogenesis are the combination of SCF, interleukin-3 (IL-3), and SDF-1α [62] (Figs. 6.3 and 6.4). Of great interest is that we were able to replace SCF with either macrophage-CSF (M-CSF) or Flt-3 ligand (Flt-3 L), suggesting that there is functional redundancy in the activation of receptor tyrosine kinases for these ligands (i.e., c-Kit for SCF; CSFR1 for M-CSF; and Flt-3 for Flt3L) that work in conjunction with IL-3 and SDF-1α as well as their receptors (IL-3Rα and CXCR4, respectively) to support the morphogenesis response [62]. Thus far, we have not identified substitutes for IL-3 and SDF-1α. Importantly, these combinations of hematopoietic stem cell cytokines are able to control vascular tube morphogenesis as well as facilitate pericyte recruitment to stabilize tubes by controlling maturation events (as evidenced by narrow tubes and basement membrane matrix assembly) [62]. Although these studies were performed with human ECs, they were confirmed using embryonic quail venous explants in which the same combination of SCF, IL-3, and SDF-1α was demonstrated to induce vessel sprouting and tubulogenesis [62]. Furthermore, the receptors for these ligands were found to be expressed by these vessels during quail vascular development (embryonic ECs from other species express the receptors also). Furthermore, inhibitors delivered in vivo (directed to the ligands or receptors) resulted in the blockade of quail vascular development with vessel abnormalities and hemorrhage [62].

Additional studies were performed with human ECs to assess how these factors might control EC sprouting behavior in 3D matrices. As discussed previously, VEGF and FGF failed to induce sprouting on their own or in combination [62]. Thus, VEGF activation of VEGFR2, long considered to be the key event in EC sprouting [137], is insufficient on its own to control EC tip cell formation and EC invasive behavior. When VEGF is added with SCF, IL-3, and SDF-1α, these combinations of factors strongly facilitate EC sprouting [62]. Thus, VEGF alone is insufficient in our serum-free-defined models utilizing human ECs and 3D matrices to stimulate angiogenic sprouting.

Since VEGF may not directly affect EC tube morphogenesis and sprouting, the question was raised in terms of what its major influence is. As discussed earlier, VEGF is an important regulator of arterial specification and formation during vascular development. One possibility is that VEGF is actually required for EC specification in general and thus, without VEGF, ECs do not properly develop or undergo morphogenesis. This issue has not been investigated in sufficient detail in part because it has generally been assumed that its primary influence is on vascular morphogenesis. To further address potential roles of VEGF and FGF in our serum-free-defined in vitro model, additional experiments were performed. We considered

the interesting possibility that VEGF may actually act upstream of hematopoietic stem cell cytokines to prepare or "prime" ECs for morphogenic events that are driven by the hematopoietic factors. Our studies strongly suggest that this may represent the major biological influence of VEGF and FGF, as both singly and in combination the factors strongly prime ECs for both tube formation and sprouting events (Table 6.1) [62]. In fact, after VEGF and FGF priming, hematopoietic factors markedly stimulate not only tube morphogenesis but also sprouting events in 3D collagen matrices. In contrast, if VEGF and FGF are utilized to prime the ECs and then VEGF and FGF were added during the morphogenesis assay (in the absence of hematopoietic cytokines), this combination of factors does not allow for morphogenesis or sprouting responses. If hematopoietic factors are added first as primers, followed by the addition of VEGF and FGF in the morphogenesis assay, the ECs fail to form tubes or survive in 3D matrices. This critical experiment shows that VEGF and FGF act upstream to facilitate EC responses to downstream promorphogenic hematopoietic stem cell cytokines, while reversing their order does not promote tube morphogenesis or sprouting responses (Table 6.1) [62]. Thus, VEGF and FGF stimulate EC morphogenesis responses to hematopoietic stem cell cytokines, but not vice versa. Importantly, we demonstrated that VEGF and FGF act as priming agents in part due to their marked ability to induce upregulation of hematopoietic cytokine receptors, including c-Kit, IL-3Rα, CXCR4, CSFR1, and Flt-3, on ECs (Table 6.1) [62]. This remarkable induction occurs on both human ECs and embryonic quail vessels, which accelerate the ability of ECs to form tubes and sprout during morphogenic events. Both types of ECs respond to the hematopoietic factors and undergo morphogenesis even in the absence of VEGF/FGF priming due to the fact that the hematopoietic receptors are expressed normally by ECs.

These new findings illustrate a point that has not been sufficiently addressed in the vascular biology field: that is, the necessity of critically assessing the functional role of relevant growth factors and cytokines that have been implicated in developmental and pathological vascularization events (Table 6.1). Only by using very well-defined systems in vitro and in vivo can such questions be appropriately addressed. The problem with the use of in vivo models here is that it is not possible to eliminate growth factors that are endogenous to the tissue. So, when it was concluded that VEGF was the primary promorphogenic factor that stimulates tubulogenesis and sprouting, it was not possible to determine when it acts to affect these processes or whether it acts in conjunction with other unknown factors. In fact, our detailed studies in vitro clearly demonstrate that VEGF and FGF combinations under defined serum-free conditions fail to stimulate tubulogenesis or sprouting in the absence of hematopoietic stem cell cytokines [62]. These new advances in our understanding of growth factor and cytokine control of vascular morphogenesis have many implications, particularly in the area of vascular therapeutics in cancer, retinal disease, and diabetes. For example, blockade of hematopoietic stem cell cytokine signaling (to affect vascular morphogenesis) may be crucial to properly interfere with vascularization within tissues [62] and could be added in addition to antagonists directed toward VEGF to properly regulate or interfere with these processes in a disease context.

Table 6.1 Emerging functional roles for cytokines, growth factors, and peptides during vascular morphogenesis

Cytokine/growth factor/peptide	Functional role during vascular morphogenesis	References
VEGF-A/FGF-2	A recent study reveals a major role for VEGF and FGF-2 in priming ECs to respond to hematopoietic cytokines which directly stimulate vascular tube morphogenesis. VEGF/FGF-2 upregulates hematopoietic cytokine receptors on ECs to facilitate subsequent tube morphogenic responses	Stratman et al. [62]
SCF/IL-3/SDF-1α	Combinations of hematopoietic stem cell cytokines have been demonstrated to support human EC tube morphogenesis in serum-free defined media and in 3D extracellular matrices. FGF-2 facilitates their action when added in combination with them. Under the same conditions, but without hematopoietic cytokines, VEGF-A and FGF-2 combined together fail to support these events	Stratman et al. [62]
BMP4/BMP2	Recent work suggests a role for BMPs, including BMP-4 and BMP-2, in controlling angiogenic sprouting events. Evidence was presented suggesting a role for BMPs (BMP2b in Zebrafish) in sprouting from venous vessels	Wiley et al. [146] Stratman et al. [62]
SDF-1α/Apelin	Recent work suggests a role for SDF-1α and its receptor, CXCR4, as well as the bioactive peptide, apelin, and its receptor, APJ, in controlling angiogenic sprouting events. SDF-1α also promotes EC sprouting and tube morphogenesis in conjunction with other hematopoietic cytokines such as SCF and IL-3	Saunders et al. [37] Del Toro et al. [135] Strasser et al. [144] Stratman et al. [62]

Molecular Control of Angiogenic Sprouting

Angiogenesis, the sprouting of ECs from preexisting vessels to facilitate the formation of new vessels, is a major step in vascular morphogenesis. Current models of angiogenic sprouting focus on signaling from the EC that leads the invasive front, a cell termed the EC tip cell [137]. EC stalk cells which follow behind tip cells during invasion are known to form the lumen compartment and, thus, control tube assembly during angiogenic sprouting [1, 14]. One of the more interesting aspects of angiogenic sprouting events is that only a subset of ECs undergoes morphogenesis while others stay behind in EC monolayers in a vessel wall. Recently, the term phalanx cell was coined for an EC quiescent cell in the vessel wall which is stabilized and is not undergoing morphogenesis [138].

Increasingly, it is becoming apparent that these events are highly dynamic and that ECs can assume different functions at varying times during the morphogenic

process [20, 139]. Interestingly, angiogenic sprouting events occurring during development result from vessels that are newly formed (e.g., intersegmental vessel sprouting in developing zebrafish) and which are not stabilized. This is likely to be distinct from angiogenic sprouting events from a stabilized vessel (such as in adult animals) with an intact basement membrane matrix that needs to be degraded to initiate the sprouting process. A fascinating aspect of angiogenic sprouting is that the process rapidly creates EC heterogeneity in a population of cells that was relatively uniform in the wall of a vessel. How ECs assume the tip cell position during this process is not well understood. Also, the mechanisms that control how cells follow this invasive cell and develop as stalk cells and other specialized cells as the vessel sprouts, forms a lumen and matures, need to be investigated in much greater detail [140]. How many types of ECs are necessary to form such sprouts with an appropriate connection to the parent vessel? This question is important to answer and needs to be addressed in more molecular detail. EC tip cells have been described as having high VEGFR2 or VEGFR3 expression, secretion of Notch activators such as DLL4, and an inability to form a lumenal structure [140, 141]. Thus, they have been defined as cells that follow gradient paths of ECM- or cell-associated VEGF to control the direction and magnitude of the sprouting response [14]. Importantly, two negative regulators of these events, DLL4 and soluble VEGFR1 (which traps and blocks the activity of VEGF), have been identified that inhibit EC sprouting events [12, 53, 140, 142, 143]. It is clear that these molecules are important, but they are insufficient to explain the complexity of EC sprouting responses. As stated previously, using defined serum-free models of vascular tube morphogenesis and sprouting, VEGF is not capable of directly controlling these responses except as a priming agent that prepares ECs for morphogenesis, a process controlled by other promorphogenic cytokines such as SCF, IL-3, and SDF-1α (Table 6.1) [62].

Along these lines, a number of recent DNA microarray analyses of EC tip cells or invading ECs and have identified new apparent markers, such as the SDF-1α receptor, CXCR4, and the apelin receptor, APJ (Table 6.1) [135, 144, 145]. Interestingly, we first showed that SDF-1α induced human EC invasion and sprouting into a 3D matrix using a model that also required the addition of phorbol esters to support EC survival [37]. In addition, recent work reveals a role for BMP4 and BMP2 in angiogenic sprouting responses in vitro and in vivo (Table 6.1) [62, 146]. Other studies by several laboratories demonstrate a critical requirement for proteinase activity, as well as integrin-dependent ECM interactions, for EC sprouting [37, 84, 97]. In particular, MT1-MMP controls EC sprouting into 3D collagen or fibrin matrices [37, 97], while the integrin α2β1 controls sprouting into collagen matrices [84] and the α5β1 and αvβ3 integrins control sprouting into fibrin matrices [84]. Blockade of MT1-MMP using protein or chemical inhibitors or siRNA suppression completely interferes with invasion into 3D matrices [37, 84]. Furthermore, siRNA suppression experiments demonstrated that Cdc42 and Rac1 were necessary for EC sprouting while suppression of RhoA did not block sprouting [58]. In addition, the Cdc42 effectors, Pak2 and Pak4, were demonstrated to be critical for EC sprouting responses in 3D collagen matrices [58]. Thus, there are many important molecules that control EC sprouting activity. There is a clear need to investigate these events

in much greater molecular detail than have been described to date. It is important to discern the molecular and signaling differences that characterize EC tip cells from stalk ECs or other ECs that are not invading at the leading front of sprouts. The molecular characterizations of EC tip cells recently reported using DNA microarray analyses are important additional steps to define these differences.

Notch signaling also has been strongly implicated as a fundamental regulator of EC sprouting since disruption of Notch signaling leads to increased numbers of EC tip cells, suggesting that more tip cells are generated if inhibitory signals are missing [12, 53, 143]. How this occurs is not clear at the level of cytoskeletal-, integrin-, Rho GTPase-, or MMP-dependent signaling, which are also necessary during these events. Additional investigation is necessary to understand how Notch-DLL4 signaling interfaces with the other signals that actually mediate the EC invasion response. Importantly, the Notch pathway is a modulator of sprouting, while other molecules and signaling pathways are required to directly control EC sprouting. Recent studies have also implicated Ephrin–Eph signaling in EC sprouting events, and these events have been linked with arteriovenous development [42, 147, 148]. Interestingly, in zebrafish embryos, VEGF and EphrinB2 suppress ventral sprouting to inhibit vein formation and promote arteriogenesis, while EphB4 expression promotes ventral sprouting events to promote the formation of the first embryonic vein (i.e., caudal vein) [42]. Importantly, bidirectional signaling from ECs expressing either EphrinB2 or EphB4 [149] is necessary to create the ventrally placed caudal vein, which sprouts from the dorsal aorta. It is important to characterize these responses in greater molecular detail and also to understand how ECs are able to respond to these factors to form separate vessels that are required for vascular development and embryonic life. Finally, Wnt signaling has been implicated in EC sprouting events (by regulating DLL4-Notch signaling) [150], and this area is also important to assess in conjunction with other modulators and required molecules during these processes [25, 151].

Molecular Basis for Arteriovenous Specification and Interconnectivity

The findings that arteries form first, followed by veins, and then lymphatics in sequence [1], and second that specific molecules such as Notch isoforms (Notch 1 and 4), Ephrin B2, and neuropilin-1 characterize arterial fate while EphB4, neuropilin-2, VEGFR3, and CoupTFII characterize venous fate [9, 13, 21], were major advances in our understanding of how arteries, capillaries, and veins arise during vascular development [1]. Importantly, a fundamental signaling pathway involving the sequential action of SHH, VEGF, and Notch was shown to be required for arteriogenesis, which is necessary for all vessel types to properly form [9, 124]. Another important observation was that Notch signaling, which is activated through ligands such as DLL4 and Jagged, leads to Notch-dependent transcriptional mechanisms through Hey and Hes transcription factors in arterial ECs, while this occurs to a

much lesser extent in venous or lymphatic ECs [9, 13, 21]. Interestingly, disruption of SHH, VEGF, or Notch leads to decreased arterial fate and concomitantly increased venous fate [9, 124]. These data suggest that arterial and venous specification signals appear to be opposing. In support of this concept, suppression of the venous EC transcription factor, CoupTFII, which represses Notch expression in venous ECs, leads to embryonic lethality due to a lack of venous specification and concomitant increase in arterial specification [124, 152].

Interestingly, arteries are marked by EphrinB2, while veins are marked by EphB4 [9, 110, 112, 147]. Ephrins and Ephs are major signaling receptors that control the development of many tissues and cell types, including the vascular and nervous systems [15, 111, 153–155]. Ephrins and Ephs exhibit both forward signaling in which Ephrins act as ligands to activate Ephs, and reverse signaling in which Ephs activate Ephrins [111, 149]. Cell–cell interactions are necessary for these signaling events to occur. Knockout of either of these markers in zebrafish or mice leads to an embryonic lethal phenotype characterized by abnormal interconnections between the developing arterial and venous tube networks. Of great interest is that ECs in these knockout situations are able to form EC tube networks but they are unable to properly communicate through abnormal interconnections. Thus, the development of arterial and venous EC fate does not appear to depend on EphrinB2 and EphB4 directly. Tubulogenesis can occur [9], but with defects in appropriate arteriovenous communication. As mentioned previously, tubulogenesis creates vascular guidance tunnels in which homotypic EC–EC interactions occur. Thus, both forward and reverse signaling through Ephrins and Ephs should be possible within these matrix spaces (mimicking a 2D matrix surface) to regulate proper arteriovenous communication events. Importantly, repulsive cell–cell interactions could be critical in facilitating the appropriate distribution of EphrinB2 expressing ECs to the arterial side and EphB4 expressing ECs to the venous side. Interestingly, at early stages of vessel assembly, ECs expressing both of these markers are intermixed in the first arteries and then sort themselves to develop the venous system [42].

The expression of ephrinB2 in the arterial system appears to be Notch-dependent, and when Notch is inhibited, venous markers such as EphB4 and VEGFR3 are induced [9]. Notch activity plays an important role in controlling the specification of arteries as well as the patterning of these vessels. Like ephrins, Notch does not control the positioning of the developing aorta and cardinal vein; it controls the specification of these lineages and their ability to properly connect and intercommunicate during development. This suggests that the arteriovenous specification pathway is primarily concerned with patterning and interconnections rather than with basic processes such as EC motility, invasion, and tubulogenesis [9]. Stating this point in a different way, the Ephrin–Eph and Notch pathways appear to be regulators and modulators rather than required molecules for the important EC behaviors necessary to form vascular tube networks. Interestingly, Notch is known to inhibit EC proliferation [53] and sprouting, particularly through the action of DLL4, which is also primarily expressed in arteries [143]. As with Ephrin/Eph signaling, Notch signaling is controlled by cell–cell interactions [13]. The critical role of Notch signaling in the vasculature appears to be primarily mediated by Notch1,

while Notch4 plays a secondary role working in conjunction with Notch1 [12, 13, 140, 156]. Notch4 is expressed in arteries, like Notch1, but shows a much more EC-restricted expression pattern compared to Notch1 [156]. Important questions for the future are how Notch1 and Notch4 work together to control arteriovenous differentiation during development and how such interactions affect vascularization responses in the context of disease.

Another critical point is that Notch signaling is controlled downstream from SHH and VEGF [124]. These two factors play a more fundamental role in EC specification, and therefore they show greater effects on vascular morphogenesis, compared to Notch and Ephrins, and arterial specification. SHH and other hedgehog isoforms, such as IHH, which are produced from endoderm, act to control EC specification and vascular morphogenesis [157]. A number of studies have implicated SHH as being important in EC tubulogenesis at this stage of development [157]. The influence of SHH on tubulogenesis may be due to EC specification issues rather than direct effects on tubulogenesis signaling. Such issues need to be pursued further. Also, VEGF isoforms clearly influence vascular morphogenesis in terms of sprouting and tubulogenesis, particularly when evaluated in vivo. Local administration of VEGF during avian vascular development has been shown to induce marked vessel–vessel fusion creating large mispatterned vessels [158]. Different VEGF isoforms affect vascular patterning during development as well as during pathologic angiogenesis [159]. In general, VEGF isoforms that strongly interact with ECM (i.e., VEGF165, VEGF188) induce more complex and branched vasculatures, while VEGF121, which shows less affinity for ECM, induces larger, dilated vessels [107, 159]. It has been suggested that VEGF's ability to bind heparan sulfate facilitates its ability to be presented in a gradient fashion by ECM to affect cell surface interactions during developmental events (i.e., such as the retina where it controls EC sprouting during postnatal development) [137]. Interestingly, VEGF can also be cleaved by proteinases such as MMPs and plasmin, and this cleavage is capable of creating a VEGF121-like product that no longer associates with heparan sulfate [107]. Thus, ECs and other cells such as tumor cells can modulate the activity of VEGF through proteolytic action. Also, when VEGF associates with ECM components, it appears to have distinct biological activities that affect vascular morphogenesis [106].

In summary, VEGF is a key regulator of arteriogenesis that acts upstream of Notch signaling to control arteriovenous specification as well as vascular morphogenesis. A recent study revealed that VEGF's primary role may be to prime (or prepare) ECs for vascular morphogenesis in response to other factors [62]. In some respects, this concept is similar to that suggesting that VEGF is upstream of Notch and Ephrin signaling. Thus, VEGF action prepares ECs to express, encounter, and signal through key downstream factors and receptors to control artery formation as well as proper arteriovenous interconnectivity. It is intriguing that with all of the factors discussed, there are necessary positive and negative signals that require a delicate balance of inputs to properly form and pattern the vasculature. When this balance is disrupted in either direction, vascular abnormalities appear, a concept which suggests considerable complexity, particularly in the context of targeted

therapeutics to the vasculature. Various proangiogenic or antiangiogenic treatments may shift the balance too far in one direction. A treatment regimen may alleviate a particular abnormality in the diseased tissue but may then inadvertently create a new abnormality within the vasculature. A very important future direction is to study in detail the molecular balances that control vascular development, arteriovenous specification, and angiogenic responses in diseases such as cancer, tissue regeneration, and diabetes.

Transcriptional Control of Vascular Development and Postnatal Vascularization

Considerable new information exists concerning how the EC lineage develops and how transcriptional regulators control EC specification and differentiation along its distinct lineages [19, 160]. A major direction that has become increasingly utilized by investigators is to identify EC-specific genes and to determine how these molecules affect EC function [19, 160–166]. In addition, the transcriptional controls that affect the expression of these EC-specific genes have been investigated in detail. Promoter elements that control EC-specific gene expression have been identified and a 44-bp consensus transcriptional enhancer is found in the majority of these genes [19, 161]. Within this DNA sequence, there are overlapping binding sites for Ets family and Forkhead family transcription factors [19, 160]. In particular, Etv2 of the Ets family and FoxC2 (and also FoxC1) bind to this consensus sequence to be able to control this expression [19, 161]. Ectopic expression of these factors in various species and cell types leads to EC-specific gene expression. Some key EC-specific genes that are typically examined are VE-Cadherin, CD31 (PECAM), von Willebrand factor, and VEGFR2. Etv2 is found to be expressed at very early stages of vascular development and then is rapidly shut off, suggesting that it plays a key role early in specification of the EC lineage. Knockout of Etv2 leads to lethality by E9.5 with a complete loss of EC specification and vascular morphogenesis [161]. After E9, other genes must take over to control these genes and to maintain their expression during later vascular development and in postnatal life. Other Ets members, such as Erg, have been shown to control expression of genes such as VE-cadherin and VEGFR2 [167, 168], and this gene is expressed in ECs during vascular development from embryogenesis into adulthood. Using Etv2 (i.e., Etsrp in zebrafish), for example, a series of new EC-specific genes have been identified in various species [161, 162, 164, 165]. Knockdown of Etv2 reduces these genes while increased expression of Etv2 increases expression of such genes. A similar approach is ongoing with Erg, which has allowed for a molecular dissection of similar and distinct genes that Etv2 and Erg control. It is of great interest to examine if genes such as Etv2 are reactivated during pathologic angiogenesis and whether this is in part responsible for abnormalities that might exist under such conditions, particularly if the expression patterns of Etv2 are altered (i.e., it is not properly downregulated). One fascinating question is why Etv2 is downregulated and whether it is

functionally relevant. For example, downregulation may be important for later steps in vascular maturation, such as response to flow forces, pericyte recruitment, and EC–pericyte intercellular communication events necessary to promote vascular stabilization.

Other key transcriptional regulators of EC development in the blood vasculature are Tal1 (SCL) (a marker of angioblasts), Lmo2, the Forkhead proteins, FoxC1/C2 (which work in conjunction with Etv2), and FoxO1, Hey 1/2 (Notch activated transcriptional regulators), the Kruppel-like factor, KLF-2, Sox7 and Sox17, and finally, CoupTFII, a key regulator of venous EC development [19, 160]. Mouse knockouts and, in many cases, zebrafish morpholino suppression experiments have revealed critical roles for each of these transcription factors at various stages of vascular development. A few other transcription factors, such as the Ets factor, Fli1, Gata2, and Runx1, have interesting overlapping effects on both vascular and hematopoietic development [19, 169], the latter process being the more affected in knockout animals of these genes. Fli1, Gata2, and Runx1 appear to be expressed in angioblasts, which may give rise to either ECs or hematopoietic stem cells. There is considerable new evidence showing that hematopoietic precursors can arise from ECs within the wall of the dorsal aorta as well as other locations in the developing embryo [17, 18, 169]. Another important EC transcription factor is Prox1, a regulator of lymphatic vascular development [170].

One of the important concepts in the transcription factor field is the influence of combinatorial effects of these factors to control very specific transcriptional events spatially and temporally in the right cells at the right time [19, 161]. ECs are clearly distinct within different tissues due to the influence of adjacent cells in the parenchyma of different tissues through influences of growth factors/cytokines/other mediators and/or unique heterotypic cell–cell interactions. The ECs are also distinct due to their anatomical locations in conjunction with differences in the flow and pressure forces that are applied. When flow is altered or disturbed as a result of a disease such as atherosclerosis, genes such as VCAM-1, which promotes monocyte adhesion and the downstream sequelae of atherosclerotic plaques, are induced [77]. But interestingly, flow-induced transcription factors such as KLF-2 [74] are induced in ECs to respond to these abnormal flow conditions [77]. It is very important to understand how these transcription factors control each of the different stages of vascular development and then to understand which of these are relevant to our understanding of EC function in postnatal life. The vasculature is frequently abnormal in diseased tissues, leading to the critical question of whether reactivation of embryonic EC transcriptional programs acting inappropriately in terms of the timing and spatial locations of their expression and activity could be responsible. Because of the issue of combinatorial transcriptional control, and because many of these transcriptional pathways overlap, it is possible that under disease conditions transcriptional abnormalities may be present in ECs responding to the diseased tissue. These types of analyses will be essential in future approaches to understand vascular abnormalities that directly and indirectly affect disease initiation or outcome.

Another fascinating question in this regard is how the EC-specific transcriptional regulatory program contributes to the individual steps that create the functional vasculature. For example, to what extent do EC-specific genes control processes such as: (1) proliferation; (2) arteriovenous identity; (3) cell invasion directly related to sprouting responses; (4) tubulogenesis to form vascular networks; (5) EC–EC junctions to stabilize tubes, control vascular permeability, and respond to flow; and (6) ECM remodeling, such as vascular basement membrane matrix assembly? These questions largely remain unanswered but there are examples of EC-specific genes that directly affect these processes. Thus far, most of these genes are involved in EC–EC junctional interactions that relate to vessel stability in response to flow and pressure, a unique property of ECs compared to other cell types. The role of EC-specific genes in the other processes is much less clear. For example, Etv2 controls the expression of VE-cadherin and PECAM, two EC–EC adherens junction proteins, claudin-5, an EC–EC tight junction protein, VEGFR2, a key receptor for VEGF and a protein, along with VE-cadherin and PECAM, that regulates flow-induced signaling in EC monolayers in the vessel wall, and Klf-2, which also controls flow-induced EC signaling. VE-cadherin and claudin-5 also play a key role in the molecular control of vascular permeability [171] to regulate this critical process during developmental and pathologic vascularization. Interestingly, the important role of growth factor receptors such as VEGFR2 and Tie-2 (which certainly are expressed at high levels in ECs, but can be expressed in a few other cell types also) in vascular development are well known and these certainly relate to various functions of ECs including proliferation, survival, permeability, priming, vessel stability, and morphogenic responses. Much additional work is necessary to examine how EC-specific genes control specific vascular functions and how transcription factors influence normal developmental and postnatal vascularization events, as well as pathologic neovascularization. However, it is important to point out that many critical regulators of vascular development that control events such as proliferation, arteriovenous specification and interconnectivity, tubulogenesis, sprouting and maturation, are not mediated by EC-specific genes.

Finally, there has been considerable attention placed on examining the role of microRNAs (miRNAs) in EC and mural cell function [172–176]. It is clear that in many biological contexts that miRNAs are fundamental regulators of cell behavior and function. In many cases such as with miR126, there is cell-type specific expression of miRNAs. This miRNA, located in intron1 of the EC-specific gene Egfl7 (and thus its expression is controlled by the Egfl7 promoter) [177, 178], is selectively expressed in ECs. Interestingly, knockout of miRNAs in mice that affect vascular cell behaviors (ECs or mural cells) have not led to embryonic lethality [172]. The vasculature of miR-126 knockout mice is leaky and clearly abnormal, and angiogenesis is impaired [177, 178]. In zebrafish, the expression of miR-126 is interestingly flow-dependent and morpholino knockdown is associated with lethality [179]. In general, miRNAs downregulate gene expression and typically do so by repressing the expression of multiple genes, thus they are complex regulators of cell function. In the cases of EC-expressed miRNAs, such as miR-126 [177, 178], miR-218 [180], miR-132 [181], miR-221/222 [182], and the miRNA cluster composed

of miR17-92 (particularly miR-92a) [183], these miRNAs appear predominantly to modulate postnatal angiogenic events (i.e., inhibit angiogenesis) [172, 182]. With each of these miRNAs, understanding of their gene targets is a critical question. In the case of miR-126, it appears to suppress the expression of Spred1 (a sprouty-like inhibitor of tyrosine kinase signaling) and PIK3R2, an inhibitory subunit of PI-3 kinase [172, 177, 178]. Receptor tyrosine kinase activation in conjunction with activation of PI-3 kinase are known to play critical roles in fundamental processes related to EC function, including proliferation, survival, and morphogenesis. miR-218 inhibits Slit-Robo signaling and is expressed in an intron involving the Slit1 and Slit2 genes [180]; miR-132 inhibits p120RasGap to suppress Ras/Raf signaling [181]; miR-221/222 suppresses c-Kit to affect EC stem cells [184] and morphogenic responses, and miR-92a suppresses integrin $\alpha 5$ and αv expression [183], which have been implicated in both developmental and postnatal angiogenic responses. Thus, it appears that a series of EC-expressed miRNAs modulate EC responses during both developmental and postnatal injury responses. The important point is that these appear to be primarily modulators and do not appear to be strictly required. As discussed previously, vascularization responses are clearly a balancing act of negative and positive signals [185, 186], and this molecular balance is a fundamental aspect of not only vessel formation and maturation events, but also of most, if not all, biological responses.

Interestingly, the miR-143/145 cluster has been identified to be expressed in mural cells [172] and, in particular, has been shown to be involved in vascular smooth muscle differentiation and function. Furthermore, it has been reported to affect blood pressure control (although knockout of these miRNAs does not interfere with vascular development) [187, 188]. This miRNA cluster is known to be expressed through the serum response factor/myocardin pathway that is responsible for smooth muscle development and differentiation [187]. It appears that miR-145 has the dominant influence and is capable on its own of inducing vascular smooth muscle differentiation [172]. It is known to suppress the expression of a variety of genes, including Rho GTPases, slingshot homolog 2, cofilin, and adducin [172]. Interestingly, miR-145 was also detected in brain pericytes and was able to affect migratory behavior on 2D surfaces [189]. Finally, various miRNAs that affect cardiovascular function have been detected in plasma and, furthermore, they have been shown to be expressed at varying levels in human diseases such as coronary artery disease and diabetes [190, 191]. It is not clear whether these circulating miRNAs are biologically active, but it is certainly possible that endogenous carriers of miRNAs exist, and they may allow uptake into cells of miRNAs that then regulate gene expression of autocrine or paracrine factors. Also, there is considerable interest in these molecules as therapeutic agents in either anti-miR configuration (which pairs with and blocks the miRNA) or the miRNA itself to suppress gene expression. Similarly, there is great interest in siRNA agents as therapeutic agents within the vasculature. As is well appreciated, it is quite difficult to appropriately target such agents to the correct cells at the right time and place. Again, the difficulty here is that the vasculature is properly balanced by a series of positive and negative signals, and it is quite easy to cause abnormalities by affecting the pathways too far in either direction.

Conclusions and Future Directions

Dramatic advances in our understanding of how blood vessels form, mature, and are altered in human diseases have occurred over the past two decades. It is clear that major reasons for this are the combined use of techniques and models using both in vitro and in vivo approaches. The development of in vivo models, particularly using zebrafish, has strongly enhanced our molecular understanding of pathways that control vessel formation and processes such as arteriovenous specification and transcriptional control of vascular development. Mouse knockout models have demonstrated the critical importance of growth factors such as VEGF and angiopoietins as well as their receptors during these events and have contributed greatly to our understanding of the role of mural cells in vascular function. In vitro approaches have contributed to identifying many critical EC or mural cell molecules that control various cell behaviors relevant to these processes. Great progress has occurred in developing in vitro models that allow for EC tubulogenesis, pericyte recruitment, and EC–pericyte interactions that lead to vessel maturation events. More and more evidence demonstrates that particular in vitro 3D morphogenesis models very accurately predict the molecular mechanisms that control these fundamental events in vivo. It has been repeatedly shown that molecules and signaling pathways that act in vitro also are operative in vivo during vascular tube morphogenesis and stabilization. Importantly, as with all systems, it is critical that in vitro and in vivo systems be developed in a systematic and detailed manner and that the work is validated using multiple approaches. Overall, in vitro approaches represent invaluable tools that are necessary to dissect out the complex molecular interactions that occur within ECs and mural cells during vascular wall assembly. It is possible to dissect out the contribution of each cell separately and then in combination to understand their complex interactions necessary to form vessels and then maintain them. Increasingly, in vitro models will be necessary to elucidate issues such as these where technologies in vivo are more limited and interpretations made difficult when multiple molecules and signaling pathways need to be examined. Finally, it should be emphasized that laboratories with expertise utilizing in vitro and in vivo systems should be increasingly working together to elucidate the molecular basis for these events. The speed by which such discoveries can be made and translated into new molecular therapies for vascular disease will be markedly accelerated through such collaborative efforts.

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Chapter 7
Therapeutic Angiogenesis for Critical Limb Ischemia: Complex Mechanisms and Future Challenges

Yihai Cao

Introduction

Critical limb ischemia (CLI) is defined as ischemia that threatens the survival of limbs which occurs when the arterial blood flow is not sufficient to meet the metabolic demands of the resting tissue and muscle. CLI is the most common indication for revascularization surgeries and interventions, including bypass and endovascular revascularization [1, 2]. CLI occurs in 1–2% of patients greater than 50 years old with peripheral arterial disease (PAD) [3]. In contrast to the relatively benign course of mild to moderate claudication associated with PAD, CLI leads to amputation unless arterial perfusion is recovered. Rest pain, ischemic ulcers, and gangrene are additional major clinical manifestations of CLI [4, 5].

Functional recovery of ischemic limbs in patients suffering from CLI is entirely dependent on establishing collateral arterial networks to sufficiently reperfuse oxygenated blood to the affected tissues. Sufficient reperfusion in ischemic muscle tissues is accomplished by the process arteriogenesis and angiogenesis. The former refers to remodeling and perfusion of preexisting arterioles, whereas the latter is defined as the process of growing new blood vessels [6, 7].

In response to an ischemic insult, angiogenesis and arteriogenesis occur simultaneously and are triggered by hypoxia-induced angiogenic and vascular remodeling factors [8–10], such as vascular endothelial growth factor (VEGF), which is known to be highly upregulated in ischemic tissues [11–17]. While VEGF promotes angiogenesis and arteriogenesis [16, 18], it also potently induces vascular permeability [19, 20], which is potentially harmful for functional recovery of the damaged muscular tissues. Consistent with this notion, delivery of VEGF as a proangiogenic factor to CLI patients has not yielded promising outcomes [21–26]. One of the

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reasons for this may be the dual activity of VEGF. It has been reported that the angiogenic and vascular permeability signaling by VEGF is divergent [27, 28]. So it may be possible that separating the VEGF-induced angiogenesis from its permeability-inducing effect will allow for the future success of proangiogenic therapy. This issue might be resolved by genetically modifying the VEGF molecule to enhance its angiogenic activity and to deplete its permeability effect. Alternatively, combinations of VEGF with other vascular remodeling factors such as platelet-derived growth factor-B (PDGF-B) and angiopoietin-1 (Ang-1) may provide better options for future therapy [29, 30].

In addition to VEGF, many other growth factors and cytokines are involved in promoting angiogenesis and remodeling the existing vasculature [6, 10, 31–33]. While many of these angiogenic modulators target vascular endothelial cells (ECs), several others are specific for perivascular cells, including pericytes (PCs) and vascular smooth muscle cells (VSMCs), which are crucial for vascular maturation and remodeling [34]. The processes of angiogenesis and arteriogenesis involve many detailed steps regulated by distinct sets of growth factors or cytokines, discussed below. Therefore, combinations of various angiogenic factors may provide an optimized rationale for therapeutic development [32]. In support of this view, several preclinical studies have already demonstrated that combinations of angiogenic and arteriogenic factors produce superior beneficial effects compared to monotherapy [10, 30, 32], although combination therapy needs to be validated in human trials.

Delivery of proangiogenic factors to human ischemic muscles have not produced the benefits seen in preclinical models [7, 18, 21, 31]. Almost all these trials were conducted as monotherapy, delivering a single angiogenic protein or achieving overexpression of their corresponding genes in the ischemic muscle. Although the fundamental concept of proangiogenic or arteriogenic therapy remains undisputed and the approach is straightforward, it has not successfully been translated into clinical use. Why does this approach produce beneficial outcomes in various animal models of CLI but not in human patients? What are the differences between animal models and human patients? In this review, I discuss these critical issues and reasonably speculate on plausible mechanisms that may underlie the differences between humans and animal models in revascularization of ischemic muscle tissue.

Mechanisms of Angiogenesis and Arteriogenesis in Ischemic Tissue

In the setting of CLI, several tissue responses, including angiogenesis, vascular leakage, and inflammation, are triggered by hypoxia in the affected muscle (Fig. 7.1). Hypoxia potently induces expression of transcription factors, including hypoxia-inducible factor-1α (HIF-1α), which target several downstream angiogenic factors, vasodilators, and hematopoietic modulators in various cell types, including ECs and VSMCs [35–38]. For example, the transcription factor HIF-1α directly targets the VEGF promoter to induce its expression [11, 39]. Thus, the endogenous level of VEGF is elevated in the affected skeletal muscle tissue. The high level of VEGF not

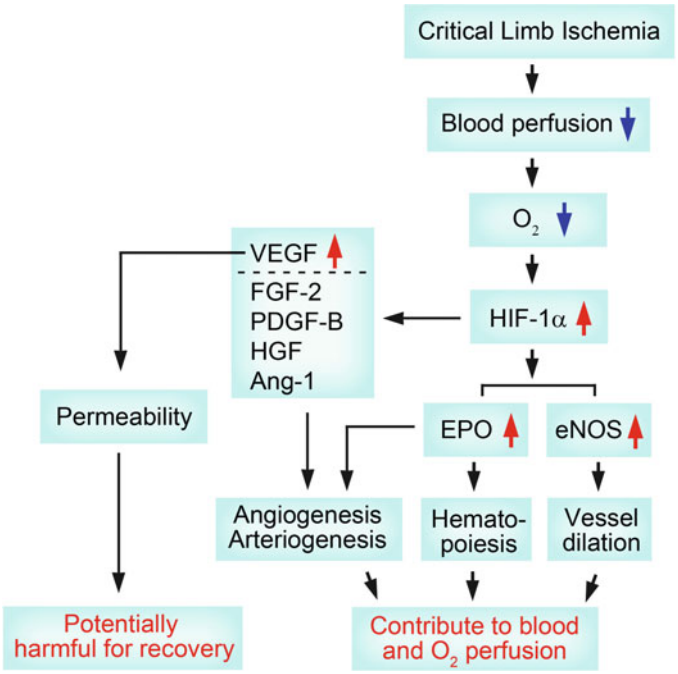

Fig. 7.1 Hypoxia-induced vascular compensatory mechanisms. Hypoxia in the ischemic tissue induces expression of the hypoxia-inducible factor (HIF)-α, which targets VEGF and several other angiogenic factors to induce angiogenesis. Hypoxia-induced EPO and eNOS stimulate erythrogenesis and vasodilation, respectively. While these compensatory responses are beneficial against ischemia, VEGF-induced vascular permeability is potentially harmful for functional recovery of the ischemic tissue

only triggers an angiogenic response but also potently induces vascular permeability, which increases tissue edema and may potentially further damage muscle tissues.

Another compensatory mechanism against tissue hypoxia during CLI is to increase blood perfusion by dilation of the existing vasculature, especially the arterioles that perfuse the muscle. Dilation and remodeling of the existing vessels are probably the most rapid and effective mechanisms against acute tissue ischemia [7, 18, 31, 32]. It is known that tissue hypoxia induces expression of several vascular remodeling factors, including members in the PDGF, hepatocyte growth factor (HGF), and angiopoietin families [40–43]. While most studies demonstrate that activation of HIF-1α-mediated transcriptional regulation is required for hypoxic upregulation of these arteriogenic and vascular remodeling factors [44–48], several studies show mechanisms of HIF-1α-independent activation of signaling systems [47, 49, 50]. For example, a recent study demonstrated that hypoxia decreases the expression levels of tyrosine kinase phosphatases, leading to constitutive activation of the receptor signaling systems of PDGF receptor-β (PDGFR-β) [50]. These factors promote vascular remodeling and maturation by recruiting pericytes and

VSMCs onto the nascent angiogenic vessels [33, 51, 52]. The well-studied PDGF isoform, PDGF-BB, promotes vascular maturation by interaction with PDGFR-β expressed on pericytes and VSMCs. It should be emphasized that angiopoietin 1 and angiopoietin 2 also bind to the same Tie2 receptor expressed mainly on ECs, but cause opposing effects in promoting vascular remodeling and maturation [52]. Angiopoietin 1 recruits pericytes and VSMCs onto the angiogenic vessels, but angiopoietin 2 ablates these perivascular cells from blood vessels, leading to the primitive and immature phenotype of the blood vasculature.

Endothelial nitric oxide synthase (eNOS) is also upregulated by tissue hypoxia [53, 54]. In ECs, eNOS synthesizes nitric oxide, which causes relaxation of VSMCs in arteries by paracrine regulation [55]. Relaxation of VSMCs in arterial vessels leads to vessel dilation, which permits increased blood flow and reduces tissue hypoxia.

A recent study in zebrafish has revealed a new mechanism of the host response to hypoxia [56]. Exposure of adult zebrafish to hypoxia led to circulation of blood cells within the lymphatic system, which under physiological conditions is devoid of blood cells. Perfusion of the lymphatic system with blood cells is accomplished by opening the arterial-lymphatic conduit, a process tightly controlled by VSMCs [56]. Under tissue hypoxia, increased levels of nitric oxide lead to relaxation of VSMCs and ultimately allow blood cells to travel through the arterial-lymphatic conduits into the lymphatic system. If a similar mechanism for lymphatic vessel perfusion exists in mammals, it would probably be the most effective, rapid, and economic mechanism of the host response against tissue hypoxia.

Another tissue response to hypoxia is to generate more red blood cells to help deliver sufficient amounts of oxygen to the ischemic muscle tissue. Hypoxia induces production of erythropoietin (EPO) via HIF-1α transcriptional activation of the EPO promoter, and EPO subsequently stimulates erythrogenesis [35, 36, 57, 58]. Under physiological conditions, EPO is produced from peritubular interstitial cells [35, 59]. To exert biological functions, EPO binds to its receptor (EpoR) leading to activation of the JAK2 signaling cascade [59]. EPO increases or sustains hematocrit by two mechanisms: (1) protecting erythrocyte precursors by inhibiting apoptosis, and (2) increasing the erythrocyte precursor proliferation and differentiation. For example, EPO is required to support erythrocyte precursor survival in colony forming unit-erythroid (CFU-E) and burst forming unit-erythroid (BFU-E) erythrocyte precursor assays [60]. EPO can also act directly on ECs to induce angiogenesis via interaction with the EPO receptor expressed on ECs [61–63]. In addition, according to recent findings, this enhanced hematopoiesis might increase the population of circulating endothelial precursor cells that participate in neovascularization [64–67].

While angiogenesis is a relatively well-characterized process in ischemic tissues, the mechanism of arteriogenesis—the process of remodeling of existing arteriolar networks—remains poorly understood. In CLI, establishment of a functional collateral network in the ischemic muscle tissue is crucial to rescue myoblasts and recover muscle function. There are two mechanisms to reestablish functional arteriolar networks: (1) remodeling of the existing arterioles and (2) outgrowth of new arterioles. In humans, the extent of preexisting arterioles varies greatly among individuals and thus arteriogenic responses in CLI patients in response to an

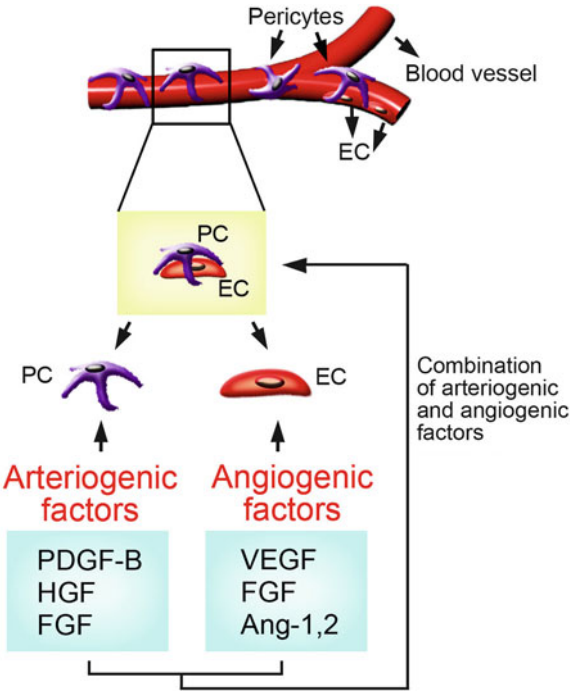

Fig. 7.2 Rationale of combination therapy. The arterial wall consists of endothelial cells (ECs) and pericytes/vascular smooth muscle cells (PCs). Therefore, therapeutic treatment with a combination of growth factors that target each of these cell types could theoretically enhance revascularization. Future clinical trials based on this combination principle need to be validated

equivalent ischemic insult may vary considerably [68]. It is known that variation of coronary collateral arteriole distribution also exists in the human heart [69]. Thus, it is interesting to speculate that genetic variation among individuals may play a critical role in determining collateral arteriole presence and function. From the therapeutic point of view, it is difficult to modulate the existing variation of arterial distribution and function among patients. However, manipulation of arteriole growth should be achievable if the mechanisms are understood and appropriate approaches are applied.

The arteriole wall is composed primarily of two cell types: ECs and perivascular cells (i.e., pericytes and VSMC) (Fig. 7.2). Thus, to induce arteriogenesis a combination of arteriogenic and angiogenic factors that target each vascular cell type should be considered. Generally speaking, it is difficult to define arteriogenic factors as a completely separate class of growth factors, since many of these factors also promote angiogenesis [10, 70]. PDGF-BB, HGF, and FGF-2 have been shown to potently induce proliferation and migration of VMSCs [51, 71–73]. Additionally, in vivo experiments show that these factors markedly improve pericyte and VSMC coverage in blood vessels, remodel the existing vasculature toward arterial differentiation, and promote maturation of immature vessels [51, 71–73]. Thus, it is reasonable to define these factors as arteriogenic factors, whereas EC-specific factors, such as members in the VEGF and angiopoietin families, are defined as angiogenic factors (Fig. 7.2).

Without arteriogenic factors, the primitive vascular networks remain unstable and are functionally less productive [10, 74, 75]. For example, the VEGF-induced vascular network usually consists of disorganized, leaky, and primitive vascular plexuses that do not sufficiently supply oxygenated blood to the ischemic tissues [10, 74, 75]. Thus, delivery of VEGF alone to the ischemic muscle tissues has not yielded significant beneficial effects in patients [10, 74, 75]. In the setting of CLI, perhaps the hypoxia-induced endogenous VEGF level is sufficiently elevated to trigger an angiogenic response, whereas exogenously administered VEGF might cause more tissue damage due to its permeability-inducing activity that may counteract its beneficial angiogenic effects. In any case, hypoxia-induced elevated levels of VEGF are always present regardless of the choice of therapeutic molecule(s). Thus, it is important to consider the mechanistic relationship between the selected therapeutic factor(s) and VEGF when two or more factors are delivered simultaneously to the ischemic tissues.

Another important alteration in the ischemic muscle tissue upon CLI is the inflammatory response, which leads to activation and infiltration of inflammatory cells, including neutrophils and monocytes/macrophages, into the affected tissues [76–81]. These activated inflammatory cells produce multiple growth factors and cytokines, including VEGF, TNF-α, IL-1β, and IL-6, which act on ECs to modulate angiogenesis and vascular remodeling. In addition, inflammatory cytokines can induce lymphangiogenesis, which may modulate tissue functions by drainage of the extracellular fluids [82–84]. Lymphangiogenesis might be beneficial for functional recovery of ischemic limbs because drainage of excessive extracellular fluids reduces tissue edema. Lymphangiogenesis might be particularly important to counteract the hyper-vascular permeability induced by high levels of VEGF. VEGF itself has been reported to act as a potent lymphangiogenic factor [85, 86]. However, the functional properties of VEGF-induced lymphatic vessels remain poorly understood. Similar to blood vessels, the formation of a functional lymphatic network may also require intimate interplay between different lymphatic factors. In support of this view, multiple lymphangiogenic factors have been identified, and they seem to display overlapping and yet distinct functions [70, 75, 87–94]. It should be emphasized that cytokines induced by inflammation can specifically promote arteriogenesis but not angiogenesis. For example, interleukin-20 has been reported as an arteriogenic-specific factor that promotes collaterogenesis and the functional recovery of ischemic limbs [95]. Currently, our understanding of the molecular mechanisms of inflammatory cytokine-induced angiogenesis and arteriogenesis remains limited.

Preclinical Models and Proangiogenic Therapy of Critical Limb Ischemia

Several animal models have been developed to recapitulate clinical CLI. These include mouse, rat, rabbit, cat, pig, dog, and primate models that utilize surgical ligation of collateral arteries to generate severe ischemia in the hind limbs (Table 7.1)

Table 7.1 Summary of preclinical animal models of CLI

Factor	Species	Model	Vehicle	Reference
VEGF	Mouse	Femoral artery ligation	Ad-VEGF	[96]
		Femoral artery ligation	PLGA polymer-VEGF	[97–99]
		Femoral artery ligation	ZFP plasmid VEGF	[100, 101]
	Rat	Femoral artery ligation	AAV-VEGF	[102, 103]
		Ligation of the common iliac artery	Ad-VEGF	[104]
	Rabbit	Femoral artery ligation	Ad-VEGF	[105–107]
		Femoral artery ligation	VEGF-NP-Fibrin	[108]
		Femoral artery ligation	Plasmid VEGF	[109]
		Femoral artery ligation	ZFP plasmid VEGF	[110]
FGF	Mouse	Femoral artery ligation	Plasmid FGF-2	[111]
	Rabbit	Femoral artery ligation	Fragmin/protamine microparticles containing FGF-2	[112]
		Proximal right common iliac artery ligation	FGF-2	[113]
		Ameroid constrictors	FGF-2	[114]
HGF	Mouse	Femoral artery ligation	Plasmid HGF	[115]
		Femoral artery ligation	HGF with alginate-sulfate microbeads	[116]
	Rat	Femoral artery ligation	Plasmid HGF	[117]
	Rabbit	Femoral artery ligation	Plasmid HGF	[118]
HIF	Mouse	Femoral artery ligation	Ad-HIF-1α	[119]

VEGF vascular endothelial growth factor, *FGF* fibroblast growth factor, *HGF* hepatocyte growth factor, *HIF* hypoxia inducible factor, *Ad* adenovirus, *PLGA* poly(lactic-*co*-glycolic acid), *ZFP* zinc finger protein, *AAV* adeno-associated virus, *NP* nanoparticle

[23, 120–125]. Two commonly used ligation methods are simple ligation of the femoral artery, which does not usually produce sustainable reduction of resting blood flow [124, 125], and a two-step ligation method in which the common iliac artery and its branches are ligated first, with subsequent ligation of the femoral artery [124, 125]. Although the two-step ligation method requires complex and tedious operative procedures, it usually produces a more desirable reduction of resting blood flow in animals and more closely resembles CLI in humans. However, persistent reduction of resting blood flow in various animal species varies considerably [124, 125].

Delivery of proangiogenic molecules for therapeutic assessment in animal models of CLI is usually achieved using one of the following two approaches: (1) delivery of angiogenic proteins, or (2) delivery of genes encoding angiogenic factors in an expression vector [6, 18]. Therapeutic effects of various angiogenic or arteriogenic molecules in different animal models are usually assessed by measuring the growth of new collaterals and improvement of blood perfusion [33, 124, 125]. In small rodents, such as mice, angiographic analysis of collaterals can be problematic. However, angiography is routinely used in large animals and this method allows direct visualization of collateral networks in the ischemic region of the

tissue [7, 10, 33, 126]. Most angiogenic factors have been assessed using both delivery systems in different animal models, and according to published reports they usually improve angiogenesis and hind limb functions [6, 13, 18]. Many excellent review articles that discuss these studies have been published [23, 127–129].

Therapeutic Rational for Using Proangiogenic and Proarteriogenic Factors in Combination

ECs and perivascular cells are both essential for vascular remodeling and stability. Therefore, the choice of therapeutic factors to be used should include growth factors that target both cell types. Among known angiogenic factors, the mechanisms of actions, signal transduction, and therapeutic implications of members of the VEGF family are well characterized. However, VEGF primarily targets ECs, and VEGF-induced angiogenic vessels consist of disorganized and leaky microvascular networks usually lacking perivascular coverage [74]. Thus, VEGF-induced primitive vascular networks need extensive remodeling to form a mature and functional vascular system. To achieve this, other factors involved in vascular remodeling, maturation, and stability should be used simultaneously to achieve therapeutic efficacy. Consistent with this notion, delivery of only VEGF to CLI patients in double-blind, randomized trials has not generated conclusive clinical benefits [7, 18, 31].

The molecular and cellular mechanisms underlying VEGF-induced angiogenesis, vascular permeability, and arteriogenesis are complex. VEGF-induced vascular effects are mainly mediated by VEGFR2, the functional receptor for the VEGF signaling system [130]. With respect to an angiogenic response, VEGF directly induces EC proliferation, migration, and tube formation in the absence of other angiogenic factors via activation of VEGFR2 [74]. Thus, VEGF is a potent direct angiogenic factor in experimental settings [75], both in vitro and in vivo. It is also known that VEGF is a potent inducer of vascular permeability. Three possible mechanisms that involve both VEGFR1 and VEGFR2 signaling systems likely mediate this effect. The first of these is the formation of vascular fenestrations [74]. In experimental settings, VEGF has been shown to induce vascular fenestrations in vivo and the fenestrated endothelium has also been shown to mediate VEGF-induced vascular permeability. This effect is mediated by activation of VEGFR2 signaling in ECs [74]. The second mechanism involves opening of junctions between ECs. This effect involves VEGF/VEGFR2-regulated cadherin (VE-cadherin) expression in ECs [131]. Finally, disassociation of perivascular cells such as pericytes and VSMCs from blood vessels may be involved. A recent study demonstrates that VEGF significantly ablates pericytes from the retinal vasculature via activation of VEGFR 1 receptor [132].

VEGF-induced arteriogenic activity is dependent in part on other signaling systems. For example, the Delta-like ligand 4 (Dll4)-Notch 1 and Dll4-Notch 4 signaling systems restrict VEGF-induced arterial sprouts. When the Dll4-Notch signaling system is activated, VEGF is insufficient to trigger an arteriogenic response.

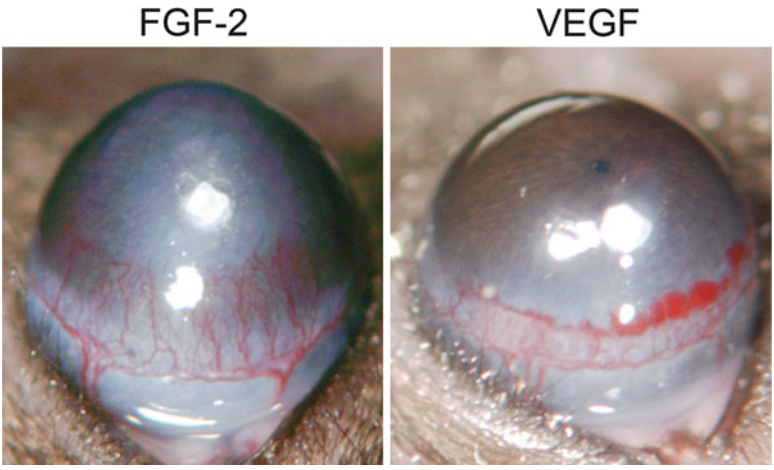

Fig. 7.3 FGF2- and VEGF-induced vascular responses in the mouse cornea. Implantation of FGF2 or VEGF protein into the mouse cornea induces an angiogenic response. While the FGF2-induced vascular network consists of well-defined microvessels, VEGF induces only the formation of premature and sinusoidal vascular plexuses

However, suppression of the Dll4-Notch system may permit VEGF-induced arteriogenic signaling, leading to establishment of an arterial network [133–135].

Fibroblast growth factors (FGFs), especially FGF1 and FGF2, are also potent angiogenic factors [75, 136, 137]. Unlike VEGF, FGF1 and FGF2 target a broad spectrum of cell types, including ECs and vascular VSMCs [138–141]. Thus, these angiogenic factors should promote more mature and well-structured vascular networks [10, 75]. In animal angiogenesis models, FGF1 and FGF2 induce the formation of relatively mature vascular networks that are markedly different from those induced by VEGF. For example, in a mouse corneal angiogenesis model, FGF2 induces the formation of angiogenic vessels that are well separated from each other (Fig. 7.3) [10, 75]. In contrast, VEGF-stimulated microangiogenic vessels are fused into sinusoidal vascular plexuses [142]. However, despite induction of well-structured vascular networks, FGF2 is unlikely to sustain its own vascular networks without other vascular remodeling factors such as PDGF-BB [10, 75]. Thus, vascular stability requires an active process of recruitment of pericytes/VSMCs onto the newly formed vascular network.

Several growth factors (including PDGF-BB) and HGF that primarily target pericytes/VSMCs have been tested in ischemic disease models [143–145]. As expected, these arteriogenic factors significantly improve therapeutic outcomes in various animal models [10, 146, 147]. Interestingly, both PDGF-BB and HGF are angiogenic factors, which induce the formation of relatively mature blood vessels. However, the angiogenic activity of these factors is less potent compared to FGF2 and VEGFA, and delivery of these factors alone to the ischemic muscle tissue produces only modest therapeutic outcomes [10, 75, 146, 147].

Clinical Relevance of Monotherapy Versus Combination Therapy

The concept of proangiogenic therapy for the treatment of ischemic disorders in general is straightforward, and delivery of angiogenic molecules to the ischemic region of affected cardiac or skeletal muscle in preclinical animal models has been rigorously tested during the last couple of decades [6, 7, 18, 31, 32]. In most experimental settings, a single angiogenic or arteriogenic molecule was delivered either as a protein or derived from a gene in an expression vector [6, 7, 18, 31, 32]. Strikingly, accumulation of a large body of research evidence shows that delivery of a single angiogenic molecule to ischemic skeletal muscles in animals has been sufficiently potent to induce collateral growth, leading to improved blood perfusion and functional recovery of the affected tissue (Table 7.1). Encouraged by these preclinical findings, clinical trials based on these preclinical findings have been designed and carried out in human patients (Table 7.2). Unfortunately, in almost all large, double-blind, randomized, and placebo-controlled clinical trials, delivery of a single angiogenic factor to the ischemic region of either cardiac or skeletal muscle tissues has not produced persistent beneficial effects [91, 130, 132].

To better understand these disappointing results, two crucial issues need in-depth mechanistic investigation: (1) Is the choice of angiogenic factors for therapy suboptimal? (2) How relevant are the preclinical models to human patients? Regardless of the choice of angiogenic factors, all therapeutic approaches in human trials are based on monotherapy. This may be because of pharmaceutical company intellectual property issues, or because of resistance by the U.S. Food and Drug Administration to test or approve two or more new molecules for combination therapy. Recent studies demonstrate that combinations of angiogenic and arteriogenic factors could produce superior and synergistic effects on collateral growth. For example, a combination of FGF2 and PDGF-BB synergistically induces angiogenesis and vascular stability by reciprocally inducing expression of their specific receptors in ECs or pericytes/VSMCs [10]. It appears that FGF2 induces PDGFR expression in ECs, whereas PDGF-BB induces FGFR-1 expression in VSMCs [51]. Thus, simultaneous expression, even at low levels, of both factors in the same environment may elicit a robust angiogenic and arteriogenic response. The use of VEGF plus PDGF-BB is another example of synergism between angiogenic factors, which in combination have been shown to promote angiogenesis and arteriogenesis in a therapeutic preclinical model [30].

Based on preclinical experiences in animal models, it is reasonable to speculate that an optimal combination of angiogenic and arteriogenic factors is needed for producing robust clinical benefits. Despite numerous difficulties in clinical practice and drug development, future designs of clinical trials should be based on biological principles rather than commercially based interests.

Table 7.2 Clinical trials with proangiogenic factors

Trial stage	Factor and vehicle	Route of delivery	Patient no. (treated/placebo)	Endpoint	Outcome	Reference
Phase II	Adenoviral and plasmid–liposome $VEGF_{165}$	IA	17–18/19	3 mo	Increased overall vascularity (Ad and P–L) Increased ischemic zone vascularity (Ad)	[148]
	Plasmid $VEGF_{165}$	IM	27/27	100 day	Decreased amputation rate Hemodynamic improvement Improved skin ulcers Decreased rest pain	[149]
	Adenoviral $VEGF_{121}$	IM	32–40/33	26 wk	No effect	[150]
	Plasmid FGF-1	IM	51/56	25 wk	Decrease amputation rate Decrease risk of death	[151]
	rhFGF-2	IV	16/8	12 wk	No effect	[152]
	rhFGF-2	IA	61–66/68	180 day	Increase change in peak walking time Increase ankle-brachial index	[153]
	Adenoviral-FGF-4	IM	10/3	12 wk	Decreased rest pain	[154]
	Plasmid HGF	IM	25–27/26	12 mo	Increase transcutaneous oxygen tension Increase change in ulcer size	[147]
	Plasmid Del-1	IM	52/53	180 day	No effect	[155]
Phase III	Edifoligide (E2F decoy)	Ex vivo graft	563/575	1 yr	Primary endpoint negative Increased secondary graft potency	[156]
	Plasmid FGF-1	IM	259/266	18 mo 36 mo (safety survey)	Primary endpoint negative Safety issues	[157]

VEGF vascular endothelial growth factor, *(rh)FGF* (recombinant human) fibroblast growth factor, *IA* intra-arterial injection, *IM* intramuscular injection, *IV* intravenous injection, *mo* month, *wk* week, *yr* year, *Ad* adenovirus, *P–L* plasmid–liposome

Outlook for Clinical Therapeutic Treatment of Chronic Limb Ischemia

The concept of promoting angiogenesis and arteriogenesis for the treatment of ischemic diseases such as myocardial ischemia and CLI is straightforward and remains an undisputed principle. Why has this simple and straightforward approach not worked for the treatment of ischemic diseases in human patients? What are the fundamental hurdles preventing the clinical success? Given the fact that genetic variation exists among human individuals, the same degree of ischemic insult may result in diverse vascular responses in individual patients. In fact, the ability to reconstitute or dilate existing collaterals in human patients is the key determinant of functional recovery. Angiogenesis, vascular remodeling, vascular stability, and arteriogenesis are all essential to facilitate functional recovery of the ischemic muscle tissue by increasing blood perfusion. Tissue responses to an ischemic insult often lead to harmful reactions, such as increased vascular leakage and inflammation, which further increase damage to affected tissues or limbs. Thus, functional separation of desirable vascular-promoting activities from the tissue damaging effects of angiogenic and arteriogenic factors should be considered during therapeutic treatment in animal or human subjects. As multiple mechanisms are involved in reconstitution of a collateral network, it seems unlikely that a single angiogenic or arteriogenic factor would be sufficient to achieve this goal. However, delivery of a single proangiogenic factor to ischemic tissues in current animal models seems to achieve functional recovery (Table 7.1). It is plausible that animal ischemia models are artificial in that they may represent only an acute ischemic situation. In human patients, CLI is a chronic condition that may involve different mechanisms to achieve functional recovery. Thus, development of new clinically relevant CLI animal models will be crucial for the future evaluation of possible therapeutic treatments for patients with CLI.

The future success of proangiogenic or arteriogenic therapy based on combination therapy for the treatment of CLI or other ischemic disorders in human patients needs to be validated in appropriate new animal models so that combination therapy can be moved into clinical trials. A combination treatment regimen to inhibit angiogenesis has been successful for the treatment of cancer patients [158, 159]. Delivery of a combination of two or more angiogenic factors may produce more meaningful clinical outcomes for CLI patients as well.

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Chapter 8
Modulating the Proliferative Response to Treat Restenosis After Vascular Injury

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A Brief Overview of Stenting

Percutaneous coronary intervention (PCI) has become the most widely used strategy for the treatment of patients with coronary artery disease since its introduction by Grüntzig et al. in 1977 [1]. Angioplasty was plagued with multiple problems in the balloon catheter era, including acute collapse and dissection of the treated artery and recurrent luminal obstruction (restenosis), a pathological process that forced target vessel revascularization in 25–50% of cases, typically within 2–12 months post-PCI. A second revolution in the field of interventional cardiology materialized with the introduction of balloon-mounted stents, which consist of a self-expandable stainless-steel mesh that acts as a scaffold that maintains radial support to neutralize elastic recoil. Palmaz and colleagues introduced in 1985 the use of bare metal stents in peripheral arteries of dogs [2]. Schatz et al. then developed the first commercially successful stent, the Palmaz-Schatz stent [3]. In 1987, Sigwart et al. provided the first evidence that implantation of bare metal stents in patients with iliac, femoral, and coronary artery disease may offer a safe and useful way to prevent subacute occlusion and dissections and limit the occurrence of restenosis [4]. Following these

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Table 8.1 Current limitations and adverse events attributed to drug-eluting stents

Pathophysiological event	Presumed cause	Reference
Impaired reendothelialization	Drug/polymer	[10]
Delayed healing with persistent inflammation/hypersensitivity	Drug/polymer	[11]
Late stent malapposition	Drug/polymer	[12]
Late/very late stent thrombosis	Drug/polymer	[13]

pilot studies, the first Palmaz-Schatz stent was approved for use in the USA, and different bare metal stents platforms developed over the next decade confirmed the benefits of stenting compared with conventional transluminal balloon angioplasty, leading to the era of elective stenting. However, restenosis after base metal stents implantation still affected about 15–30% of patients, causing in the Western world an estimated annual cost exceeding $1 billion USD. After the identification of excessive proliferation of vascular smooth muscle cells (VSMCs) as a key feature of experimental and clinical neointimal thickening postangioplasty and the results of numerous animal studies demonstrating the utility of antiproliferative strategies to prevent this pathological process (reviewed in [5, 6]), a new era in interventional cardiology began nearly a decade ago with the advent of drug-eluting stents (also referred to as "coated" or "medicated" stents) that locally deliver high doses of antiproliferative drugs. Pilot studies using the sirolimus-eluting Bx Velocity™ stent demonstrated negligible neointimal thickening at follow-up [7, 8]. The superior performance of several drug-eluting stents platforms versus bare metal stents has been irrefutably confirmed in large multicenter clinical trials demonstrating dramatic reductions in restenosis rates, in target lesion revascularization, and in major adverse cardiac events [9]. Although the initial clinical trials with drug-eluting stents did not report significant adverse effects, recent case reports in real life patients have recognized an increased risk of late stent thrombosis potentially due to a mismatch between the vessels and the stent (late stent malapposition), hypersensitivity, or incomplete reendothelialization consider changing to "due to" to the cytostatic and cytotoxic effects that the active drugs exert on the underlying and neighboring ECs or the proinflammatory effects of the biostable polymeric coatings (Table 8.1) [9, 14]. The current clinical guidelines therefore recommend prolonged potent antiplatelet and antithrombotic adjunctive therapies in patients receiving drug-eluting stents. Because of these shortcomings, further research is essential in order to improve the long-term safety and efficacy of drug-eluting stents.

Etiopathogenesis of In-Stent Restenosis and Cell Cycle Control in Mammalian Cells

Stenting can result in acute damage to the endothelial cell (EC) monolayer, triggering a chronic inflammatory response that may promote exuberant neointimal hyperplasia (Fig. 8.1) [5, 15]. Localized platelet activation and thrombosis accompanied

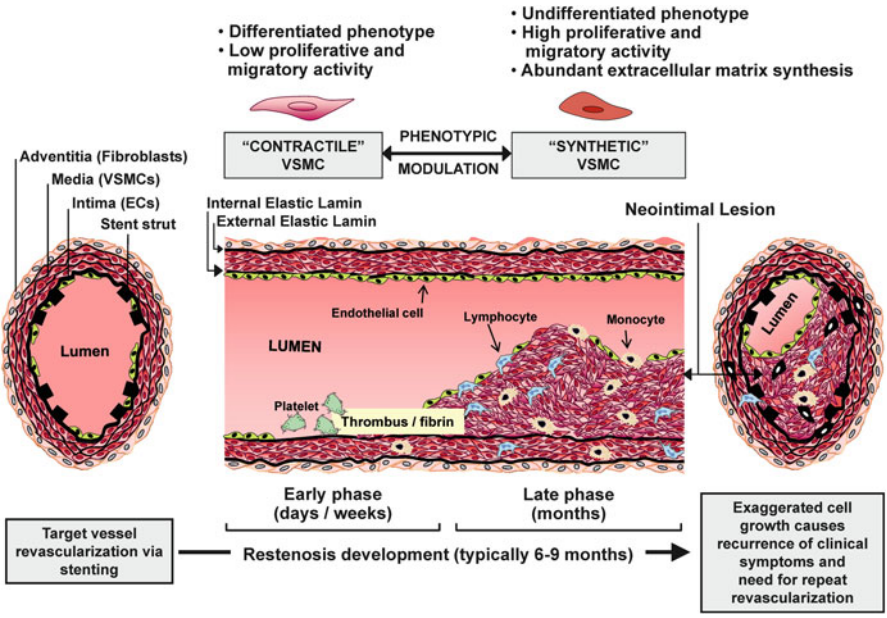

Fig. 8.1 Mechanisms of in-stent restenosis. The *left* and *right images* show cross sections through a stented artery immediately after intervention and at a late time point showing excessive neointimal lesion development, respectively. The scheme between both images represents a longitudinal section through the vessel wall (for simplicity, neither the native atherosclerotic plaque that compromised blood flow before performing angioplasty nor the stent struts are depicted). Platelets are recruited into the damaged vessel wall and provoke thrombi formation. Blood-borne leukocytes adhere to thrombi via selectins and integrins and, driven by locally produced chemokines, they migrate across the fibrin-platelet layer toward the intimal area. Medial VSMCs exhibiting a differentiated so-called contractile phenotype revert to a "synthetic" less-differentiated phenotype characterized by abundant extracellular matrix protein synthesis and high responsiveness to mitogenic and migratory stimuli. Activated VSMCs migrate toward the growing neointimal lesion and proliferate very actively, thus contributing to neointimal thickening

by recruitment of circulating monocytes, neutrophils, and lymphocytes into the intimal area characterize the acute early phase of restenosis. Numerous chemotactic and mitogenic factors produced by neointimal cells provoke a first hyperplastic response of medial VSMCs, which migrate toward the growing neointimal lesion where they maintain high proliferative activity. Compared with VSMCs in normal adult arteries, which are fusiform and display a differentiated so-called contractile phenotype characterized by reduced proliferative activity and motility, activated VSMCs within the injured vessel wall exhibit an undifferentiated "synthetic" phenotype characterized by broader and flatter shape, expression of embryonic isoforms of contractile proteins, high responsiveness to growth and chemotactic

stimuli, and abundant synthesis of extracellular matrix components. Accumulating evidence indicates that recruitment of bone marrow-derived and adventitial VSMC progenitors and adventitial myofibroblasts also plays a role in neointimal lesion development, but the relative contribution of this phenomenon to restenosis remains unclear [15]. At later stages, resolution of inflammation is associated with restoration of the "contractile" phenotype of neointimal VSMCs and normalization of the composition of the extracellular matrix, which more closely resembles the undamaged vessel wall. Consistent with the complexity of restenosis, numerous animal and human studies have identified a plethora of candidate regulators of neointimal hyperplasia, including signal transduction pathways (e.g., MEK/ERK and PI3K/Akt signaling cascades), transcription factors (e.g., AP-1, YY1, Gax, NF-κB, E2F, c-myb, c-myc), growth factors (e.g., PDGF, FGF, TGFβ, VEGF, IGF, EGF), inflammatory cytokines (e.g., TNFα), chemotactic factors (e.g., MCP-1, CCR2), thrombogenic factors (e.g., thrombin receptor, tissue factor), cell adhesion molecules (e.g., VCAM, ICAM, Mac-1, LFA-1), metalloproteases (e.g., MMP-2, MMP-9), and cell cycle regulatory proteins (e.g., CDK2, CDC2, cyclin B1, PCNA, pRb, p27, p21).

Neointimal hyperplasia following PCI can thus be viewed as the arterial wall's healing response to acute mechanical injury, which encompasses excessive hyperplastic growth of VSMCs. The proliferation of mammalian cells requires a series of sequential events that constitute the mitotic cell cycle (Fig. 8.2). Under normal conditions, most differentiated cells are maintained in a nonproliferative state (G0 phase). After stimulation with growth factors, cells enter the first gap phase (G1), during which proteins necessary for DNA replication are synthesized and/or activated. In the subsequent synthesis phase (S) the DNA is replicated, then cells enter a second gap phase (G2) that allows the synthesis and activation of proteins required for mitosis (M phase). Cell cycle progression is orchestrated by the activation of various holoenzymes composed of the regulatory subunit cyclin and a catalytic cyclin-dependent protein kinase (CDK). Several mechanisms sequentially activate distinct CDK/cyclin complexes during different phases of the cell cycle, including the periodic synthesis and degradation of cyclins and the phosphorylation/dephosphorylation of CDKs and cyclins. Another important level of cell cycle regulation is the inhibition of CDK/cyclin holoenzymes through their interaction with CDK inhibitory proteins (CKIs) of two families: Cip/Kip (CDK interacting protein/kinase inhibitory protein: $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) and Ink4 (inhibitor of CDK4: $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, $p19^{Ink4d}$) [16]. Cip/Kip proteins bind to and inhibit many CDK/cyclin complexes, while Ink4 proteins specifically interact with and inhibit cyclin D-associated CDKs (Fig. 8.2). The rates of synthesis and degradation of CKIs, as well as their redistribution among different CDK/cyclin heterodimers are modulated by mitogenic and antimitogenic stimuli. The tumor suppressor p53 and other proteins modulate CKI expression and function to ensure that cell cycle progression is halted if environmental conditions are not appropriate and/or cells accumulate genetic damage. CDK/cyclin activity regulates E2F/DP- and retinoblastoma protein (pRb)-dependent transcription of target genes involved in cell cycle control and DNA biosynthesis (Fig. 8.2). In nonproliferating cells, low CDK/cyclin activity

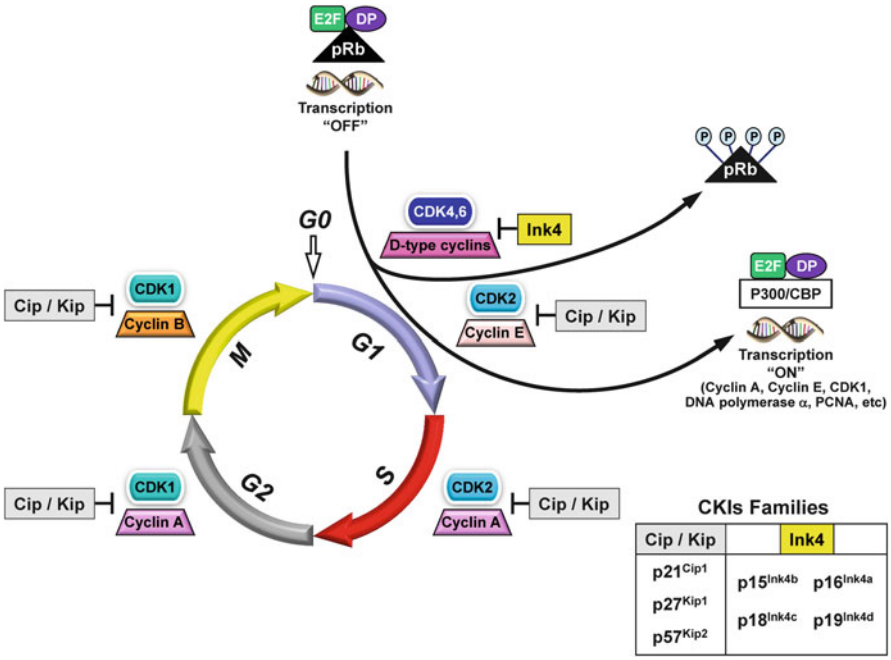

Fig. 8.2 Cell cycle regulation in mammalian cells. Activation of specific CDK/cyclin complexes drives progression through the different phases of the mammalian cell cycle (*G1* Gap 1, *S* synthesis of DNA, *G2* Gap 2, *M* mitosis). Advance through G1/S is orchestrated by CDK/cyclin-dependent hyperphosphorylation of pRb, which allows the transcriptional activation of E2F/DP target genes that are required for cell proliferation. CDK inhibitory proteins (CKIs) of the Cip/Kip and Ink4 families interact with and inhibit the activity of CDK/cyclin holoenzymes. Cip/Kip proteins bind to and inhibit a wide spectrum of CDK/cyclins, while Ink4 proteins are specific for cyclinD-associated CDKs. CDK1 is also known as CDC2

keeps pRb in its hypophosphorylated form, which binds to and inactivates the dimeric transcription factor E2F/DP. In contrast, high CDK/cyclin activity in proliferating cells causes the accumulation of hyperphosphorylated pRb during late G1-phase, thus leading to the release of E2F/DP and transactivation of various target genes necessary for cell cycle progression.

MicroRNAs (miRNAs) represent an additional layer of the complex regulatory network that controls cell cycle progression, and evidence is accumulating that they may be of particular therapeutic interest in the context of pathological vascular remodeling [17]. For instance, miRNA-221 and miRNA-222 have been shown to limit VSMC proliferation by targeting the CKIs $p27^{Kip1}$ and $p57^{Kip2}$, and the growth-factor receptor c-Kit [18, 19]. Importantly, knockdown of these microRNAs inhibits arterial cell proliferation and neointimal formation in the rat carotid artery injury model [19].

Pharmacological Antiproliferative Strategies to Limit Neointimal Thickening After Mechanical Injury of the Vessel Wall

The recognition that excessive VSMC proliferation is a hallmark of restenosis postangioplasty in animal models and humans has fueled extensive research into the molecular mechanisms that control the cell cycle in these cells in vitro and in vivo. Moreover, numerous preclinical studies have been conducted to assess whether antiproliferative strategies are efficient at limiting neointimal lesion development, including gene therapy and drug-based approaches. Although gene therapy targeting cell cycle regulatory factors (e.g., inhibition of positive cell cycle regulators and overexpression of growth suppressors) has shown undisputable therapeutic efficacy in animal models of restenosis [5, 6], its clinical use awaits the overcoming of current limitations of gene therapy in humans. We have therefore focused our discussion on drug-based strategies that limited neointimal lesion development in animal models of angioplasty, some of which have demonstrated clinical benefits when administered in drug-eluting stent platforms.

Animal models are critical to provide mechanistic insight into neointimal thickening associated with balloon angioplasty and stenting, and to establish safety margins, efficacy, and toxicity [20–22]. The rat carotid model of balloon angioplasty has been extensively used to gain insight into the molecular mechanisms that provoke neointimal thickening induced by mechanical injury; however, the porcine and the rabbit models are considered standard for the evaluation of drug-eluting stents prior to human use [20–22]. Nevertheless, there are shortcomings associated with animal models that limit their biological significance. Ideally, drug-eluting stents should be tested in atherosclerotic arteries to more closely resemble the clinical situation; however, preclinical studies are generally performed in atherosclerosis -free vessels. Moreover, neointimal responses associated with stent deployment are exaggerated in pigs and rabbits, and the time course of healing is reduced compared to humans (about 4–6 weeks in swine and rabbits compared to roughly 6–9 months in humans). It is also noteworthy that the rabbit model does not offer the possibility of coronary stenting due to its anatomical size; thus, the aorta or the iliac arteries are used for stent placement in rabbits. Albeit the site of stenting can be considered as a critical limitation of the rabbit model, it resembles more closely than the pig the healing process observed in humans and is therefore widely used to examine inflammatory, proliferative, and thrombotic processes subsequent to vascular injury and stenting [20].

Inhibitors of Mammalian Target of Rapamycin

The mammalian target of rapamycin (mTOR) protein is a member of the phosphoinositide 3-kinase (PI3K)-related proteins kinases (PIKK) family that forms the catalytic subunit of two different complexes: mTOR complex 1 (mTORC1) and mTOR

complex 2 (mTORC2) [23, 24]. Signaling through the mTOR pathway links cell cycle activity with energy and nutrient availability and therefore plays a key role in maintaining homeostasis. A potent inhibitor of mTOR is rapamycin (also known as rapamune, sirolimus), a fungal macrolide produced by *Streptomyces hygroscopicus* that impairs mTOR complex assembly via sequestration of the intracellular receptor FKBP12 [23, 24]. Treatment of VSMCs with sirolimus upregulates $p27^{kip1}$, inhibits pRb phosphorylation, and limits cell proliferation and migration in vitro [25–29]. These findings are in agreement with the observation that the $p27^{kip1}$/CDK/pRb pathway regulates VSMC proliferation and migration in a coordinated manner [30, 31].

Preclinical studies in different animal models have demonstrated the utility of sirolimus to limit neointimal thickening induced by arterial injury. Oral or intramuscular application of sirolimus reduces neointimal thickening in porcine and rat balloon injury models [32–34]. As in cultured VSMCs [28, 29], the reduction in neointimal proliferation observed in the porcine coronary model is associated with increased $p27^{kip}$ expression and reduced pRB phosphorylation [33]. Sirolimus eluting-stents have also demonstrated protection against neointimal thickening in porcine [35–37], rabbit [38], and rat [39] models. Likewise, Pires and colleagues reported that sirolimus-eluting cuffs placed around the femoral artery significantly reduce intimal thickening in both normocholesterolemic wild-type mice [40] and atherosclerotic hypercholesterolemic apoE*3-Leiden transgenic mice [41] with no systemic adverse effects or effect on cuffed contralateral femoral arteries. However, evidence has been presented demonstrating that sirolimus has unfavorable in vitro and in vivo effects on ECs. Barilli et al. demonstrated that prolonged treatment of human ECs with sirolimus impairs cell viability (increased apoptosis and necrosis) and function (reduced proliferation and mobility and increased actin stress fiber formation), possibly through mTORC2 inhibition [42]. Suppression of reendothelialization and revascularization by sirolimus also correlates with increased EC mortality via apoptosis and autophagy [43], a process activated in response to cellular damage and nutrient deprivation that mediates the degradation of cellular components in lysosomes [44]. Using a porcine model of epicardial coronary artery stenting, Frey and colleagues noted delayed vascular healing (endothelialization) with slow-release sirolimus-eluting stents compared with bare metal stents and extended-release sirolimus-eluting stents [45]. Sirolimus treatment might also delay reendothelization through induction of endothelial progenitor cell senescence, possibly due to increased expression of $p27^{kip}$ and inactivation of telomerase [46]. Moreover, sirolimus suppresses the coordinated proadhesive and proinflammatory gene expression that normally occurs in renal artery segments subjected to mechanical injury, which in turn may reduce the recruitment of leukocytes and hematopoetic progenitor cells that participate in vascular healing [47]. These adverse effects of sirolimus on mature ECs and endothelial progenitors might contribute to increased risk of late stent thrombosis in patients receiving sirolimus-eluting stents.

Several sirolimus derivatives have been developed with the goal of optimizing mTOR inhibitory therapies. Everolimus exhibits a shorter half-life and reduced unwanted side-effects compared with sirolimus if delivered systemically, yet both drugs elicit similar protection against neointimal formation in a porcine coronary artery model [48, 49]. Zotarolimus exhibits increased retention in the arterial wall and

reduces neointima development after stent deployment in a porcine coronary artery model [50]. Finally, compared with sirolimus-eluting stents, a polymer-free stent coated with the sirolimus analog biolimus A9 has demonstrated equivalent early and superior late reduction of intimal proliferation in a porcine model [51]. Remarkably, delayed arterial healing with biolimus A9 was minimal, and there was no increased inflammation at 180 days compared with implantation of sirolimus-eluting stents.

Taxanes

Taxol (paclitaxel) is a microtubule stabilizing drug that impairs centrosomal function, induces abnormal mitotic spindles, and suppresses spindle microtubule dynamics during mitosis causing G2/M-phase arrest [52]. In vitro treatment of VSMCs with paclitaxel upregulates both p53 and its downstream target $p21^{Cip1}$, and also disorganizes cytoskeleton structures and increases apoptosis. These effects are associated with a significant inhibition of processes that promote restenosis, including cell proliferation and migration and extracellular matrix production [27, 53–55]. Accordingly, oral paclitaxel treatment markedly reduces neointimal lesion formation after rat balloon angioplasty without causing significant toxicity [53, 56], and local delivery of paclitaxel to the balloon angioplasty site in rabbit carotid artery disorganizes microtubules and inhibits neointimal thickening [57]. Likewise, studies in the porcine coronary artery model have demonstrated long-term effects of paclitaxel-eluting stents [58, 59]. However, the cytotoxic effects of paclitaxel may partly explain its reduced long-term efficacy and safety compared with sirolimus [60]. Wessely and colleagues demonstrated that both drugs efficiently block VSMC proliferation, but paclitaxel has more deleterious effects on ECs, such as more potent antiproliferative and proapoptotic activities [27]. Moreover, paclitaxel-eluting cuffs placed around the femoral artery effectively prevent neointimal thickening on the atherosclerotic plaques of hypercholesterolemic apoE*3-Leiden transgenic mice, but high concentration demonstrated adverse vascular pathology and transcriptional responses (e.g., increased mRNA level of the proapoptotic factors FAS, BAX, and caspase 3), suggesting a narrower therapeutic range of this drug [41]. Given the high cytotoxicity of paclitaxel, major efforts are underway to improve the safety of this drug while maintaining therapeutic benefits. Such strategies include programmable drug release [61], addition of paclitaxel to contrast media [62], or drug coating of the angioplasty balloon rather than the stent [63].

Estradiol

Estradiol, the most abundant sex hormone in humans, has numerous effects in vascular cells, including modulation of cell proliferation and migration, which are for the most part mediated by its binding to the estrogen receptors α and β [64, 65]. Upon binding of estradiol, these intracellular receptors act as transcription factors that

modulate the expression of a large number of genes [64]. In cultured cells, estradiol inhibits VSMC proliferation and migration and, conversely, promotes EC proliferation [66]. Mechanistically, the effects of estradiol appear to be mediated by changes in the activity of various signaling proteins, including the mitogen-activated protein kinases p38 and ERK1/2 [66–68] or the GTPase Rac1 [69]. Therefore, estradiol may be effective at preventing vascular restenosis after arterial injury with low risk of late stent thrombosis, as it would be predicted to enhance reendothelialization. Supporting this notion, a number of preclinical studies have demonstrated the protective function of this hormone against vascular injury. For example, systemic delivery of estradiol in rodents and rabbits accelerates reendothelization after vessel denudation [70–73], reduces neointimal thickening in the injured carotid artery [74–81] and aorta [82], and inhibits VSMC proliferation in vivo [75, 76, 82, 83]. Similarly, catheter-mediated local delivery of estradiol in balloon-injured porcine coronary artery reduces VSMC proliferation and neointimal thickening [84], and estrogen-coated stent implantation reduces neointimal formation in a similar porcine model [85]. These preclinical studies demonstrate that both systemic and local delivery of estradiol prevent adverse vascular remodeling after arterial injury and provide rationale for the assessment of the therapeutic potential of estradiol-eluting stents in humans.

Other Drugs

Based on reported capacity to inhibit VSMC proliferation and neointima formation in different animal models of vascular injury, other drugs might prove effective at inhibiting clinical restenosis. For example, the 3-hydroxi-3-methilylglutaryl-CoA (HMG CoA) reductase inhibitor cerivastatin is one of the most promising compounds owing to its pleiotropic effects, which include inhibition of cell proliferation and improvement of EC function [86]. Preclinical assessment in a rat carotid model has revealed that cerivastatin-eluting stent deployment limits neointima formation [87]. Treatment of VSMC cultures with cerivastatin increases $p21^{Cip1}$ and $p27^{Kip1}$ levels, downregulates cyclin A and D1, and decreases CDK2 activity and pRb phosphorylation, leading to reduced cell proliferation, and all these effects of cerivastatin are less pronounced in ECs [87]. Therefore, local application of statins might limit restenosis while decreasing the risk of late stent thrombosis associated with defective reendothelialization.

Another compound of potential therapeutic interest in the setting of restenosis is flavopiridol, a synthetic CDK inhibitor that induces VSMC growth arrest in parallel with increased levels of the growth suppressors $p21^{Cip1}$, $p27^{Kip1}$, and p53 and decreased accumulation of hyperphosphorylated pRb [88, 89]. Accordingly, both oral and stent-mediated administration of flavopiridol significantly reduce neointima formation after rat carotid injury [88, 89].

Some antioxidants, such as carvedilol and probucol, have also demonstrated strong antiproliferative properties in the arterial wall. Oral treatment with carvedilol inhibits VSMC proliferation and blunts neointima formation in the rat carotid injury

model [90, 91], and carvedilol-coated stents inhibit neointima hyperplasia in pigs [92]. In contrast, the results with the related antioxidant probucol are conflicting. On one hand, oral administration of probucol in rabbits decreases neointima formation and the number of lesional proliferating VSMCs in balloon-injured carotid artery [93] or abdominal aorta [94], and some studies suggest that probucol also promotes reendothelization [94, 95]. However, other studies do not find any protective effect of probucol against neointimal thickening following balloon angioplasty in the rat carotid artery [96] or stent deployment in porcine coronary artery [97]. Similarly, probucol-coated stents fail to demonstrate beneficial effects in lumen area, neointimal area, or arterial cell proliferation in a porcine coronary injury model [92].

Cilostazol is a novel and potent inhibitor of phosphodiesterase in platelets and VSMCs that exerts both antithrombotic and antiproliferative properties, and is therefore a promising therapeutic candidate in the setting of restenosis. Cilostazol inhibits mitogen-induced VSMC proliferation by increasing the concentration of cyclic adenosine monophosphate [98], resulting in activation of the p53-$p21^{Cip1}$ axis [99]. Notably, oral cilostazol treatment inhibits neointima formation in the rat carotid balloon angioplasty model [100], and cilostazol-coated stents reduce neointimal thickening in porcine coronary arteries [101].

Some antidiabetic drugs have also demonstrated their effectiveness at reducing adverse vascular remodeling. Thiazolidinediones (e.g., rosiglitazone, pioglitazone) are peroxisome proliferator-activated receptor γ [PPAR-γ] agonists originally developed as insulin sensitizers, but also exhibit vascular protective properties. For example, among other beneficial effects in the arterial wall, thiazolidinediones inhibit VSMC proliferation via ERK inactivation and induction of GSK-3β-dependent signaling [102]. Studies in rodents have demonstrated that rosiglitazone treatment prevents neointimal thickening after mechanical injury of the carotid artery [102–104]. Similar beneficial effects of thiazolidinediones have been observed in balloon injury [105] or stent implantation [106] rabbit models, and stenting in porcine coronary arteries [107].

Tranilast is an inhibitor of TGF-β-dependent signaling that attenuates VSMC proliferation in vitro [108–111] by a complex mechanism that involves inhibition of ERK1/2 [110], downregulation of the transcription factor c-myc [109], and upregulation of $p21^{Cip1}$ [112]. Studies in rabbits and rodents have demonstrated that oral administration of tranilast reduces neointimal growth after photochemical or balloon injury of the arterial wall [113–116], and similar results have been obtained in pigs after coronary artery stenting [117, 118].

Antiproliferative Strategies for the Treatment of Clinical Restenosis Using Drug-Eluting Stents

PCI is the preferred therapeutic option to treat symptomatic coronary artery disease in the majority of cases. Interventional cardiology, as well as special areas of interventional angiography such as stent- or balloon-based treatment of complex lesions

Table 8.2 Indications for drug-eluting stents—current evidence

Lesion characteristics	Reference
Long lesions (≥20 mm)	[120]
Chronic total occlusions	[121]
Acute myocardial infarction	[122]
Small vessels (≤2.75 mm)	[123]
Bare metal stent restenosis	[124]
Bypass grafts	[125]
Patient characteristics	**Reference**
Diabetic disease	[126]
Chronic renal failure	[127]
Transplant vasculopathy	[128]

of the superficial femoral artery or below-the-knee arteries that inevitably carry a high risk of restenosis, have greatly benefited from the introduction of drug-eluting interventional devices. The use of drug-eluting stents has now even paved the road to safely and reliably treat complex coronary artery disease even in cases that had been previously considered to be a domain of bypass surgery, such as left main coronary artery and multivessel disease, including in diabetic patients [119]. To date, numerous lesion and patient characteristics have been identified to benefit from the usage of drug-eluting stents (Table 8.2). Predictors of restenosis include stent length and the number of stents per lesion, lesion length and complexity, small vessel diameter (≤2.75 mm), residual diameter stenosis, and certain clinical scenarios (e.g., previous restenosis and diabetes mellitus), while premature antiplatelet therapy discontinuation, renal failure, bifurcation lesions, diabetes, and low ejection fraction have been identified as predictors of thrombotic events associated with drug-eluting stents deployment [14]. The diagnostic gold standard for restenosis is coronary angiography, but noninvasive diagnostic tools are being developed (e.g., computerized tomography, cardiac magnetic resonance tomography).

To date, the two major classes of pharmacological compounds used in clinical interventional cardiology are the mTOR inhibitors (referred to as "limus drugs") and paclitaxel, which inhibit VSMC proliferation and migration, two key processes that contribute to neointimal thickening during in-stent restenosis (Fig. 8.3). The term "limus drugs" is confusing since pimecrolimus and tacrolimus are calcineurin inhibitors that only exhibit immunosuppressive activities and have yielded unsatisfactory results when used in drug-eluting stent platforms to prevent restenosis [129, 130]. By contrast, pivotal studies a decade ago using sirolimus- and paclitaxel-coated stents have shown a dramatic decrease of late lumen loss, the pathoanatomical correlate of angiographic and clinical restenosis, compared to uncoated bare metal stents [131]. Meta-analysis and recent clinical head-to-head trials have implicated superior performance of mTOR-inhibitor-eluting stents [132]. Interestingly, the first clinically available drug-eluting stents, Cordis' sirolimus-eluting stent, has been unsurpassed in terms of clinical safety and efficacy as is becoming evident in recent randomized comparisons presented at large international meetings as well as

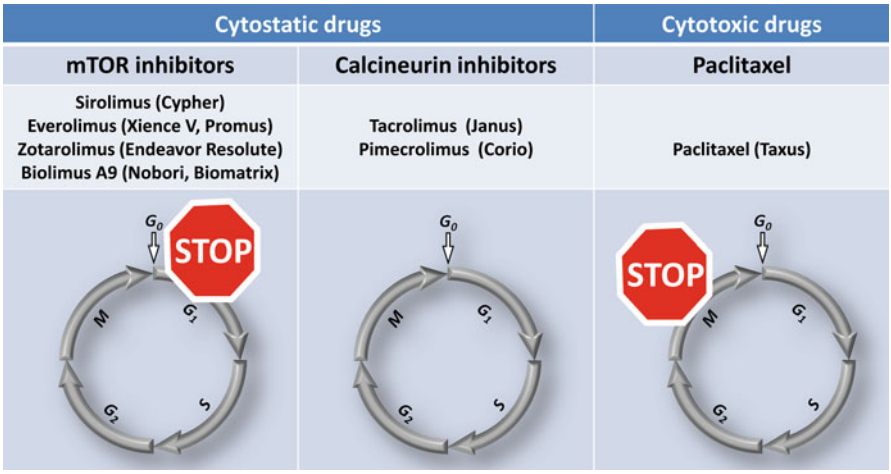

Fig. 8.3 Overview of drugs currently used on the vast majority of drug-eluting stents approved for human use. The name of the stent platform is provided in *parenthesis*. A large reduction in restenosis and need for target vessel revascularization has been conclusively demonstrated with drug-eluting stents that deliver mTOR inhibitors and paclitaxel, two unrelated families of drugs which cause cell cycle arrest in G0/G1-phase and M-phase, respectively. Tacrolimus and pimecrolimus are calcineurin inhibitors which only exhibit immunosuppressive properties and have yielded unsatisfactory clinical results in drug-eluting stents platforms

peer-reviewed publications [133]. However, due to potential improvements in stent design, Abbott's everolimus-eluting Xience V stent is the most frequently used stent in contemporary interventional cardiology.

Several studies investigated the use of dual drug-eluting stents to inhibit the rate of restenosis. Most of the combinatorial approaches revealed no beneficial effect. Examples include the combination of paclitaxel and pimecrolimus [130] or sirolimus and estradiol [134]. Interestingly, a combination of sirolimus and probucol on the ISAR platform revealed a positive effect [135]. However, replication of clinical results by independent groups is not yet available.

Current Limitations of Drug-Eluting Stents and Optimization

As in many instances, medical devices such as drug-eluting stents do not exclusively alleviate clinical problems such as restenosis but are associated with limitations that merit further optimization. The major shortcomings associated with current FDA-approved drug-eluting stent platforms that can be associated with the development of late (between 1 and 12 months after stent placement) and very late (12 months and later) stent thrombosis are listed in Table 8.1. Since cell cycle

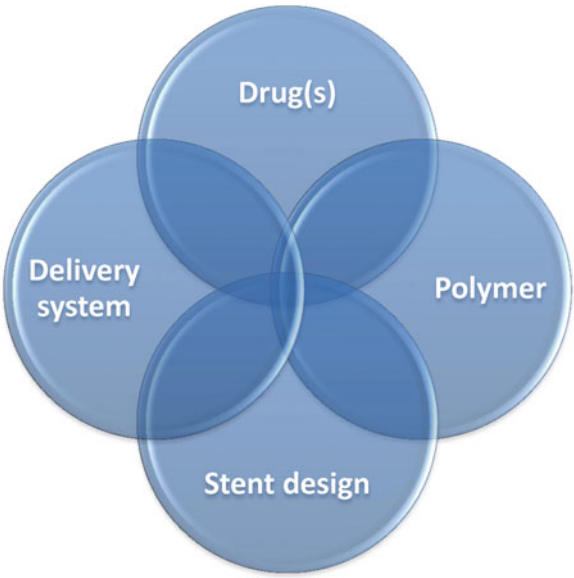

Fig. 8.4 Major components of a typical drug-eluting stent platform. Research endeavors to improve the efficacy and safety of drug-eluting stents include the identification of new drugs, new stent design, optimization of delivery systems (e.g., use of bioabsorbable stent platforms), and improved biodegradable polymers

inhibitors do not selectively inhibit proliferation of their main target cells, namely VSMCs, but also inhibit proliferation of other cells, most importantly ECs, cell cycle inhibitors can delay healing processes and thus precipitate acute and subacute, life-threatening events, in particular stent thrombosis. Whereas early stent thrombosis that occurs during the first 30 days after stent placement is generally associated with problems linked with PCI itself or shortcomings attributable to concomitant antithrombotic pharmacotherapy such as drug resistance or patient incompliance, late/very late stent thrombosis is often related to risks associated with ongoing local inflammatory processes and delayed arterial healing, thus leading to a prothrombotic milieu (Table 8.1). Since stent thrombosis is associated with considerable mortality, it has been the focus of many clinical investigations. Thus, current guidelines recommend prolonged dual antithrombotic therapy of at least 12 months after drug-eluting stent implantation, exceeding the 4-week recommendation for bare metal stents [136].

The major components of a typical drug-eluting stent platform that can be optimized to increase efficacy and safety of drug-eluting stents include the polymer, the delivery system, stent design, and the drug itself (Fig. 8.4) [137]. All current FDA-approved drug-eluting stents carry a nonerodible polymer to avoid boost release and retard drug delivery to the vascular wall, since prolonged drug release of several weeks is considered to be of pivotal importance for effective inhibition of restenosis. Thus, the issue of polymeric coating is of integral importance for the development of novel drug-eluting stent platforms. Yet, virtually all polymers are able to precipitate proinflammatory processes in the vascular wall and are therefore

considered to be a major cause for late and very late stent thrombosis. To circumvent this important clinical dilemma, several possible solutions have been proposed and are currently under investigation. The major focus is now on biodegradable polymers such as a polylactic acid polymer that biodegrades into carbon dioxide and water over time, as it is used on the biolimus A9-eluting Biomatrix and Nobori drug-eluting stents platforms. A fairly large clinical trial has shown noninferiority of this stent platform compared to the current gold standard, the sirolimus-eluting stent [138]. Other approaches to limit or abstain from surface polymer coating are microporous stents [139], reservoir-based drug delivery [137], and bioactive surface technology [140].

Major attention has been recently drawn to bioabsorbable stent platforms. The rationale behind this intriguing approach is the limited presence of a vascular scaffold in the coronary artery. However, first-in-man clinical trials using these approaches revealed rather disappointing results, eventually leading, for example, to the cessation of the magnesium bioabsorbable stent program from Biotronik [141]. However, a polymer-based, fully erodible coronary stent that delivers everolimus has recently shown encouraging results in a limited number of patients [142]. Albeit widespread clinical use of this particular stent platform is not currently foreseeable, the interest and expectations regarding this technology remain high in the interventional cardiology community.

Conclusions

In the last two decades, numerous studies in animal models have conclusively demonstrated that inhibiting cell proliferation within the damaged vessel wall is a suitable strategy to limit neointimal thickening after angioplasty. Nowadays, the majority of coronary interventions utilize drug-eluting stents that deliver locally high doses of antiproliferative drugs, such as sirolimus (and derivatives) and paclitaxel. These medical devices have revolutionized the field of revascularization owing to a dramatic reduction in the incidence of restenosis, target lesion revascularization, and major adverse cardiac events. However, both efficacy and safety of drug-eluting stent platforms need to be improved to reduce the need for repeated revascularization and the development of late stent thrombosis due to delayed reendothelialization, which forces prolonged oral dual antiplatelet therapy. Major areas of drug-eluting stent research include the development of new drugs, approaches to limit or even avoid the presence of polymers (e.g., biodegradable polymers, microporous stents, reservoir-based drug delivery), use of antithrombotic coatings, bioactive surface technology to promote vascular healing (e.g., antibody-, peptide-, and nucleotide-coated stents), and development of bioabsorbable stent platforms. By combining different strategies, next-generation drug-eluting stent platforms may consist of polyvalent devices that embrace the three foundations of stent-based lesion therapy: antirestenotic, prohealing, and antithrombotic. Another goal will be

to develop drug-eluting stents tailored to some patient or lesion subgroups (e.g., diabetics, patients presenting with acute myocardial infarction) and lesion characteristics. Achieving these ambitious objectives will certainly require the close interaction of specialists in different biomedical and medical disciplines.

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Chapter 9
Cardiovascular Nanomedicine: Challenges and Opportunities

Biana Godin, Ye Hu, Saverio La Francesca, and Mauro Ferrari

Introduction

Nanomedicine is a multidisciplinary field that integrates concepts of nanotechnology and medicine, aiming at precise control over the biological processes that take place on the submicron scale. Being on the interface of the two areas of research, nanomedicine possesses exceptional social and economic potential arising from the synergistic combination of specific achievements in the respective fields.

The science of nanotechnology was envisioned by the Nobel Laureate Richard Feynman in his eminent talk at the annual meeting of the American Physical Society in 1959 titled "There is a plenty of room at the bottom." In this visionary and, at that time, "science-fictionary" speech, he described his dream of manipulating and controlling objects on a submicron scale. Almost four decades later, Richard Smalley, who received the Nobel Prize in 1996 for the seminal discovery in nanoscience and nanotechnology of carbon-60 molecules, aka buckyballs, claimed "human health has always been determined on the nanometer scale; this is where the structure and properties of the machines of life work in every one of the cells in every living thing." Following the 1968 discovery in the Bell Laboratories of molecular beam epitaxy, a method enabling deposition of single crystals and creation of structures with electrons confined in space, and the invention of the scanning tunnel microscope (STM), nanoscience progressively developed into a strong discipline [1, 2].

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A fulfillment of Dr. Feynman's old dream was enabled by prominent technological advances in electronics, optical systems, the auto industry, environmental engineering, and medicine. Though for many of the end-users "nano" became just a fashionable term, the practical applications of sophisticated nanotechnology in durable surface coating, intelligent materials, faster electronics, biosensors and nanoparticles in a variety of our everyday products clearly demonstrate its colossal potential to enhance our lives.

Nanotechnology is beyond mixing together a bunch of nanoscale objects—it requires the ability to manipulate and control them in a useful way. Nanotechnology is defined as the science of engineered synthetic objects that are nanoscale in dimensions or have critical functioning components of such a size, and that therefore possess special emerging properties [3]. This is a general and operational definition involving the following interrelated constituents: (1) nanoscale dimensions of the whole system or its vital components; (2) man-made nature; and (3) the unique characteristics of new material that arise due to its nanoscopic size, with each element being equally essential for an object to be described as nanotechnological. Another vital component in this description is that the unique features and emerging properties of the nanomaterial have to be sustained by proper mechanism of action (e.g., mathematical modeling). Other definitions of nanotechnology can be found in the literature and according to some the term "nanoscale" should be interpreted to encompass the range of 1–100 nm. For example, the National Cancer Institute defines (http://www.cancer.gov/dictionary) nanotechnology as: "the field of research that deals with the engineering and creation of things from materials that are less than 100 nm (one-billionth of a meter) in size, especially single atoms or molecules."

Nowadays, nanotechnology is a rapidly growing, multidisciplinary field involving support from scientists in academia, industry, and the regulatory/federal sectors. As an example, the National Nanotechnology Initiative was established in 2001 to coordinate federal nanotechnology research and development (www.nano.gov). The 2009 budget request included $1.5 billion for the National Nanotechnology Initiative, with major investment in nanotechnology research and development over the past decade that reflects broad support by the United States Congress for this program.

Challenges and unmet needs in medicine provide an opportunity to develop conceptually new, nanoscience-enabled sophisticated technologies. One of the prominent challenges facing contemporary medicine is the personalization of treatment. Personalized medicine can be defined as the tailored treatment strategy individually developed from results of the patient's clinical samples, including sophisticated diagnostic imaging, genomic and proteomic analysis. Due to its ability to direct processes on the subcellular level, nanomedicine is considered one of the main enablers of personalized therapy [4, 5].

There are numerous challenges in cardiovascular disease diagnosis and treatment that may be met by nanomedicine. These challenges are depicted in Fig. 9.1, along with the types of nanotechnology that may be applied to meet the challenges.

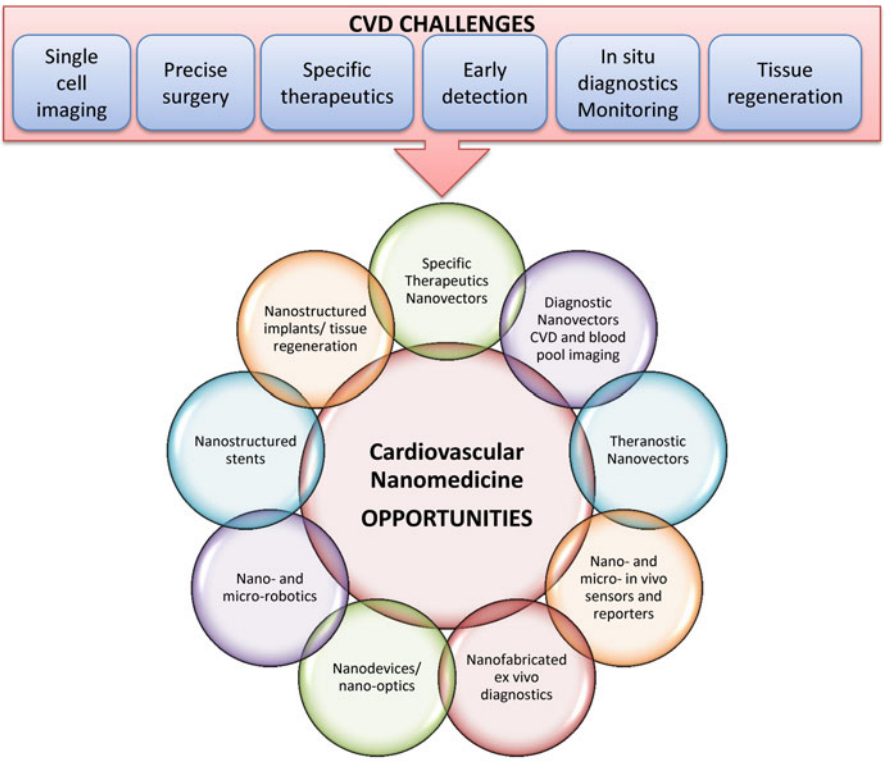

Fig. 9.1 Challenges in cardiovascular disease diagnosis and treatment that may be meet by opportunities provided by nanomedicine

In this chapter we will present a brief overview of nano-based technologies in medicine, with a special focus on cardiovascular nanomedicine. We begin with a snapshot of the challenges in management of cardiovascular disorders (CVDs), focusing on the opportunities for nanotechnology in the field. Nanovectors taxonomy and its potential to improve CVD diagnosis and therapy will be discussed. Further, an overview of other nanotechnology-based platforms, such as diagnostic tools and nanotextured devices, is described. The chapter concludes with a clinical perspective on cardiovascular nanomedicine.

Cardiovascular Disease and Nanomedicine

Nanomedicine is a far-reaching field that spans many different scientific disciplines. Most of the efforts in nanomedicine have focused on the treatment of cancer. The vascular system was thus viewed as a mere conduit for the nanoparticles,

which would try to interact in the most passive way possible with the system and its circulating components while *en route* to their target, the leaking tumor endothelium. In light of this, most of the main concepts driving the successful navigation and delivery of the particles and their payloads needed to be readdressed when targeting the cardiovascular system.

In 2003, The National Heart, Lung, and Blood Institute (NHLBI) called together a working group of scientists to analyze the challenges and opportunities offered by nanotechnology for CVDs (http://www.nhlbi.nih.gov/meetings/nano_sum.htm). The working group, chaired by Dr. Mauro Ferrari (currently the CEO of The Methodist Hospital Research Institute), included physicians, engineers, chemists and biologists sharing ideas of how to apply nanotechnology concepts to overcome challenges associated with therapy and diagnosis of heart, lung, and blood-related disorders. The working group consensus was that nanotechnology is expected to have a very pronounced impact in the near future in the following fields: nanotherapeutics, molecular imaging, point-of-care diagnostics, biosensors, and tissue engineering/biomaterials. The major findings of the committee were reported in a seminal paper in the field of cardiovascular nanomedicine titled "Recommendations of the National Heart, Lung, and Blood Institute Nanotechnology Working Group" [6].

The primary recommendation of the working group was to facilitate interdisciplinary research between the nanotechnology/nanoscience communities and researchers working on CVDs and lung disorders. As a result, in 2005 the NHLBI created a program of excellence in nanotechnology (PEN) with the specific goal of bridging the gap between clinicians and academics to enable the translation of cutting-edge discoveries in nanoscience and nanotechnology research to the diagnosis, treatment, and management of an array of CVDs. Given the progress made since the first round of funding in 2005, in the fall 2010 NHLBI awarded $65 million to renew its Programs for Nanotechnology Research. The four current awards involve teams from 17 institutions (http://www.nhlbi-pen.net/centers/gatech.html) and are focused on clinical translation of technological advances accomplished in the prior years. As an example, one of the PENs, initially awarded in 2005 and re-awarded in 2010, unifies the efforts of scientists from six institutions, namely Washington University in St. Louis, Texas A&M University, The University of California, Berkeley, The University of California, Santa Barbara, and Southwestern Medical Center in Dallas. The main goal of this center of excellence is to produce nanomaterials tailored with specific sizes, shapes, and compositions for improved imaging and treatment of acute lung injury and atherosclerosis [7–9]. Other centers of excellence with home institutions at Massachusetts General Hospital, Georgia Institute of Technology and Mount Sinai Medical School/Massachusetts Institute of Technology are developing nanoparticle-based tools to image and deliver therapeutics to atherosclerotic plaque [10], to enhance stem cell repair of damaged heart tissue [11], to create a point-of-care system for the rapid detection of pulmonary infections and CVDs [12], to treat atherosclerosis, and to prevent heart attack, and to deliver regeneration factors to repair damaged heart tissue.

Nanovector Design for Cardiovascular Disease Therapy

Targeted Drug Delivery and Nanovector Taxonomy

At the beginning of the twentieth century, Paul Ehrlich, considered by many to be the father of pharmacology, proposed the "magic bullet," a concept that diseases in the body could be treated by chemical substances with a high affinity for each particular disease [13]. At that time, the idea appeared too avant-garde since no agents and molecular disease targets had yet been discovered. However, in the past few decades, remarkable progress has been made to shed light on the pathological processes and identify specific molecular targets for various disease processes. As an example, the FDA has approved more than 30 anticancer drugs for clinical use in the past decade [14], as well as a vast variety of other therapeutic agents for a wide range of conditions, from cardiovascular disease to inflammation. The therapeutic potential of these and many other agents on the molecular scale is unarguable. These advances in pharmacology and medicine provide us with an opportunity to design specific and capable drug-delivery nanovectors that can utilize both the biology of the disease and the physics associated with the disease locus to better treat it.

There are several roadblocks that hamper clinical translation and success of new therapeutic agents. First, the physicochemical properties of some therapeutics prevent them from being efficiently administered in the molecular form. As an example, the polycyclic nature of most drugs makes them insoluble in aqueous environments [15]. Drugs such as paclitaxel and dexamethasone have water solubility values of 0.0015 mg/mL [16] and 0.1 mg/mL [17], respectively, which makes them unacceptable for intravenous injection in aqueous media. Additional impediments are associated with the presence of multiple biological barriers that effectively prevent the administered drug or imaging agent from reaching its target tissue. The disease tissue accumulation of a molecularly targeted and specific agent administered in a solution is extremely low, with only 1 in 10,000 to 1 in 100,000 molecules reaching their intended site of action [18]. As a result, significantly higher doses of an agent have to be administered to achieve an adequate therapeutic response, thus making the range between efficacy and toxicity very narrow [19]. Doxorubicin, which possesses prominent cardiotoxic effects, represents an example of such an agent [20].

The ideal drug, in contrast, should possess a multitude of characteristics such as increased stability, solubility, and targeting to the site of action. It is clear, though, that a single molecule is frequently unable to meet these needs. This reality presents the fundamental driving force behind the concept of nanotherapeutic drug delivery to enable and enhance drug function regardless of poor intrinsic physicochemical, pharmacological, and biodistribution properties.

A "nanovector" is a nanoscale particle or system, having nanoscale components, that is used for the delivery of therapeutic or contrast agents. Nanovectors are being investigated and developed as carriers for personalized therapeutic and imaging contrast agents based on the anticipated advantage of enhanced homing to the

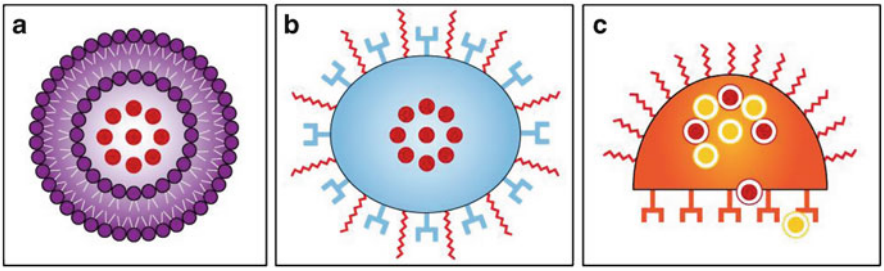

Fig. 9.2 Nanovector design and evolution. (**a**) First-generation nanovectors, such as currently used therapeutic liposomes, comprise a container (*purple lipid bilayer*) and an active principle (*red dots*). They localize in the tumor by enhanced permeation and retention (EPR), or the enhanced permeability of the inflamed/tumor vasculature. (**b**) Second-generation nanovectors target their therapeutic action to specific cells or locations via antibodies and other biomolecules (*blue* and *red surface projections*), remote activation, or responsiveness to the environment. (**c**) Third-generation nanovectors, such as multistage agents, are capable of more complex functions, including time-controlled deployment of multiple waves of active nanoparticles (*red* and *yellow encapsulated dots*), deployment across different biological barriers, and deployment to different subcellular targets. Modified from [4, 24]

diseased site (such as atherosclerotic plaque, cancer lesions, etc.). This homing behavior relies on the nanoparticles' ability to cross various obstacles, the so-called "bio-barriers," located between the administration site and the target organ. Historically, oncology represents the field of medicine to which nanotechnology has made the most prominent contributions. During the last 15 years, nanocarriers occupied an important niche in treatment of cancer patients, with liposomes being the first commercially available drug nanovector for injectable therapeutics [2, 21, 22]. Liposomal doxorubicin was granted FDA approval in the mid-1990s for use against Kaposi's sarcoma. Since then, a diverse array of therapeutic nanovectors with various compositions, physicochemical characteristics, geometry and surface functionality have completed various stages of development [23, 24]. This has generated a gigantic "toolbox" of nanovectors to consider when developing a carrier for a specific drug or disease condition. The goal is to enhance disease targeting by designing a nanovector to account for physiological and biochemical changes in the disease processes and site, such as differences in hemodynamics, molecular/cellular targets, impairment in the integrity of endothelial layer, etc.

Nanovectors can be divided into the three main subcategories or generations that reflect the order of their development, as shown schematically in Fig. 9.2 [4, 5, 21, 24]. The first generation of nanovectors includes nanoparticles that reach the disease site using passive mechanisms. The main subclass in this category is the liposomes [25], which have been utilized clinically for several decades. In the case of cancer, liposomes utilize the enhanced permeability of the neovasculature to localize into the disease site via the so-called enhanced permeation and retention (EPR) mechanism [26, 27]. The extravasation of liposomes is favored

by this mechanism due to the presence of the large (several hundred nm) vascular fenestrations in the newly formed vessel. The carriers in this subclass may possess surface modifications with, for example, polyethylene glycol (PEG), making the nanovectors hydrophilic, with a large number of water molecules adsorbed on the surface, effectively shielding the nanovector surface and thus preventing their uptake by the cells of reticuloendothelial system (RES). These "stealth" particles have substantially prolonged circulation time, effectively increasing the likelihood of reaching the tumor [25, 28–30]. Significant strides in the fields of chemistry and materials science have yielded several other nanosized vectors with immense potential for drug delivery, including macromolecular assemblies, such as polymer–drug conjugates [31], polymer micelles [16], and dendrimers [7]. Polymers are macromolecules composed of repeating linear, cross-linked structural units, while dendrimers refer to repeatedly branched, roughly spherical macromolecules. Based on the physicochemical characteristics of the repetitive units in these macromolecules, it is possible to design systems with beneficial properties, such as enabling the solubility of hydrophobic substances in aqueous environments and protecting sensitive therapeutics from degradation.

These first-generation nanovectors are carriers with no active mechanisms of disease site homing. The second-generation of nanovectors includes delivery systems that incorporate an additional functionality [32–36]. This functionality can be of two origins: (1) specific molecular recognition moieties on the nanovector to cognate receptors overexpressed on the tumor cells or adjacent blood vessels (e.g., monoclonal antibody-conjugated liposomes) [37], or (2) active or triggered release of the nanovector payload at the disease location (e.g., magnetic liposomes) [38]. Superior to their precursors, second-generation nanovectors incorporate additional complexities such as targeting moieties, remote activation, and environmentally sensitive components to enhance drug delivery and efficacy. However, the second-generation vectors predominantly represent a simple a progressive evolution of the first-generation nanovectors.

The fundamental problem of various obstacles that block therapeutic agents from reaching their target has led to a paradigm shift in the design of nanovectors, and the emergence of a third generation of vector. We strongly believe that further developments of nanovectors for personalized therapy will rely on the third generation of carriers, so-called logic embedded vectors (LEVs) [39, 40]. LEVs are therapeutic multicomponent constructs in which the functions of biorecognition, cytotoxicity, and biobarrier avoidance are decoupled, yet act in efficacious operational harmony. LEVs will help to meet the ideal therapeutic strategy that is capable of navigating through the vasculature after intravenous administration to reach the desired disease site at full concentration, and to selectively affect target cells with minimal harmful side effects.

Below we provide examples of nanovectors belonging to the three above-mentioned generations for advanced therapy and imaging of cardiovascular disease. These nanovectors demonstrate immense potential for enhanced drug delivery, which will undoubtedly have a great impact on the future of personalized medicine.

Effect of Geometry on the In Vitro and In Vivo Performance of Nanovectors

Clinically used nanovectors as well as the majority of the vectors under investigation have a spherical shape. Recent progress in microfabrication techniques, as well as micro- and nano-particle chemical synthesis, has enabled the production of nanovectors of different geometry, including hemispheres, discs, and rods [41–43]. Nanovector geometry (i.e., size and shape of the vector) plays a fundamental role in the process of intracellular uptake and biodistribution. Recent work emphasizes that nanovector geometry is an important design parameter that defines its transport in the vasculature and its adhesion to the target receptors, as well as the mode of nanovector–cell interactions such as endocytosis, vesiculation, and phagocytosis [24, 41, 44–56]. The effect of carrier geometry on intracellular delivery can be explained in part by the different effects that particles with variable sizes and shapes have on membrane remodeling. This process can be related to the number of binding receptors and is important in endocytosis, vesiculation, and vesicle transport through the endoplasmic reticulum and Golgi apparatus, protein sorting, and other vital cellular functions. In general, membrane remodeling is highly dependent on membrane curvature, normally affected by proteins such as actin, dynamin, and coatomer protein [57], through curvature-mediated attractive interactions [58]. As an example, the cell's formation of a coordinated actin cup is crucial to phagocytosis and probably follows the local geometry of the particle. Because actin remodeling is a metabolically intensive process, it may provide the basis for the fact that particles requiring only gradual expansion of the actin ring are phagocytosed more effectively [52].

The interaction of nanovectors of various geometries with different cells lines has been described. In vitro studies in macrophages (phagocytic cells present in atherosclerotic plaques) indicate that IgG-coated polystyrene spherical particles (200 nm to 2 μm) are internalized by multiple intracellular delivery pathways. Nanosized particles are internalized by clathrin-mediated endocytosis, but larger microspheres undergo a classic endosome-mediated phagocytotic trafficking to the lysosomes [59, 60]. The tube-shaped nanovectors, such as carbon nanotubes, were shown to behave quite differently with respect to cellular uptake. These lightweight structures do not produce a pressure force sufficient to initiate intracellular delivery processes. In a publication on the rate of intracellular uptake of length-fractionated single-walled carbon nanotubes (SWNTs), the authors assessed single-particle tracking by rate of their intrinsic photoluminescence. It was suggested that nanosized particles must aggregate on the cell membrane to form a cluster sufficient in size to generate an enthalpic contribution to overcome the elastic and entropic energy barriers associated with membrane vesicle formation. Interestingly, the rate of endocytosis for nanotubes was three orders of magnitude higher than that for spherical gold nanoparticles, whereas a similar rate of exocytosis was observed for poly(D,L-lactide-*co*-glycolide), single-wall nanotubes, and gold nanoparticles in different cell lines [61].

In endothelial cells, the effect of carrier geometry on the rate of endocytosis and lysosomal trafficking was demonstrated in a study testing the intracellular uptake of various sizes of ICAM-1-targeted spherical and elliptical polymeric nanoparticles (100 nm to 10 μm). It was shown that discoidal particles exhibited higher targeting specificity. An increase in particle size to above 1 μm caused particles to accumulate and remain longer in pre-lysosomal compartments, whereas submicron nanovectors trafficked to lysosomes more readily [53]. Doshi and Mitragotri have reported that spheres, elongated, and flat particles with an effective diameter of 0.5–1 μm and varying surface charges affected endothelial cells differently. An impairment in cell membrane functions related to cell spreading and motility was observed with needle-shaped vectors, whereas spherical and elliptical disc-shaped particles did not have an impact on these functions [54].

Dynamic manipulation of particle geometry can be used as a tool to control particle–cell interactions [62]. Yoo and Mitragotri have designed polymeric particles that switch shape in a stimulus-responsive manner. The shape-changing behavior was a result of a fine balance between polymer viscosity and interfacial tension. Particle shape could be modified based on external stimuli (temperature, pH, or chemicals in the medium), and elliptical particles that were previously not internalized by the cells could then be phagocytosed [63]. These results clearly emphasize the importance of size, shape, and surface physicochemical properties on the rate of uptake of nanovectors.

The geometry of the particle is a physical parameter that can be precisely controlled through bioengineering. Hence, the process of the cell uptake has been the subject of extensive mathematical modeling to achieve rational nanovector design. A number of mathematical models and design maps have been proposed [55]. In one of these models, the rate of uptake is described through a first-order kinetic law in which the intracellular concentration $C_i(t)$ increases with time, according to the relationship (1):

$$\frac{\mathrm{d}\tilde{C}_i(t)}{\mathrm{d}t} = k_{\mathrm{int}}[\chi - \tilde{C}_i(t)], \quad k_{\mathrm{int}} = \tau_{\mathrm{w}}^{-1}, \tag{1}$$

where τ_{w} is the characteristic time for the nanovector to be wrapped by the cell membrane, and it is related to the nanovector geometry (size, shape) and surface chemistry (zeta potential, specific ligands). Based on this model, which accounts for energy analysis of receptor-mediated internalization, there is a threshold minimal particle radius needed to enable intracellular uptake. Uptake of particles smaller than this threshold size is energetically unjustified, and thus cannot occur spontaneously. Similar analysis shows that the surface physicochemical properties of the nanovector can substantially increase or decrease the rate of uptake [44, 64]. This rate for ellipsoidal particles was found to be dependent on their aspect ratio, with spherical or oval particles more rapidly internalized by cells, in comparison with elongated particles.

Modeling of all the processes that a particle of specific geometry undergoes from the moment of administration to the arrival at the target requires integration of the

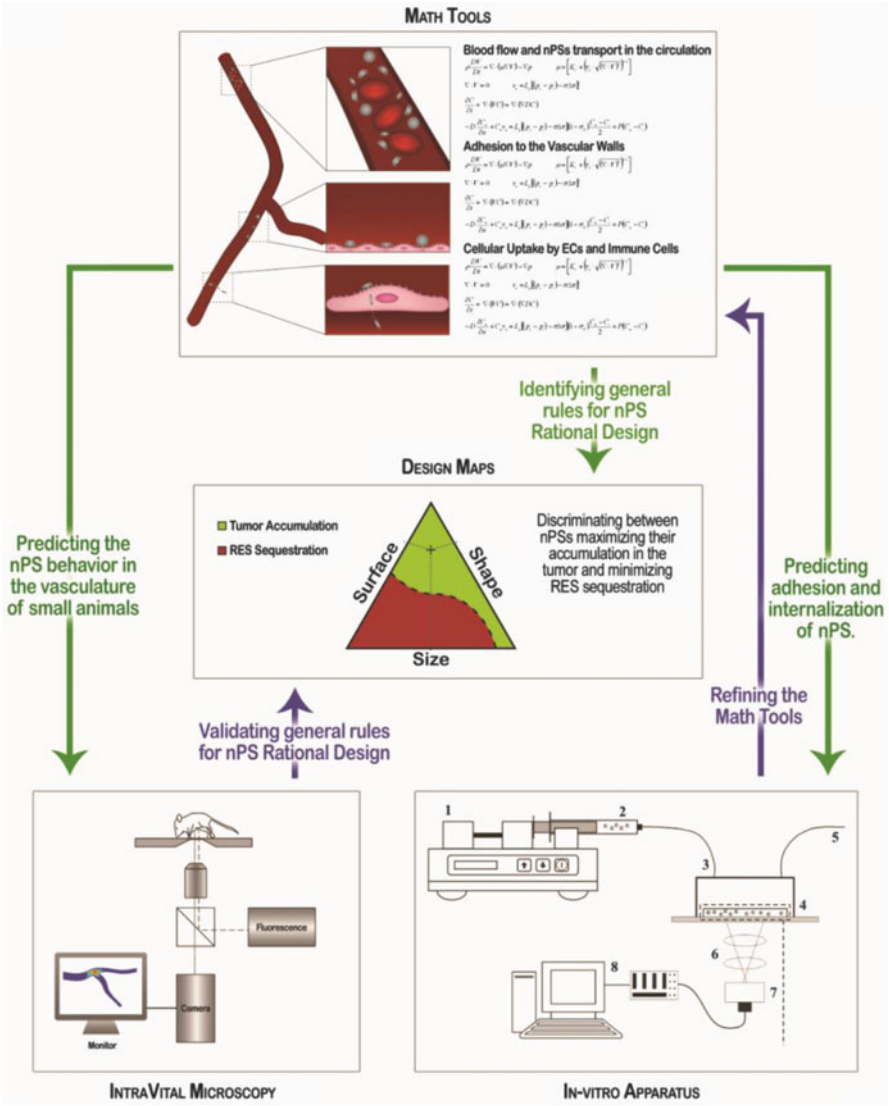

Fig. 9.3 Diagram of the integrated approach to develop design maps based on the data from in vitro, in vivo, and in silico studies. *nPS* nanoparticle(s), *RES* reticuloendothelial system. Reprinted from [1] with permission from Elsevier

effect of geometry on the intracellular uptake with other geometry-affected biological processes (e.g., margination, vascular transport, and adhesion to vessel walls). These phenomena can be evaluated both in vitro and in vivo, and data can be used to formulate in silico models. The process of model development is depicted in Fig. 9.3 [1]. This data integration enables the generation of design maps that

combine various components of nanovector performance, such as margination from the blood flow, recognition and specific binding to target receptors, and intracellular uptake as a function of the design parameters and physiologic and biophysical conditions. For example, such design maps for spherical particles (the simplest case) account for specific versus nonspecific interactions of the vector with the substrate. These interactions are dependent on steric and electrostatic surface interactions between the particle and the cell, and the ratio of the number of ligand molecules bound to the nanovector versus the number of receptor molecules expressed on the cell membrane. Based on these design maps, the probability of an intravenously administered nanovector of binding to a cell surface receptor at the target site and to be internalized by endothelial cells, tumor cells, or phagocytic cells can be estimated [45].

Nanotherapeutics for CVDs

Numerous nanovector-based drug-delivery systems have been and are being developed for applications in cardiovascular diseases. These systems have various features, multiple functionalities [2, 24, 55, 65], and exhibit differences in surface functionalization, with a broad range of electrostatic charges and conjugations. As with cancer applications, active targeting of nanovectors for CVDs is expected to enable local or facilitated delivery into the target cells, prolonged effect of the drug, and reduction of the shear effects of blood flow.

The main nanovector categories investigated as therapeutic and theranostic (combined therapeutic and diagnostic) agents for restenosis are lipid vesicles, polymeric nanocarriers, perfluorocarbon nano-emulsions, and cross-linked iron oxide (CLIO) particles conjugated to therapeutic molecules [66–70]. These classes of nanovectors are exemplified in Table 9.1 [75].

The major focuses of nanotechnology application in cardiovascular research have been the directed imaging and therapy of atherosclerosis, restenosis, and other cardiovascular conditions. During the progression of CVDs, such as atherosclerosis, there are a number of disease stage-specific molecules expressed on or in the plaque or newly formed neointima. These molecules include α (alpha)$_v\beta$(beta)$_3$-integrin [76], vascular cell adhesion molecule 1 [77], and the pentapeptide tyrosine-isoleucine-glycine-serine-arginine (YIGSR) [78], all of which can be used for active targeting of CVDs. In general, the in vitro studies that target CVDs are similar to those designed for tumor neovasculature. In both cases, the growth of new blood vessels and inflammation are characteristic features of the disease site.

Multiple nanoplatforms are under investigation for treatment of atherosclerosis and restenosis. The nanovector-delivered agents tested for prevention of restenosis included siRNAs [79], low-molecular-weight heparin [80] cytotoxic agents inhibiting smooth muscle cell proliferation (e.g., rapamycin [81] paclitaxel, cytarbine, etoposides, and doxorubicin), antagonists of platelet-derived growth factor (PDGF) receptor (e.g., tyrphostins [82]), inhibitors of the inflammatory/immune response (e.g., bisphosphonates [83], sirolimus [84]), and antibiotics (e.g., fumagillin [85]).

Table 9.1 Examples of nanocarriers for therapy of cardiovascular disorders

Nanocarrier	Example of agent	Experimental model	Outcomes	References
Neutral liposomes	Bisphosphonates (clodronate, alendronate, etc.)	Injured rat carotid artery	Macrophage depletion, reduced inflammation	[66]
Cationic liposomes	Chloramphenicol acetyl transferase (CAT) encoding gene [67]	Balloon injured Yorkshire pig artery, local delivery	Increased CAT expression	[67, 68]
Hemaglutin virus of Japan (HVJ) liposomes	Tissue factor pathway inhibitor gene	Porcine models	Reduced neointimal hyperplasia, inhibited thrombosis, and attenuated vascular remodeling and luminal stenosis after angioplasty	[71]
Perfluorocarbon nanoparticles		Up to phase III clinical studies [72]	Blood substitutes	[72]
Polymeric (PLA or PLGA) nanoparticles	AG-1295 and AGL-2043	Balloon injured rat carotid artery	Inhibition of restenosis	[73, 74]

Several biologically active agents such as nucleic acid-based materials [siRNA, DNA and energy substance-adenosine triphosphate (ATP)] undergo prompt enzymatic degradation in plasma by endogenous nucleases. Thus, nanovectors were used to protect these substances from degradation and to enable an efficient delivery to a disease location in CVDs. As an example, in studies focusing on ATP delivery from immunoliposomes conjugated to anti-myosin antibodies to treat myocardial infarction [37], it was shown that liposomal encapsulation significantly increased circulation time and enabled targeting of injured myocardial cells. Improvements in systolic and diastolic function as well as myocardial histomorphology in animals with induced myocardial infarction have shown that ATP-immunoliposomes effectively protected myocardium from ischemia and reperfusion-induced injury [25, 86].

Nanotechnologies for Early Detection of CVDs

In the field of modern cardiovascular medicine, our ability to achieve the goal of providing personalized, timely and highly sensitive early-stage diagnoses has been constrained by the lack of biosensors and molecular probes capable of rapidly recognizing the distinct molecular profiles of the diseases involved. Although significant advances have been made in management and treatment of CVDs, the molecular mechanisms responsible for conditions such as plaque formation remain largely unsolved. As a result, early detection is difficult, leading to a high rate of morbidity and mortality. The development of nanovector architectures and materials that have the ability to interact with cells, cellular organelles and biomacromolecules (proteins and genetic material) at the nanoscale could potentially extend subcellular and molecular detection beyond the limits of conventional diagnostic modalities. These novel nanoparticles or nanotextured devices would be able to directly interact with biologically significant molecules and convert those interactions into significantly amplified electrical or electromagnetic signals. These devices could potentially provide personalized information that could be used to assess the risk of developing specific conditions and could aid in the optimization of therapy for each patient.

The emerging applications of nanomedicine have enabled a new generation of diagnostic techniques for early-stage CVDs.

Nanoscale contrast agents that can be detected by conventional clinical imaging techniques have emerged as multifaceted modalities that identify and characterize early disease stages prior to the development of gross disease. Contrast-generating nanomaterials for cardiovascular imaging include fluorescent, radioactive, paramagnetic, superparamagnetic, and electron-dense and light-scattering particles that contain iron oxide, gadolinium (Gd), iodine, and fluorescent or radioactive probes. Currently, nanovectors approved by the U.S. Food and Drug Administration for imaging are limited to three iron oxide formulations targeted to the gastrointestinal tract, liver, and spleen: AMI-121 (Ferumoxsil), OMP50, and AMI-25 (Feridex). Injection of high doses of iron was shown to be nontoxic in the nanoparticle

formulation due to slow release of free iron and its subsequent assimilation into iron-containing substances in the body [87]. Thus, the door is open to further development of similar iron oxide-containing nanovectors to treat CVDs.

In this section, we review several nanotechnology and biomolecular engineering tools and methodologies to detect and analyze the formation of atherosclerotic plaques at the molecular level. All of the nanoscale biosensing materials included here were selected based on their potential to significantly impact diagnosis of CVDs, as well as their high sensitivity and capability for high-throughput screening of biological samples.

Nanoparticle-Based Bio-Barcodes

Effective clinical biomarker screening requires three important elements: (1) the ability to detect ultra-low concentrations of proteins within a complex biological sample, (2) the ability to look for several targets simultaneously, and (3) the ability to operate with minimal sample volumes. Several multiplexed assays have been developed to meet these needs, such as microsphere-based flow cytometry [88] and microarrays [89, 90]. Both techniques provide simultaneous high-throughput screening for different molecular markers of cardiovascular pathology. For example, DNA microarrays were used as a tool to study the effect of mechanical stimuli (e.g., shear stress) on endothelial cell gene expression profiles [91]. These studies showed that changes in shear stresses induce changes in gene expression and transcription profile in smooth muscle cells [92, 93]. Expression of genes, such as the endothelial transcription factor Kruppel-like factor 2 (KLF-2) which is a target for angiotensin II signaling and regulates cardiovascular remodeling, was found to correlate well with changes in shear stress and damage to vascular cells [94]. If needed, the sensitivity of biomarker detection by microarray can be increased by directly amplifying low abundance DNA or RNA target sequences using polymerase chain reaction (PCR).

In 2003, Chad Mirkin's group developed a barcode system based on the use of magnetic microparticles and gold nanoparticles [95]. As shown in Fig. 9.4, the magnetic microparticle is first loaded with recognition elements, such as primary antibodies, and the gold nanoparticle is conjugated by a second, similar recognition element, as well as large numbers of specific double-stranded DNA sequences. The two particles then sandwich a specific target that is subsequently extracted using a magnetic field. Although all of the magnetic microparticles are captured, only gold nanoparticles that have bonded to the target are captured. The double-stranded DNA immobilized on the gold nanoparticle surface is then dehybridized to release one of the strands into solution, either chemically using dithiothreitol [96], or thermally [97, 98]. That strand is quantified using chip-based DNA techniques with or without PCR. This bio-barcode technique is not only capable of multiplexed analysis of small sample volumes, but given the high ratio of DNA barcodes to target recognition elements, also provides significant signal amplification. The sensitivity of this

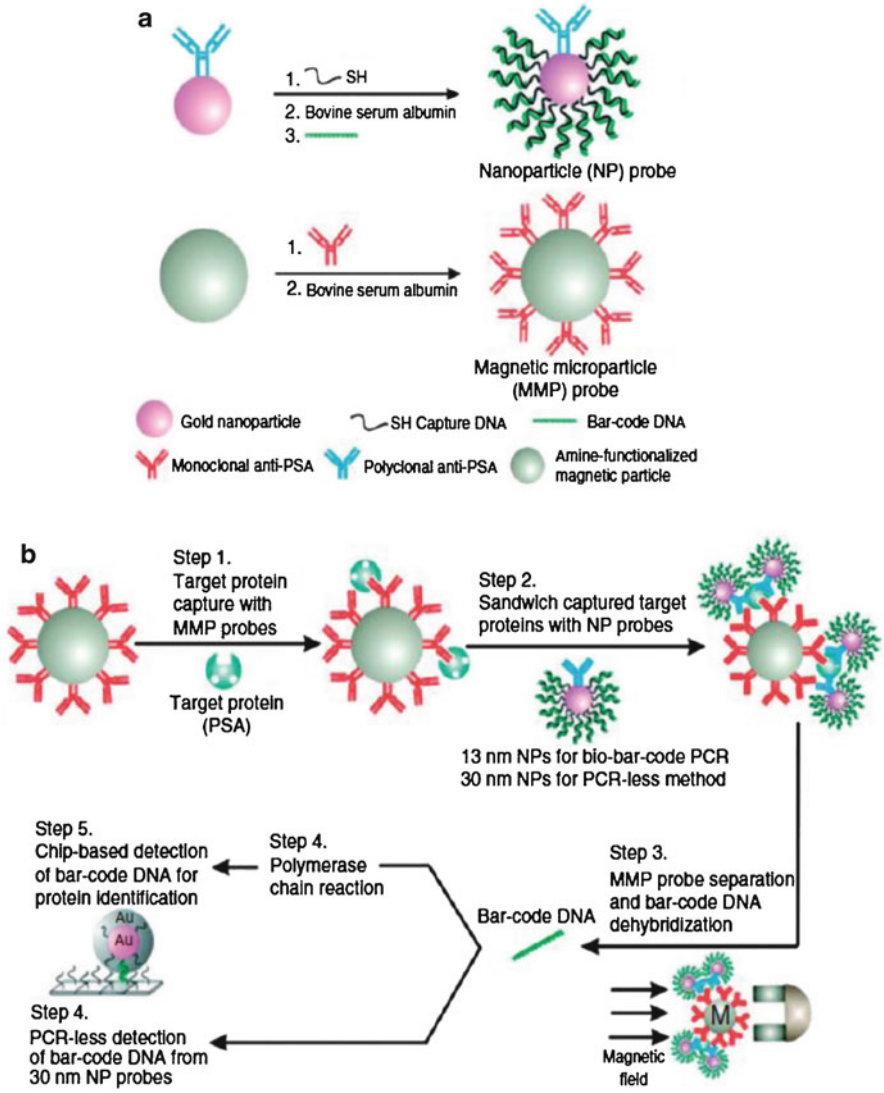

Fig. 9.4 Bio-barcode assay scheme: (**a**) probe design and preparation. (**b**) A magnetic probe captures a target using either monoclonal antibody or complementary oligonucleotide. Target-specific gold nanoparticles sandwich the target and account for target identification and amplification. The barcode oligonucleotides are released and detected using the scanometric method. Reprinted from [95] with permission from AAAS

methodology exceeds that of enzyme-linked immunosorbent assay (ELISA) by up to a million-fold, making possible the use of low abundance biomarkers whose concentrations were considered too low to be useful with previous technologies. The bio-barcode assay has been shown to successfully detect free prostate specific

antigen (PSA) at concentrations in the range of 3–300 fM. The method has also been used to demonstrate the potential of amyloid-derived diffusible ligands in cerebrospinal fluid as a marker for Alzheimer's disease [99].

Altered expression of certain genes is associated with myocardial infarction (MI) [100]. For example, expression of two important apoptosis regulating genes, FOXO3A (underexpressed in post-MI sample) and CFLAR (overexpressed in post-MI sample), differs significantly pre- versus post-MI [101]. The bio-barcode technology can be used to measure gene expression in, or genotype, samples. In this case, it could enable identification of patients who are vulnerable to processes such as apoptosis in cardiovascular tissues during long-term heart failure [102].

Plasma norepinephrine levels elevated due to sympathetic activation are considered the most informative clinical prognostic biomarker for heart failure [103]. However, this biomarker is useful only in active and well-defined disease states. Thus, emerging nanotechnologies that allow analysis of unamplified RNA derived from endomyocardial biopsies may be useful to develop new biomarkers for heart failure. For example, Heidecker et al. [104] obtained RNA from endomyocardial biopsies early in the course of heart failure from a well-characterized cohort of patients with idiopathic cardiomyopathy, and performed microarray analysis of transcript levels. They identified and incorporated into a transcriptome panel 45 differentially expressed genes. No individual gene was overexpressed more than 1.8-fold in poor prognosis patients, but together, the expression panel could predict heart failure prognosis with 90% specificity and 74% sensitivity.

The process of detecting DNA or RNA using sensitive nanotechnology begins with capturing oligonucleotide probes of interest on glass microarray slides, to which fragmented genomic DNA/RNA is subsequently hybridized. Unbound nucleic acids are removed and oligonucleotide-decorated gold nanoparticles roughly 15 nm in diameter are applied to induce a secondary hybridization event. Silver is then deposited on the gold nanoparticles to increase detection sensitivity. This method has been used without PCR amplification to identify single-nucleotide polymorphisms (SNPs) which comprise the most abundant source of genetic variation in the human genome. It has the potential to accelerate the adoption of personalized therapy in the field of cardiovascular medicine, and has recently been adapted to an on-chip system that can be read by the Verigene ID system from Nanosphere Inc [105].

Liposomes for Blood Pool Imaging

Nanoliposomes have been tested as carriers for contrast agents such as iodine with the goal to improve the sensitivity of magnetic resonance imaging (MRI) and computed tomography (CT) [106, 107]. These liposomes are efficient in preventing clearance of the contrast agent from the blood pool via the RES, thus significantly enhancing the ability to image total blood pool and cardiac functions in vivo.

In these studies, contrast efficiency was measured in Hounsfield (H) enhancement units in the descending aorta, myocardium, interventricular septum, blood-filled chamber of the left ventricle, kidney, liver, and spleen. Iodine liposomes enabled an immediate relative contrast enhancement of 900 H in the aorta with plateau levels of 800 H achieved after 2 h. These high levels of iodine in the blood pool allowed for excellent contrast discrimination between the myocardium and blood in the ventricles or major arteries and veins. Thus, this liposomal iodine formulation is a promising micro-CT agent for contrast enhancement within micro-vessels [108].

Magnetic Nanoparticle Probes for In Vivo Plaque Detection

Beginning in the early 1980s, superparamagnetic nanoparticles were developed for a variety of biological applications such as purification, separation, and detection of specific cell populations. Since then, magnetic nanoparticles like iron oxide have been proposed as agents that might enable the detection of early-onset atherosclerosis via rapid internalization by plaque-resident macrophages [109]. Several research groups have sought to use this class of nanomaterials for the development of MRI contrast agents for the diagnosis of cardiovascular diseases [110–112].

Using a strong external magnetic field, MRI first aligns the nuclear magnetization of hydrogen atoms incorporated in water or fat molecules within the body. Radiofrequency (RF) waves are then used to excite these aligned magnetizations out of equilibrium. When the excited hydrogen nuclei relax back into the aligned equilibrium positions, two characteristic relaxation times, the longitudinal relaxation time T1 and the transverse relaxation time T2, are generated and result in the emission of the excitation energy absorbed from the radiofrequency waves. Image contrast and resolution are improved with increasing external magnetic field strength, leading to shorter relaxation times. The use of iron oxide nanoparticles typically enhances T2 contrast and produces dark contrast [113]. Other magnetic nanomaterials, such as paramagnetic contrast agents (i.e., gadolinium chelates) used in the enhancement of T1 contrast, and manganese nanoparticles [114, 115], have also been introduced to provide a broader range of magnetic nanoparticles for use in cardiovascular imaging. In fact, the majority of clinically used MRI contrast agents are based on gadolinium ions, which are highly toxic in a free form and thus have to be chelated. Though chelation minimizes the toxicity of gadolinium ions, it also reduces the number of coordination sites resulting in a low relaxivity of less than 4 $mM^{-1}\ s^{-1}$ at a magnetic field strength of 1.41 T, and thus decreased contrast efficiency.

Using the multistage nanovector approach, a new category of MRI contrast enhancing agents was synthesized by loading gadolinium-based contrast agents such as a clinically used chelate (Magnevist) or $gadolinium^{3+}$-loaded carbon nanoparticles (carbon nanotubes, GDNT, and fullerenes, GF) into the nanoporous structure of discoidal or hemispherical third-generation, multistage nanovectors [43]. The resulting multistage constructs showed a significant boost in longitudinal relaxivity, resulting in up to 40 times higher values than the clinically used chelate,

Magnevist [43]. The proposed mechanism of the prominent enhancement in the MRI contrast is based on the geometrical confinement of gadolinium-contrast agents within the porous silicon nanovectors. This affects the paramagnetic behavior of the gadolinium^{3+} ions by enhancing interactions between neighboring contrast agents through reducing the mobility of water molecules and the ability of contrast agents to rotate [43].

Targets that are useful for imaging atherosclerotic plaque include the inflamed endothelium, macrophages [116], fibrin [117], collagen III [118], and markers of angiogenesis. One of the initial indicators of plaque rupture is fibrin, formed as a result of tissue factor expression. Tissue factor-targeted perfluorocarbon (PFC) nanoparticles incorporating large payloads of lipid–gadolinium chelate conjugates were used to detect picomolar concentrations of cell surface tissue factor with MRI [119]. Other ligands that target inflamed endothelium and angiogenesis such as selectins and $\alpha_v\beta_3$-integrin are also being investigated [120]. Targeting macrophages resident within vulnerable plaques was explored using high-density lipoprotein (rHDL) nanoparticles enriched with gadolinium-based amphiphiles and a targeting moiety (apolipoprotein E-derived lipopeptide, P2fA2) [121]. The results of this study indicate a significant enhancement in MRI signal of the atherosclerotic wall 24 h following injection of Gd containing rHDL-P2fA2 as compared to administration of untargeted rHDL (90% vs. 53% enhancement, respectively).

Another target useful to study atherosclerosis is vascular cell adhesion molecule-1 (VCAM-1) expressed on inflamed endothelial cells. A linear peptide sequence that binds specifically to VCAM-1 was identified by in vitro and in vivo phage display. The resulting VCAM-1 internalizing peptide, VHPKQHR, demonstrated a 20-fold increase in binding to murine cardiac endothelial cells, compared to a previously identified peptide, CVHSPNKKC (Fig. 9.5). The peptide increased uptake of Cy 5.5-labeled nanoparticles in animal models of TNF-induced inflammation or atherosclerosis in apoE$^{-/-}$ mice [122]. These authors also performed an ex vivo study on freshly resected human endarterectomy specimens incubated with iron oxide-conjugated fluorescently labeled, VCAM-1-internalizing peptide-conjugated particles. They showed that after a 24–48-h incubation, a marked decrease in T2 signal, as expected for iron oxide nanoparticles, and an increase in fluorescence signal were detected relative to the evaluated controls. These features of the particles enable continuous imaging of the atherosclerotic lesion. The use of nanoparticle probes to detect, image, and study atherosclerosis depends on achieving the desired level of sensitivity, specificity and minimization of artifact, but studies to date demonstrate the potential that these probes have to advance our understanding and treatment of this disease.

Nanoparticle Positron Emission Tomography Imaging

The development of nanoparticle contrast agents may represent the next frontier of imaging, drug delivery and personalized medicine, especially pertaining to early

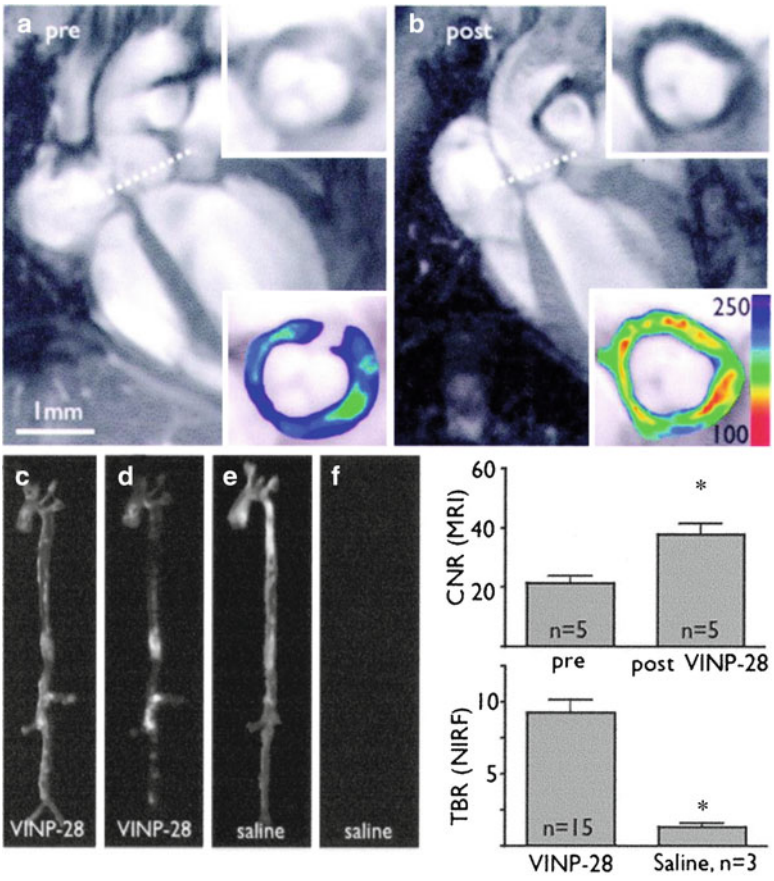

Fig. 9.5 In vivo magnetic resonance and optical imaging of vascular cell adhesion molecule 1 (VCAM-1) expression: (**a**) MRI before injection of VCAM-1 internalizing peptide-28 (VINP-28). *Dotted line* depicts location of short-axis view across the aortic root shown in the inserts. (**b**) Same mouse 48 h after injection of VINP-28. A marked signal drop in the aortic root wall was noted, as depicted in the insets. The contrast-to-noise ratio (CNR) of the aortic wall was increased significantly after injection of the probe (mean ± SD; $^{*}P < 0.05$ before versus after injection). (**c**, **e**) Light microscopic images of excised aortas. (**d**) Near-infrared image after VINP-28 injection demonstrates distribution of the agent to plaque-bearing segments of the aorta. (**f**) In contrast, a near-infrared image of the aorta of the saline injected apoE$^{-/-}$ animal shows very little fluorescent signal. Both images were acquired with identical exposure times and were identically windowed. The target-to-background ratio (TBR) was significantly higher in the VINP-28-injected mice ($^{*}P < 0.05$). Reproduced from [122] with permission from Lippincott Williams & Wilkins

diagnosis of atherosclerosis and cardiovascular pathology at the protein level [123]. For example, the application of macrophage-specific, dextran-coated nanoparticle positron emission tomography (PET) agents significantly reduced background noise and increased the specificity compared to the currently used ^{18}FDG(fludesoxiglucosa)-PET imaging agents [124]. A new agent, ^{64}Cu-TNP,

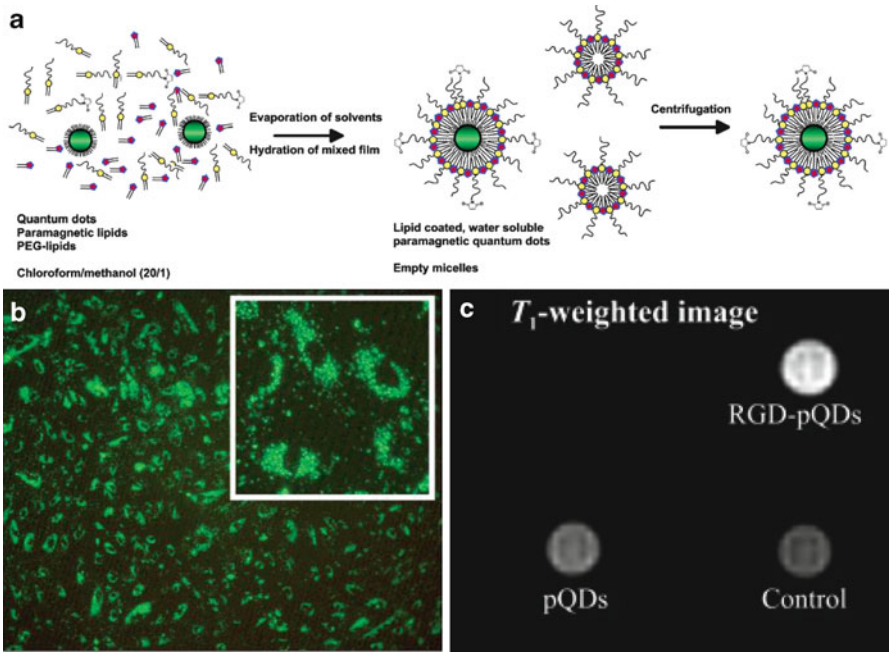

Fig. 9.6 Quantum dots may be used as the basis of multimodal molecular imaging probes. (**a**) Schematic depicting the RGD-peptide targeted, paramagnetic quantum dot. (**b**) Expression of αvβ3-integrin in human umbilical vein endothelial cells as imaged by fluorescence microscopy. The *green signal* is due to quantum dot fluorescence. The *inset* shows an enlargement of the cells. (**c**) MRI scan showing pellets of HUVEC cells (~1.5 million). The *brightest circle* originates from cells that were incubated with αvβ3-integrin-specific paramagnetic quantum dots. Reprinted from [127] with permission from American Chemical Society

composed of a magnetic nanoparticle base conjugated to chelated ^{64}Cu and a near-infrared fluorochrome, acts as a trimodality reporter useful for PET, MRI, and fluorescence imaging applications. In a mouse model of atherosclerosis, the detection threshold was 5 μg Fe/mL on T2-weighted MRI and 0.1 μg Fe/mL for PET-CT. In addition, the iron concentration used for PET imaging was 1.5 mg Fe/kg, which is lower than the maximum dose of magnetic nanoparticles approved by the FDA (2.6 mg Fe/kg). In apoE$^{-/-}$ mice, atherosclerotic plaques in the aorta can be identified with PET-CT. This study used small amounts of ^{64}Cu, which was chelated in order to control its reactivity and toxicity [125]. Based on this principle, a trimodality reporter nanoparticle using ^{18}F labeled iron nanoparticles (^{18}F-CLIO) has also been developed, offering greater PET detection sensitivity than ^{64}Cu and a shorter half-life, reducing the subject's radiation exposure and thus diminishing treatment side effects [126].

Quantum-Dot-Based Probes

A major focus of the application of nanotechnology to the cardiovascular system has been the characterization of atherosclerotic plaques. Quantum dots, a family of fluorescent semiconductors, consist of a semiconductor core encapsulated by another semiconductor shell with a typical combined diameter of 2–10 nm. By varying the size and the composition of quantum dots, the emission wavelength can be tuned from blue to infrared. Due to their tunable physicochemical properties, high photostability, broad absorption spectra, and narrow emission bands, quantum dots have been used as florescent labels to optically image a host of biological structures and processes, ranging from DNA, small organelles, and tumors to cell–cell interactions and cell signaling processes. In cardiovascular molecular imaging, the varied applications of quantum dots (Fig. 9.6) are expected to be useful in tracking disease-associated events, such as macrophage cell infiltration into arterial tissue, angiogenesis, and vascular remodeling, including the rejuvenation of the endothelial lining of the intima after cardiovascular procedures damage the vasculature. The ability of quantum dots to simultaneously tag proteins both on and inside cells may enable the identification and study of cellular changes associated with disease pathogenesis, thus providing valuable information useful for the development of novel therapeutic agents.

Use of Nanofabricated Materials for the Design of Medical Devices for CVDs

Nanomaterials are defined as materials comprised of basic components within a confined dimensionality, yielding a host of unique physicochemical properties not present in the bulk material. Realizing the full potential of nanotechnology as it pertains to cardiovascular disease diagnosis and therapy requires the ability to fabricate nanoscale devices and materials with a high degree of precision and accuracy. Eventual goals for the development of nanofabricated materials in cardiovascular medicine include control of infection and thrombosis, modification of cellular adhesion, and control of drug delivery.

Fabrication of Nanotextured Stents

Coronary artery stents have been shown to reduce restenosis following balloon angioplasty. However, in-stent restenosis still remains problematic and affects up to 60% of patients who receive a coronary stent implant [128, 129]. Restenosis after stent placement has been associated with the migration of smooth muscle

cells into the neointima, eventually resulting in the production of significant amounts of matrix proteins, such as collagen and fibronectin, and leading to a mostly collagen-containing lesion called neointimal thickening. Drug-eluting stents release compounds that inhibit smooth muscle cell proliferation and therefore restenosis due to neointimal thickening. These stents have reduced the necessity for repeat angioplasty procedures due to restenosis by 70–80% [130]. However, the antiproliferative drugs released from the stents also inhibit normal re-endothelialization of the vessel, rendering it vulnerable to thrombosis, which requires treatment with anti-platelet drugs.

Nanotextured stent coatings, such as hydroxyapatite [131] and titania [132], have been applied to enhance endothelial cell attachment and proliferation to promote re-endothelialization of vessel walls. Several such coatings have been generated using a sol–gel process, in which a colloidal suspension (sol) of metal or ceramic is applied to a surface. The preferential evaporation of the solvent after dip or spray-coating drives self-condensation of the solute into a uniform thin-film nanophase by increasing the concentration of solute in the solution until it exceeds the critical micelle concentration. This process forms a porous, highly textured coating from the solute.

Studies suggest that these coatings can significantly enhance cellular attachment. For instance, Areva et al. demonstrated a direct attachment between soft tissue and the sol–gel-derived titania coatings in vivo 2 days after implantation, whereas the titanium control implants resulted in a gap and a fibrous capsule at the implant–tissue interface [132].

The porosity of these coatings establishes them as potential candidates for drug elution. The Sridhar group at the California Institute of Technology has used nanoporous alumina and titania coatings for localized drug- and gene-delivery applications [133, 134]. As shown in Fig. 9.7, they have fabricated nanoporous alumina and titania films on metal substrates with precise control over pore size, wall thickness, and film thickness. They presented the results for the release of a model drug, doxorubicin, from different noneroding nanoporous coatings: anodic aluminum oxide (AAO) with pore diameter of 20 or 200 nm, anodic titanium oxide (ATO) with a pore diameter of 120 nm and the conventional polymer coating, or a biodegradable polycaprolactone as a control. Nanoporous surfaces in the study achieved a sustained release rate observed by in situ fluorometry over periods of several weeks without delamination or leaching. The kinetics of sustained release from these nanoporous platforms were investigated by an activated surface-density-dependent desorption model, which appears to be universal for noneroding platforms. After a rapid burst release, which was similar for all platform types, an activated surface-density-dependent desorption was observed for nanoporous templates. These findings suggest that new generations of biomedical implants and cardiovascular stents that are currently being used can perform localized elution of drug molecules to enhance the lifetime of these devices and to promote biointegration.

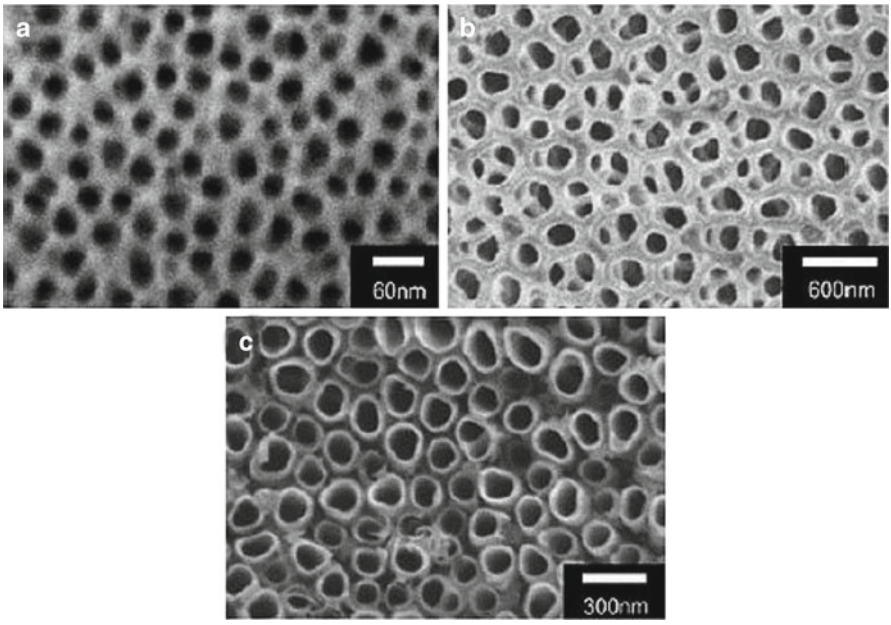

Fig. 9.7 Scanning electron microscopy images of noneroding nanoporous templates. (**a**) Anodic aluminum oxide (AAO), 20 nm pore diameter (AAO-20). (**b**) Anodic aluminum oxide, 200 nm pore diameter (AAO-200). (**c**) Anodic titanium oxide, 125 nm pore diameter. Adapted from [133, 134]

Carbon Nanotubes

Due to their intriguing electrical, physical, and structural characteristics, carbon nanotubes have applications in many biomedical fields, including diagnostics, imaging and biosensing. In 2005, scientists from the Chinese Academy of Science sought to utilize the advantages of pure carbon nanotubes, specifically their excellent mechanical properties and high aspect ratio, to improve both blood compatibility and the mechanical properties of polyurethane matrices [135]. It has been shown that when foreign materials contact blood, they cause red blood cell disruption, platelet activation and aggregation, and finally thrombus formation through a cascade of reactions mediated by nonspecific binding of plasma proteins. Therefore, blood compatibility is a key problem that limits the application of biomaterials in environments that interact with blood, such as in the cardiovascular system. With this in mind, they created a multi-walled carbon nanotube and polyurethane composite through controlled co-precipitation in a mixed solution of water and organic solvents to avoid surfactants in the polymeric matrix. Both the strength and elasticity of the polyurethane was increased considerably when multi-walled carbon nanotubes with an oxygen-containing functional group were incorporated. Studies with

this material showed that platelet activation and red blood cell disruption induced by the composite were remarkably reduced compared to plain polyurethane. This novel material will likely have many potential applications in the development of acute and chronic implant devices in cardiovascular medicine as well as other medical fields.

Nanotechnology for Ex Vivo Biomarker Harvesting and Detection

Identification of biomarkers for a particular disease is an important tool for disease screening, early diagnosis, and monitoring that can greatly improve its prognosis [136]. Sensitive, rapid, and specific assays for analysis of known biomarkers for cardiovascular disease hold potential to improve the therapeutic outcome for patients. The use of individual biomarkers of cardiovascular disease such as C-reactive protein [137], B-type natriuretic peptide [138], fibrinogen [139], D-dimer [140], and homocysteine [141] for better identification of populations at high-risk for CVD has been reported. A study by Wang et al. reported the simultaneous measurement of 10 commonly used biomarkers including high-sensitivity CRP, fibrinogen, D-dimer, plasminogen activator inhibitor-1, B-type naturetic peptide, N-atrial naturetic peptide, aldosterone, renin, homocysteine, and urine albumin excretion to evaluate the risk of major cardiovascular events and death from CVD [142]. The authors concluded that use of currently known biomarkers can only moderately enhance assessment of the disease. This finding emphasizes the need to develop more sensitive assays for the current markers, and better methods to identify new biomarkers. Tunable nanoporous materials, such as nanoporous silica films, have been proposed to meet these needs. These materials can selectively harvest low-molecular-weight proteins separating them from high-molecular-weight and highly abundant plasma proteins (e.g., albumin), thus providing a unique opportunity to detect and identify new circulating biomarkers [129, 130, 143, 144].

D-dimer is a well-known biomarker for increased blood clotting activity associated with deep vein thrombosis (DVT). A device combining impedimetric analysis nanoelectrodes and a microfluidics system was developed for high specificity immunoassay-based detection of D-dimer levels in the blood [145]. The device increased the accuracy and reliability of early assessment of patients at risk to develop DVT.

The cardiac troponins I and T, structural proteins specific to cardiac myocytes, are widely used biomarkers for MI. These markers of myocyte injury are usually assessed using immunodetection protocols [146]. A novel ultrasensitive nanoparticle-based assay for cardiac troponin I tested in a clinical setting had significantly enhanced sensitivity for Troponin I that reached pg/mL concentrations in serum, promising earlier detection of myocardial injury [147]. In another study, engineered viral nanoparticles that combine troponin antibodies and nickel nano-hairs were six to seven times more sensitive, compared to conventional immunoassays, at detecting troponin in human serum [148]. Significant improvements in the ability to detect biomarkers for myocardial damage will likely have important clinical ramifications.

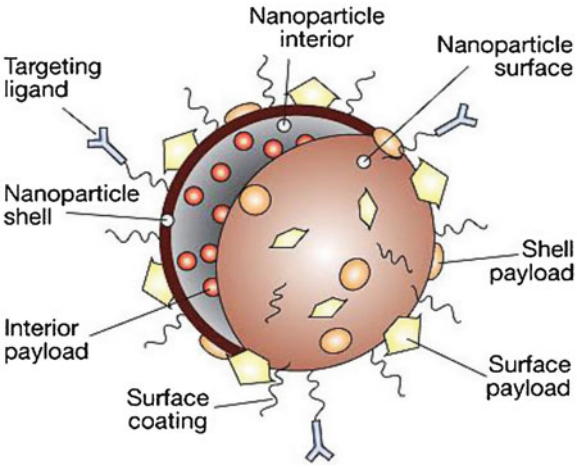

Fig. 9.8 Depiction of a multimodal nanoparticle showing the typical features and surface modifications that enable targeting and biocompatibility. Several locations are shown for the incorporation of therapeutic or imaging contrast payloads. Reprinted from [150] with permission from Nature Publishing Group

For example, cardiac troponin levels evaluated in patients with stable coronary disease by a highly sensitive detection technique, cardiac computed tomography, were found to directly correlate with the increased incidence of cardiovascular mortality and heart failure [149]. Such early detection of the myocyte injury may lead to improved therapeutic outcomes.

Multimodal Nanotracers

The design of next-generation nanoparticles for the treatment of cardiovascular disease has focused on combining properties that allow for both diagnostic imaging and delivery of therapeutic agents. A recent review provides an overview of nanoparticles that have been designed to combine imaging and therapeutic functions [150]. Figure 9.8 shows typical features and build-up of multimodal nanoparticles. Although the use of multimodal tracers in cardiovascular medicine is still at an early stage, the field is growing rapidly. Mulder et al. have generated a "smart" system in which quantum dots capped with trioctylphosphine oxide and hexadecylamine are then coated with paramagnetic and PEGylated lipids [127]. This multimodal nanoparticle presents improved bioapplicability, target specificity, and superior detectability using MRI, and has been used to visualize $\alpha_v\beta_3$-integrin expression on human umbilical vein endothelial cells. Another novel approach utilizes paramagnetic and fluorescent micelles to specifically target the macrophage scavenger receptor-B (CD36), an epitope expressed on activated macrophages which are an important cell type in atherosclerotic plaque formation and progression [116]. This approach resulted in increased MRI signal at 1 and 24 h after injection, compared to pre-contrast MRI imaging of anatomically matched areas with histologically determined, macrophage-rich plaques. Specific association

of the micelles with targeted macrophages was also revealed by fluorescence microscopy. These recent advances demonstrate that the development of multimodal nanotracers will lead to improved and personalized treatment strategies for patients with CVDs.

Conclusions and Future Perspectives

This chapter highlights the multiple applications of nanotechnology to the diagnosis and therapy of a number of cardiovascular diseases. Nanotechnology-based materials for molecular and MRI are being refined with the goal of detecting disease as early as possible. Ultimately, imaging at the level of a single cell, combined with the ability to monitor the effectiveness of therapy, may provide more accurate diagnosis and treatment at an earlier disease stage, possibly before the onset of symptoms. In addition, targeted delivery agents may allow therapy localized to only the diseased cells, thereby increasing efficacy while reducing deleterious side effects.

We have also discussed nanotechnology applications specific to certain cardiovascular diseases. Nanoscale technologies hold great potential for the development of diagnostic devices that can be applied to the analysis and detection of atherosclerotic plaque, to the specific and sensitive detection of biomarkers for early disease diagnosis, and to the monitoring of therapeutic efficacy. Hopefully, this will result in improved patient management, quality of life, and lower mortality rates associated with this disease.

Nanotechnology has advanced regenerative medicine by contributing to the understanding of the mechanisms for stem cell recruitment, activation, control and homing. In the setting of myocardial infarction, new treatments might include intelligent nanobiomaterials with the ability to attract local adult stem cells or cultured cells to the site of injury. Early treatment of myocardial infarction with stem cells or stem cell-modifying drugs could improve early rescue of injured myocardium. This may open the possibility of cell therapy for cardiac tissue regeneration, as well as new therapeutic options for patients with severe cardiac insufficiency.

The application of nanotechnology to the development of artificial blood replacements has resulted in second-generation engineered hemoglobin (Hb) by assembling Hb together with superoxide dismutase (SOD) and catalase (CAT) to form a nanodimension soluble complex of polyhemoglobin (PolyHb)-CAT-SOD [151]. Another approach is to prepare nanodimensional or “nanosized” complete artificial red blood cells that can circulate for a sufficient length of time after infusion.

Finally, new nanomedical applications will arise from the use of nanofabrication techniques adopted from other fields. For example, a coronary angioplasty balloon has been equipped with single-crystal ultra-thin silicon (100 nm). This form of stretchable silicon is completely compatible with immersion in biofluids and enables the balloon to carry an array of sensors on its surface that can monitor several parameters, such as concentration of ions, blood pressure, and concentrations of biochemical markers. Advances in the field of mechano-synthetic tools may

ultimately yield micro- and nano-robotics with functional characteristics. These tools have been under intense investigation for the past decade and include functionalization of nanomaterials to enable self-assembly, MEMS-based robotics, bacteria-driven motors, diamond mechano-synthesis, and the design of artificial red and white blood cells [152]. With continued innovations in imaging, biomaterials, tissue-targeted nanovectors, biosensors, and personalized therapies, nanomedicine can offer to cardiologists and cardiovascular surgeons new tools to diagnose and treat CVDs with higher efficiency and to ultimately improve patient care.

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Chapter 10
Endothelial Progenitor Cells in the Treatment of Vascular Disease

Gareth J. Padfield

Introduction

Since its inception by Gruentzig in 1977 [1], percutaneous coronary intervention (PCI) has revolutionized the treatment of coronary artery disease (CAD). However, the treated arterial segment is exposed to significant mechanical trauma and endovascular laceration and disruption of endothelial cell continuity as a result. The endothelium is of critical importance in regulating vascular homeostasis. Loss of endothelial function following PCI leads to impaired regulation of smooth muscle tone and endogenous thrombolysis, and potentiates vascular inflammation, predisposing the patient to in-stent restenosis (ISR) and the potentially lethal stent thrombosis, both of which remain major clinical problems [2]. Effective reendothelialization following PCI is therefore necessary to restore vascular homeostasis. Reendothelialization was previously considered to occur exclusively through the migration and proliferation of mature endothelial cells adjacent to regions of endothelial denudation [3]. However, attention has recently focused on a novel mechanism of vascular repair: endothelial progenitor cells (EPCs) bone marrow-derived cells, mobilized in response to angiogenic stress that are capable of homing to sites of vascular injury [4] and facilitating reendothelialization [5]. The discovery of the EPC launched a new era of cardiovascular research that has changed our understanding of mechanisms underlying vascular repair. The EPC offers an exciting and novel therapeutic target in the management of cardiovascular disease and the complications associated with modern revascularization strategies.

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PCI-Associated Vascular Injury

Adverse cardiac events following PCI were initially very common, with major complications occurring in up to 20% of patients undergoing stent implantation [6]. Technological advances have improved outcomes, although angiographic restenosis following PCI remains significant, with an overall incidence of 11%, but as high as 29% in higher risk populations receiving bare metal stents (BMS) [7]. Stent thrombosis, although a relatively rare complication of PCI, can still occur in up to 2% of cases, with potentially devastating and fatal consequences [8]. These complications occur, in part, as a consequence of the vascular injury that occurs during PCI [9]. High-pressure balloon inflations and forceful apposition of rigid stent struts against the vessel wall that occur during PCI invariably cause laceration of the tunica intima and disruption of endothelial continuity. Endovascular laceration may extend through the media to involve the external elastic lamina, with vessel rupture being an uncommon but well recognized potential complication of PCI. Dysfunction of endogenous fibrinolytic and vasomotor function, combined with the exposure of underlying collagen and tissue factor, leads to activation of platelets and initiation of the coagulation cascade, which may lead to acute or sub-acute stent occlusion [10]. An intense local [9] and systemic inflammatory response [11, 12] is generated, involving a rapid influx of neutrophils, and later monocytes and macrophages, into the vessel wall.

The endothelium normally provides a protective barrier for smooth muscle cells against inflammatory cytokines and growth factors, and also secretes a number of cytostatic factors that prevent smooth muscle cell proliferation. In the absence of a functional endothelium, fibroblastic activation and smooth muscle hypertrophy are potentiated, and may lead to ISR and myocardial ischaemia [13]. Rapid reendothelialization is therefore important in the restoration of normal vascular function, reduction of vascular inflammation and the prevention of adverse remodelling following PCI [2]. The process of stent integration into the vessel wall has been elegantly characterized using electron microscopy [14]. During the first 6 weeks following PCI, a thin multi-layered thrombus is present on the endovascular surface, and progressive smooth muscle hyperplasia and deposition of extracellular matrix occurs. From 6 to 12 weeks following PCI, the thrombus resolves and endothelial cells begin to cover the stented segment. At approximately 3 months, reendothelialization is complete, and there is an associated diminution in the quantity of smooth muscle cells. The use of drug-eluting stents (DESs) has dramatically reduced the incidence of ISR [10]; however rather than encouraging reendothelialization, this approach is based on the suppression of cellular proliferation. Reendothelialization is therefore also suppressed [15], with a requisite prolongation of anti-platelet therapy. A means of encouraging reendothelialization would therefore be favourable. EPCs may comprise an important component of the cellular response to vascular injury and represent a potential therapeutic target through which reendothelialization following PCI can be enhanced.

Defining the EPC

The concept that naïve precursor cells with the capacity to differentiate into mature cell types exist within the adult circulation is supported primarily by studies of bone marrow transplant recipients, within whom exist appropriately differentiated cells of donor origin that are integrated into host structures such as the heart [16], lung [17] and vasculature [18]. The formation of blood vessels by naïve precursor cells in the adult is contrary to the traditional paradigm of reendothelialization or neovascularization, whereby new endothelial cells are generated through the proliferation and migration of mature endothelial cells [3]. Although the recipient of enormous interest in the last decade, the concept of circulating progenitor cells is not so novel. In the 1930s, the formation of vascular structures arising from peripheral blood cultures was observed in vitro [19, 20]. In the 1960s, "islands" of endothelium were seen developing on the surface of implanted devices and intravascular prostheses [21–24]. The presence of circulating progenitors capable of reendothelialization was suggested by Scott et al. in 1994 following the successful reendothelialization of a vascular graft that was suspended within the aorta of a dog and therefore isolated from endothelial cells migrating from the aortic endovascular surface [25]. In 1997, Asahara et al. popularized the term EPC by isolating cell populations from peripheral blood that were capable of homing to regions of ischaemia and facilitating vascular regeneration [4]. EPC have been described as circulating [4], bone marrow [26] or tissue resident [27] cells that are mobilized in response to tissue ischaemia or vascular perturbation and possess the capacity to home to regions of injury and differentiate into mature endothelial cells or adopt an "endothelial-like" phenotype and participate in vascular repair. However, the term EPC is ambiguous, having been used to define a variety of different cell populations. While our understanding is incomplete, it is likely that the various populations so far identified comprise different components of a vascular repair system. Broadly speaking, two methods of identifying EPCs have been used: (1) specifically identifying cells bearing surface markers that indicate both cellular naïveté and an endothelial phenotype (Fig. 10.1); and (2) inferring the presence of an EPC by demonstrating the evolution of mature endothelial characteristics in a heterogeneous population of cells following a period of culture under angiogenic conditions (Figs. 10.2 and 10.3).

Identifying EPCs by Surface Proteins

Through the close spatial relationship observed between endothelial and haematopoietic cell lineages in developing embryos, it is evident that a common progenitor, the haemangioblast, gives rise to both the vascular and haematopoietic systems [30]. This contention is supported by gene deletion studies used to identify common cell surface proteins necessary for vascular and haematopoietic development [31, 32]. While previously thought to be specific to the developing embryo,

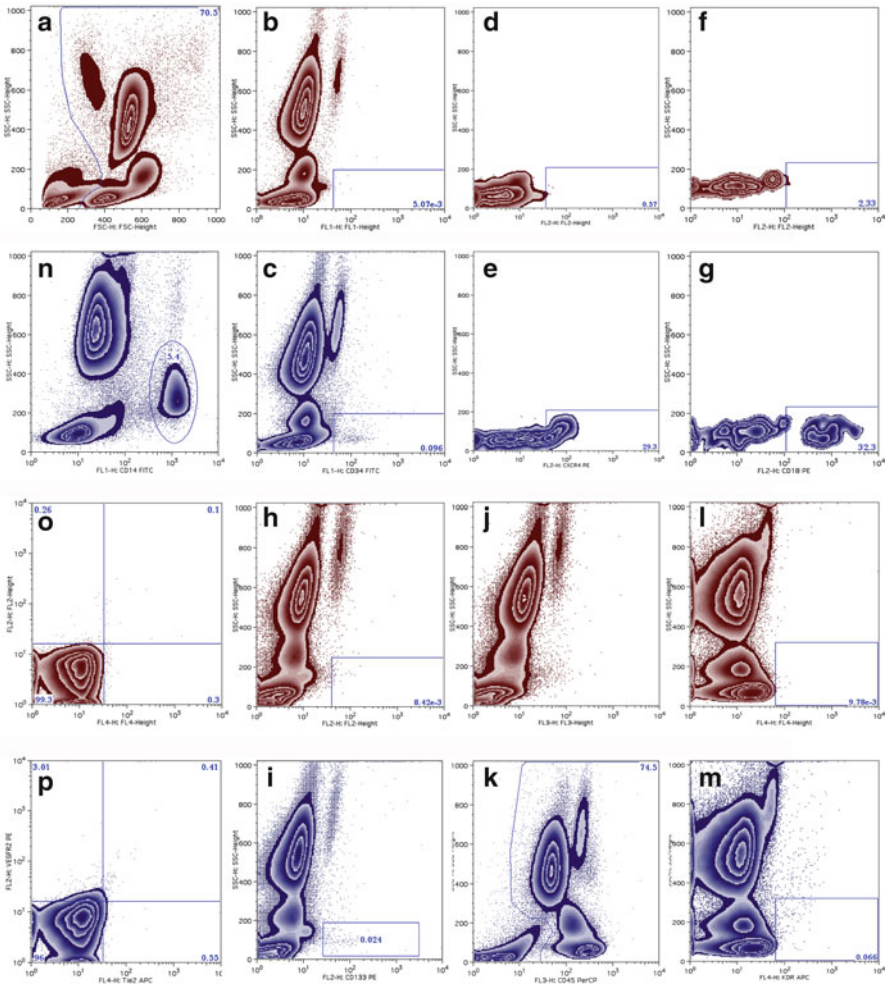


Fig. 10.1 Flow cytometric analysis of putative EPCs. Representative dot plots of negative controls and stained samples are shown in *red* and *blue*, respectively. Leukocytes can be identified on the basis of their characteristic forward and side scatter profile (**a**). CD34-FITC (**b**, **c**) expression and CD34$^+$ events expressing adhesion molecules necessary for migration and homing are shown; CD18-PE (**d**, **e**) and CXCR-4-APC (**f**, **g**). CD133-PE$^+$ (**h**, **i**), CD45-PercP$^+$ (**j**, **k**), and VEGFR-2-APC$^+$ (**l**, **m**) are shown below. CD14-FITC$^+$ events (**n**) expressing Tie-2-APC and VEGFR-2-PE can be determined using quadrant analysis (**o**, **p**). Here gates have been set on single or unstained stained negative controls

the existence of a post-natal haemangioblast is now supported by several studies [33, 34]. Efforts to define the haemangioblast have helped shape our understanding of the EPC, which were originally defined by the expression of CD34 and the extracellular domain of vascular endothelial growth factor receptor-2 (VEGFR-2), as these cell surface markers were thought to indicate cellular naïveté and a vascular

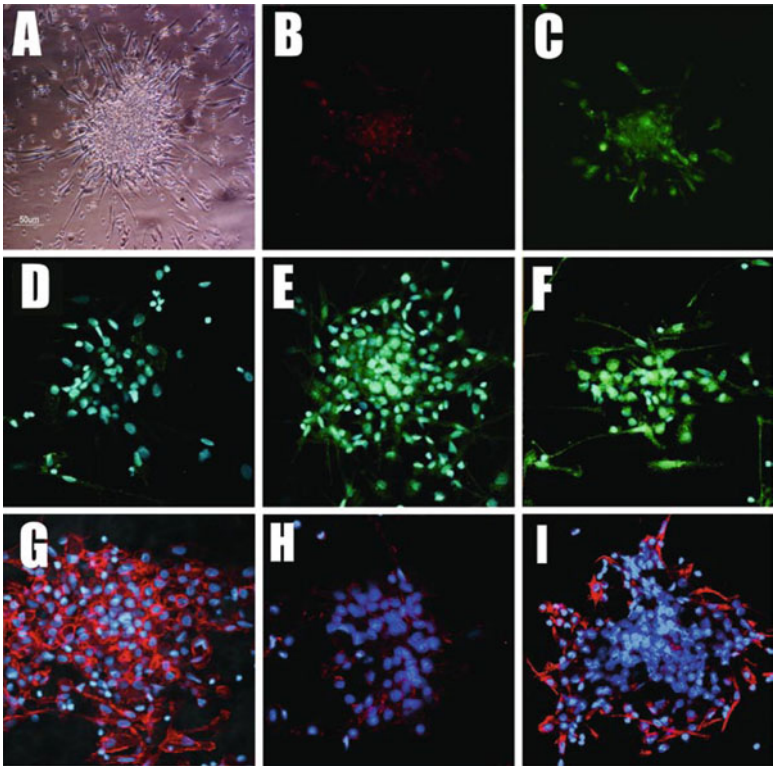
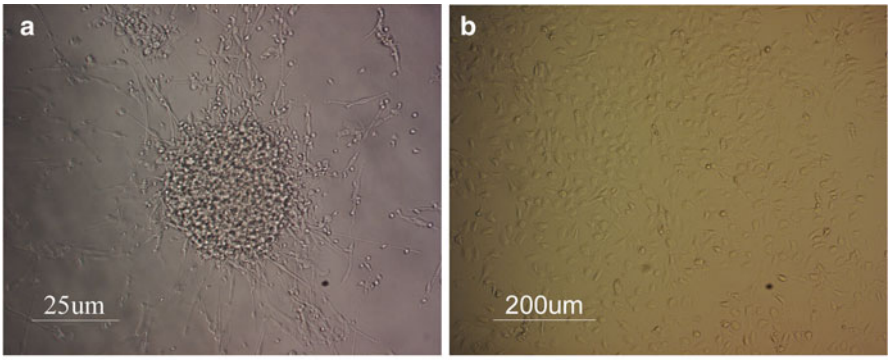


Fig. 10.2 EC-CFU characterization. Here a typical "endothelial cell colony-forming unit" (EC-CFU) is shown at ×20 magnification (**a**). Colonies displayed the classical, though non-specific characteristics of endothelial cells; uptake of Alexafluore-546 conjugated acetylated low-density lipoprotein (**b**) and FITC conjugated *Ulex europaeus* binding (**c**). Colonies also expressed endothelial nitric oxide synthase (**d**) an enzyme responsible for the production of NO by endothelial cells in response to sheer stress, Tie-2 (**e**), a tyrosine kinase receptor expressed on mature endothelial cells necessary for normal vascular development, and CD146 (**f**), a transmembrane glycoprotein responsible for endothelial intracellular adhesion. CD105 or endoglin, a constituent of the transforming growth factor-beta receptor is a marker of activated endothelium thought to be limited to proliferating cells and was widely expressed throughout the colonies (**g**). The haematopoietic nature of the colonies is evident by their widespread expression of CD45 (**h**), and most intensely of all, the macrophage marker, CD68 (**i**). (**b**–**i**) ×40 magnification. Colonies (**e**)–(**i**) are stained with primary antibodies followed by the relevant secondary antibody-fluorochrome conjugate. Nuclei are counter stained with DAPI (*blue*) [28]. EC-CFU are now recognized to be activated monocyte and lymphocyte populations rather than endothelial cells. Reprinted from [28] with permission from The American Physiological Society

phenotype, respectively. A variety of haematopoietic and endothelial surface markers have been used to identify putative EPCs (Table 10.1). Often regarded as a stem cell marker, the surface receptor CD34 is a cellular adhesion molecule necessary for normal haematopoiesis [32] and is also expressed on vascular endothelium [35]. VEGFR-2 is one of a family of transmembrane cell surface receptors for vascular



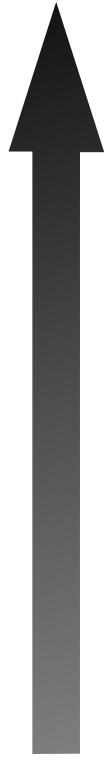
Characteristic	EC-CFU (A)	ECFC (B)
Adhesive Protein	Fibronectin	Collagen
Precursor	Monocytes/Lymphocytes $CD14^+$ & $CD2^+$	Late outgrowth EPCs $CD45^-CD34^+KDR^+$
Appearance in culture	5 -7 days	14 – 21 days
Morphology	Mass of round and spindle shaped cells	Flat cobblestone appearance
CD45 expression	Yes	No
Proliferative Potential	Low	High
Phagocytic function	Yes	No

Fig. 10.3 Characteristics of EC-CFU and ECFC [29]. (**a**) *The endothelial cell colony-forming unit assay* (*EC-CFU*). EC-CFU are generated from a non-adherent population of mononuclear cells cultured on fibronectin and appear some 5–7 days into culture. EC-CFU exhibit "endothelial-like" surface characteristics; however, it is now recognized that EC-CFU are composed of monocytes and angiogenic lymphocytes and have little capacity to form perfusing vessels or incorporate directly into vascular structures. EC-CFU exhibit phagocytic activity and avidly secrete angiogenic growth factors. EC-CFU are depressed in association with cardiovascular disorders and are mobilized in response to cardiovascular stress. EC-CFU therefore serve as a marker of cardiovascular health and possibly indicate an individual's capacity to exert a haematopoietic and angiogenic response to tissue injury. The nomenclature for this population requires revision. (**b**) *The endothelial colony-forming cell* (*ECFC*) *assay*. ECFCs are generated from adherent mononuclear cells grown on type I collagen and appear after 2–3 weeks of cell culture. ECFCs are morphologically indistinguishable from mature endothelial cells and are derived from non-haematopoietic ($CD45^-$) cells expressing CD34. ECFCs have robust proliferative potential and the capacity to form perfusing blood vessels in vitro. Reprinted from [29] with permission from Elsevier

endothelial growth factor, a pro-angiogenic cytokine. VEGFR-2 is considered a marker of endothelial lineage, due to its being widely expressed on mature endothelial cells and but also being necessary for the normal development of the vascular system and haematopoiesis in utero. Upon differentiation toward a haematopoietic lineage, the VEGF and CD34 receptors are lost, while those committed to an endothelial lineage retain expression [36, 37].

Table 10.1 Characteristics used to identify putative EPCs

Identifier	Distribution	Function
CD45	Leukocytes	A signalling molecule regulating leukocyte differentiation and proliferation
CD14	Monocytes Macrophages Some neutrophils	Endotoxin receptor regulating inflammatory cytokine production such as TNF by monocytes
CD 115	Monocytes Macrophages	Receptor for macrophage-CSF regulating myeloid proliferation and differentiation
CD117 (c-Kit)	Haematopoietic stem and progenitor cells	Receptor for stem cell factor Stimulates cellular proliferation
CD133	Haematopoietic stem and progenitor cells	Unknown
CD34	Haematopoietic stem and progenitor cells Capillary endothelium	Intracellular adhesion molecule. Binds E and L-selectins and is thought to regulate leukocyte/endothelial interactions
CD31	Leukocytes Platelets Endothelium	Adhesion molecule thought to be important for trans-endothelial cellular migration to sites on acute inflammation
Ac-LDL uptake	Macrophages Monocytes Endothelium	N/A—phagocytic process occurring in myeloid and endothelial cells
Ulex binding	Macrophages Monocytes Endothelium	N/A—histochemical stain
CD105	Endothelium Activated macrophages Smooth muscle	Constituent of transforming growth factor-beta receptor 1. Important regulator of angiogenesis
CD 141	Endothelium Smooth muscle Monocytes and Neutrophils	Binds thrombin and activates protein C and initiates anticoagulant pathways
Von Willebrand factor	Endothelium Platelets	Haemostasis



(continued)

Table 10.1 (continued)

Identifier	Distribution	Function
Tie 2 (CD202)	Endothelium Monocytes Stem cells	Angiopoietin 1 receptor Regulates vessel remodelling and maintains vascular integrity
CD 146	Endothelium Melanoma cells Dendritic cells	Intracellular adhesion molecule
E-selectin (CD62E)	Endothelium	Adhesion molecule regulating leukocyte/endothelial interactions and cell trafficking to sites of inflammation
CD 144	Endothelium	Intracellular adhesion molecule regulating endothelial permeability and proliferation
VEGFR-2 (CD 309, KDR, Flk1)	Endothelium	Regulation of endothelial adhesion and signalling. Essential for embryonic vascular development
Endothelial—nitric oxide synthase	Endothelium	Enzymatic generation of nitric oxide

Asahara et al. demonstrated that both $CD34^+$ and $VEGFR\text{-}2^+$ fractions isolated from mononuclear cells cultivated under angiogenic conditions up-regulated endothelial surface antigens (CD31, Tie-2, E-selectin, VEGFR-2) and were capable of homing to regions of ischaemia, as well as appearing to participate in neovascularization in an animal model of hind-limb ischaemia [4]. While fuelling an intense period of research, this study was far from definitive and indeed raised more questions than it answered. The purity of the cell populations used in the study was poor (CD34 ~15.7% and VEGFR-2 ~20%), and the infused cells were not specifically dual positive. Furthermore, the "endothelial" colonies that were generated from the fractions used in the study were remarkably similar to colonies subsequently demonstrated to be composed of activated monocytes and lymphocytes rather than true endothelial cells [38].

$CD34^+VEGFR\text{-}2^+$ co-expression is also a characteristic of mature endothelial cells. Therefore, additional surface markers have been used in an attempt to distinguish EPCs from mature endothelial cells sloughed from the vessel wall by vascular stress [39]. The cell surface protein CD133 is a relatively novel surface receptor with unknown function that is expressed on most $CD34^+$ cells. CD133 is quickly down-regulated on differentiation and is therefore a reliable indicator of cellular naïveté [40]. While CD133 is generally considered to be a marker of haematopoietic lineage, on account of the ability of $CD133^+$ cells to provide haematopoietic reconstitution by transplantation, $CD133^+$ cells have also been reported to give rise to cells with an endothelial phenotype in culture [41]. CD133 expression either in isolation or in combination with CD34 and VEGFR-2 has been used in variety of studies to define EPCs [2, 42–50]. $CD34^+CD133^+VEGFR2^+$ cells are, however, extremely rare in the peripheral circulation. Reliably measuring them in clinical studies is extremely difficult, and it is questionable whether they could be used in clinical practice. It should also be recognized that CD133, although a reliable indicator of cellular naïveté, still fails to adequately distinguish a vascular progenitor from a haematopoietic progenitor. Case et al. specifically compared the behaviour of $CD34^+CD133^+VEGFR2^+$ cells when used in two widely employed endothelial colony-forming assays: early and late EPCs (discussed in the "Culturing EPCs" section) and a haematopoietic colony-forming assay [51]. $CD34^+CD133^+VEGFR2^+$ cells were incapable of forming colonies in the culture assays previously used to define EPCs, but consistently formed colonies in haematopoietic assays. The majority of $CD34^+CD133^+VEGFR2^+$ cells in fact expressed the common leukocyte antigen CD45, and the capacity to form an endothelial phenotype was confined to those $CD34^+$ cells that were $CD45^-$. These findings have been supported by Timmermans et al. who also demonstrated that $CD45^-CD34^+CD133^-VEGFR\text{-}2^+$ cells are capable of forming late-outgrowth endothelial colonies (discussed in the "Culturing EPCs" section), and that a separate $CD45^+CD34^+CD133^+VEGFR\text{-}2^-$ population formed haematopoietic colonies [52]. $CD45^+$ is a powerful discriminator of pure haematopoietic and non-haematopoietic fractions and is beginning to be used to discriminate populations of EPC in clinical studies. It may transpire that the absence of CD45 may define a population of progenitor cell that ultimately forms true endothelial cells, as $CD45^-CD34^+$ cells generate colonies that more closely

resemble mature endothelial cells both functionally and phenotypically. However, as mature endothelial cells are $CD45^-$ and may also express CD34 [35, 53], the detection of $CD34^+CD45^-$ cells in the peripheral circulation may simply represent the presence of circulating mature endothelial cells rather than bone marrow-derived progenitor cells. Late outgrowth colonies may represent the same phenomenon [51].

Just as endothelial function is integral to the maintenance of normal vascular homeostasis [54], EPC regulation appears to be similarly important. Concentrations of circulating $CD34^+VEGFR\text{-}2^+$ cells are reduced in patients with traditional cardiovascular risk factors, including cigarette smoking, elevated LDL cholesterol [55], diabetes mellitus [56] and hypertension [57]. $CD34^+VEGFR\text{-}2^+$ cell concentration is markedly reduced in patients with overt atherosclerotic disease of the coronary and peripheral circulation [58]. Consistent with their putative vasculoprotective function, high circulating concentration of $CD34^+VEGFR\text{-}2^+$ cells has been associated with a lower risk of myocardial infarction, hospitalization, revascularization and cardiovascular death in patients with CAD [59, 60]. $CD34^+VEGFR\text{-}2^+$ [55], $CD133^+VEGFR\text{-}2^+$ [61], and $CD133^+CD34^+$ populations fall with advancing age, and mobilization in response to cardiovascular stress is reduced in older patients [62]. Matching for biological characteristics, however, in particular angiographic severity of coronary disease, diminishes the importance of chronological age as a determinant of EPC concentration [63]. EPC biology is in part inherited, with apparently healthy offspring of patients with CAD having reduced levels of $CD34^+VEGFR\text{-}2^+$ cells compared with the offspring of healthy controls [64].

$CD14^+$ EPCs

Even in healthy people, $CD34^+$ cells comprise a mere 0.05% of circulating leukocytes. Subpopulations co-expressing CD133 and VEGFR-2 are even more rare, and the capacity of such a small population to provide significant endovascular repair is questionable. CD14 is a membrane-bound receptor primarily responsible for the detection of lipopolysaccharide [65]. It is expressed not only in abundance on monocytes, but also at low levels on mature endothelial cells [66]. $CD14^+$ cells, approximately ten times more abundant in the peripheral circulation than $CD34^+$ cells, are known to be involved in the response to vessel injury and new vessel formation, and share many antigenic characteristics with mature endothelial cells in culture. Indeed many of the culture assays used to define EPCs have been found to express monocytic characteristics. There has therefore been much speculation and investigation into whether $CD14^+$ cells may be a source of clinically useful EPC [67–70]. Monocytes are highly plastic, with a capacity to differentiate into a variety of mature phenotypes, depending on environmental cues [71]. In particular, monocytes will up-regulate a variety of endothelial characteristics in culture [67, 70–76], and will form vascular structures in vitro under appropriate angiogenic stimulation [69, 74, 76]. Activated monocytes accumulate at sites of new vessel formation and adhere to injured endothelium. Under experimental conditions $CD14^+VEGFR\text{-}2^+$ and

$CD14^+Tie\text{-}2^+$ cells accelerate reendothelialization with demonstrable improvements in endothelial function by enhanced acetylcholine-mediated vasodilation in an NO-dependent manner [77, 78]. Such VEGFR-2 and Tie-2 expressing monocytes are also known to facilitate neoangiogenesis in the context of neoplasia [79–81]. Numerous studies, however, indicate that while monocytes are pro-angiogenic, they do not incorporate directly into the vasculature [80, 82–84]. It is possible that $CD14^+$ cells accelerate vascular repair through the secretion angiogenic growth factors rather than by differentiating into true endothelial cells.

While monocytes can enhance reendothelialization and inhibit intimal hyperplasia, the role of monocytes in vascular injury is complex. Their remarkable plasticity may result in diverse responses to vascular injury depending on the local microenvironment. Monocyte migration and adhesion is mediated by monocyte chemoattractant protein-1 (MCP-1). MCP-1 is released by endothelial cells in response to shear stress and tissue ischaemia, and augments neoangiogenesis [85]. However, MCP-1 is also significantly elevated, in both the plasma [86, 87] and in the coronary atherectomy specimens [88], of patients who develop ISR following PCI. Circulating monocyte concentration correlates strongly with the development of ISR [89]. Furthermore, anti-MCP-1 monoclonal antibodies inhibit neointimal hyperplasia following balloon angioplasty in rats [90]. These findings suggest that MCP-1 production and macrophage accumulation at the site of vascular injury may play a pivotal role in adverse remodelling following vascular injury. Circulating monocytes may contain subpopulations of bone marrow-derived progenitors capable of facilitating both reendothelialization and neointimal formation. The local microenvironment within a given patient may be a more powerful determinant of the development of neointima vs. neoendothelium.

While both $CD34^+$ and $CD14^+$ populations appear important in the regeneration of diseased and damaged arteries, it is likely that populations bearing the two receptors interact. $CD34^+$ cells, for instance, augment the incorporation of $CD34^-CD14^+$ cells into the endothelium of blood vessels in mouse ischaemic limbs [73]. Furthermore, drawing a firm distinction between $CD14^+$ and $CD34^+$ cells may be incorrect, as several lines of evidence indicate that at least a proportion of $CD14^+$ cells are in fact derived from the more naïve $CD34^+$ population. $CD34^+$ cells cultured on fibronectin in the presence of VEGF and basic fibroblastic growth factor differentiate into a $CD14^+$ population before developing endothelial characteristics, including tube formation in Matrigel®, and the expression of von Willebrand factor (vWF), eNOS and CD144 [91]. A study using a $CD34^+$ acute myeloid leukaemia cell line MUTZ-3 interrogated the differentiation of $CD34^+CD14^-CD11b^-$ progenitors through a $CD34^-CD14^-CD11b^+$ stage, into non-proliferating $CD14^+CD11b^{hi}$ progeny [92]. The presence of $CD34^+CD14^+$ cells has been confirmed by Romagani et al. who used highly sensitive antibody-conjugated magnetofluorescent liposomes to isolate a subset of $CD14^+$ cells co-expressing CD34 [93]. In peripheral blood these $CD14^+CD34^{low}$ cells constitute 0.6–8.5% of all peripheral blood leukocytes and are the dominant population among circulating $VEGFR\text{-}2^+$ cells. In the bone marrow virtually all $CD14^+$ cells were $CD14^+CD34^{low}$ double positive. Circulating $CD14^+CD34^{low}$ cells exhibit high expression of embryonic stem cell markers such

as Nanog and Oct-4, and are capable of differentiating into endothelial cells. Taken together, these studies suggest that $CD14^+$ cells are derived from $CD34^+$ cells and have the ability to develop endothelial characteristics depending on the action of external cues. The interactions among these cell types require further investigation.

Culturing EPCs

A widely used but largely inferential approach to the isolation and quantification of EPCs has been through demonstrating the development of mature endothelial characteristics in mononuclear cells following a period of culture. Broadly speaking, there are three populations of cultured EPC, sometimes referred to as functional EPCs:

1. Endothelial cell colony-forming unit (EC-CFU), also referred to as EPC-CFU, and as early outgrowth colonies on the basis of the time at which they appear in culture (5 days).
2. Circulating angiogenic cells (CACs), also at times referred to as early outgrowth colonies.
3. Endothelial colony-forming cells (ECFCs) or late outgrowth colonies based on their appearance in culture at 2–3 weeks. These have also been referred to as late outgrowth EPCs, endothelial outgrowth colonies and blood outgrowth endothelial cells.

The nomenclature has been inconsistent throughout the literature and the in vivo correlates of these assays remain unclear.

Endothelial Cell Colony-Forming Unit

The EC-CFU or early outgrowth colony assay is a modified version of the method originally used by Asahara [4]. Colonies are generated in endothelial growth medium on fibronectin. A pre-plating step at 2 days excludes initially adherent cells in an attempt to remove circulating endothelial cells from the assay. Mature colonies form at around day 5 and are comprised of clusters of small round cells with peripheral spindle-shaped cells that express endothelial characteristics such as CD105, CD146, CD31, Tie-2, VEGFR-2, CD34, E-selectin and eNOS, and are additionally characterized by Ulex Europeus Agglutinin-1 (UEA-1) binding and acetylated low-density lipoprotein uptake (Fig. 10.2). Consistent with a vasculoprotective role, EC-CFU concentration is associated with improved brachial reactivity [94] and is increased in response to tissue ischaemia and vascular injury, such as that occurring in patients undergoing coronary artery bypass surgery [95], the presence of myocardial ischaemia [96] or infarction [97, 98], and in patients undergoing angioplasty [11, 99–102]. Impaired mobilization of EC-CFU in response to ischaemic injury is associated with an adverse outcome [103]. EC-CFU concentrations are lower in

association with adverse cardiovascular risk profiles, such as in patients with type 1 [104] and type 2 diabetes mellitus, hypercholesterolaemia [94], hypertension [105], CAD [106], cerebrovascular disease [107], COPD [50], rheumatoid arthritis [47], and heart failure [108], although rather consistently not in non-ischaemic cardiomyopathy [109]. Mobilization of EC-CFU is probably specific to cardiovascular injury and not just a non-specific inflammatory response [28]. However, although EC-CFU are relevant to cardiovascular health, it is now widely recognized that the name EC-CFU is, in fact, a misnomer. They lack the capacity to form perfusing vessels in animal models of neovascularization, they widely express the haematopoietic markers CD45 and CD68 and they are composed not of mature endothelial cells, but mainly of activated leukocytes [28, 68]. Contrary to the initial description, the spindle cells that were thought to "emanate" from the central core of cells are in fact monocytes and T cells migrating toward the colonies, contributing to their formation [110]. It is likely that the EC-CFU concentration represents an individual's capacity to exert a pro-angiogenic response through the secretion of growth factors [67].

Circulating Angiogenic Cells

Unlike EC-CFU, CAC are derived from an adherent population of mononuclear cells and do not form discrete colonies. They have also been defined as EPCs on the basis of the expression of endothelial characteristics, lectin binding and uptake of acLDL, and expression of vWF, CD31, VEGFR-2, CD144, and Tie-2 [111–114]. They probably represent a population of adherent monocyte. Similar to EC-CFU, CAC and are depressed in cardiovascular diseases such diabetes, CAD and rheumatoid arthritis [47, 55, 115, 116] and are mobilized in response to acute stressors such as myocardial ischaemia [98] and coronary artery bypass grafting [95]. They promote neovascularization in animal models of acute myocardial infarction [117], but similar to EC-CFU, probably do this through the secretion of angiogenic cytokines, as they have little proliferative capacity of their own [113, 117].

ECFC or Late Outgrowth Colony

This population of cells is also generated from the culture of peripheral mononuclear cells, but in contrast to EC-CFU, ECFCs are derived from an adherent fraction of mononuclear cells grown on type I collagen. While ECFC share many phenotypic characteristics with EC-CFU [118], they do not express the haematopoietic marker CD45 or myeloid/macrophage markers such as CD14 and CD115, and do not exhibit phagocytic function. ECFCs arise from non-haematopoietic cells negative for CD133 and the panleukocyte marker CD45, but positive for CD34 and VEGFR-2 [52]. Enrichment of unsorted mononuclear preparations for $CD45^{-}CD34^{+}$

cells increases the frequency of ECFC by some 400% compared to an abolition of ECFC growth in the $CD45^+$ fraction [52]. Of the populations so far identified, the $CD45^-CD34^+$ derived ECFC more closely fulfil the characteristics of a true EPC. They conform to an endothelial phenotype and morphology, are derived from the bone marrow [119], have robust proliferative potential [118, 120], and are capable of forming perfusing vessels in vivo [68]. ECFC are mobilized within the first few hours following myocardial infarction [121, 122] and have been positively correlated with the severity of CAD in patients undergoing coronary angiography [123]. However, as previously discussed, mature endothelial cells may also express CD34 [35] and are also $CD45^-$. The growth of late outgrowth colonies from the peripheral blood may therefore represent the presence of circulating mature endothelial cells sloughed from the vasculature, rather than circulating bone marrow-derived progenitor cells. This important distinction remains to be established.

Unfortunately, across hundreds of different studies, the term EPC has been used interchangeably with reference to several different cell populations bearing different surface markers and cultured under different conditions, making it difficult to make sense of the literature. Furthermore, it is likely that many of the means of identifying endothelial cells have low specificity. For instance, Rohde et al. observed that properties such as AcLDL uptake, lectin binding, and the expression of CD31, CD105, and CD144 are also characteristics of uncultured circulating monocytes, and surprisingly, that these markers were *down*-regulated in angiogenic medium commonly used to culture EPCs [38]. Erroneous conclusions regarding the phenotype of cell populations in culture as a result of the low specificity of given endothelial characteristics may provide some explanation for the diversity of cells labelled as EPCs.

EPCs and Iatrogenic Vascular Injury

Preclinical studies have amassed a significant body of evidence to indicate that bone marrow-derived cells localize to regions of vascular injury and accelerate vascular healing by restoring endothelial function and attenuating neointimal hyperplasia (Table 10.2) [124–128, 130, 131]. Several clinical studies have specifically addressed the role of putative EPC populations following angioplasty (Table 10.3).

Most of these studies have examined the behaviour of EC-CFU. The studies have been small, and often have lacked an appropriate control group for comparison. A study of patients undergoing peripheral angioplasty reported an increase in circulating mature endothelial cells immediately after angioplasty, followed by a two- to three-fold increase in EC-CFU at 24 h [100]. These findings were confirmed by Marboeuf et al. who also correlated EC-CFU with plasma C-reactive protein concentrations following angioplasty [99]. Garg et al. described EC-CFU mobilization following PCI in patients with acute coronary syndromes [137]. However, it is impossible to determine whether EC-CFU were mobilized in response to myocardial

Table 10.2 Preclinical studies of putative EPCs following vascular injury

Reference	Model	Number	Putative EPCs	Effect
Walter et al. [124]	Murine Tie2/lacZ BM transplant recipients subjected to balloon-mediated arterial injury and pre-treatment with simvastatin or placebo	18 placebo 34 simvastatin	Dil-Ac-LDL$^+$lectin$^+$ MNCs	Simvastatin enhanced EPC mobilization following vascular injury and increased their adhesive capacity. Reendothelialization was accelerated by BM-derived cells and neointimal hyperplasia was reduced
Werner et al. [125]	Murine GFP BM transfection followed by wire-mediated arterial injury and pre-treatment with rosuvastatin or placebo	5 placebo 4 rosuvastatin	Sca1$^+$KDR$^+$ cells of BM origin	Rosuvastatin enhanced BM-derived EPC mobilization following vascular injury. Reendothelialisation was accelerated by BM-derived cells and neointimal hyperplasia was reduced
Werner et al. [126]	Intravenous cell therapy following wire-mediated murine arterial injury	6 vascular injury	Spleen derived Dil-Ac-LDL$^+$lectin$^+$ MNCs with (EPC) or without (MNC) a period of culture in endothelial growth medium	Cell therapy enhanced reendothelialization in splenectomized animals and was associated with a reduction of neointima formation but MNC were more effective than EPCs
Fujiyama et al. [127]	Intravenous cell therapy vs. saline following balloon mediated murine arterial injury	12 vascular injury 12 controls	BM CD14$^+$CD34$^-$ CD14$^-$CD34$^+$ PB CD14$^+$CD34$^-$	Compared to saline placebo, BM-derived CD34$^+$ cells and both PB- and BM-derived CD14$^+$ cells up-regulated endothelial markers, accelerated neoendothelialization and inhibited neointimal hyperplasia following activation with MCP-1
Kong et al. [128]	G-CSF vs. control prior to balloon-mediated murine arterial injury	5G-CSF 5 control	CD34$^+$KDR$^+$	G-CSF enhanced EPC mobilization following vascular injury. Reendothelialization was accelerated by BM-derived cells and neointimal hyperplasia was reduced

(continued)

Table 10.2 (continued)

Reference	Model	Number	Putative EPCs	Effect
Nowak et al. [129]	Intravenous cell therapy following balloon injury in mice using CD14/CD11b cells expressing, Tie 2^+, Tie2^-, KDR^+, KDR^-, or saline control	6 cell therapy 6 controls	Myeloid cells (CD14, CD11b) expressing KDR^+ or $Tie2^+$	$CD14^+KDR^+$ and $CD14^+Tie\text{-}2^+$ displayed endothelial characteristics in culture and enhanced reendothelialization of denuded arteries with an associated reduction in neointimal hyperplasia
Elsheikh et al. [77]	Intravenous cell therapy using GFP transduced cells following balloon mediated arterial injury in mice	10 $CD14^+KDR^+$ cells 10 $CD14^+KDR^-$ cells 6 controls	$CD14^+KDR^+$ $CD14^+$ KDR^-	Unlike $CD14^+KDR^-$ cells, $CD14^+KDR^+$ cells exhibited an endothelial phenotype in culture and contributed to neoendothelialization
Yoshioka et al. [130]	G-CSF following wire-mediated arterial injury in mice Treatment pre- and post-arterial injury vs. post-arterial injury alone	22G-CSF 20 controls	$CD34^+KDR^+$	G-CSF reduced neointimal hyperplasia in association with mobilization of bone marrow-derived EPCs and accelerated reendothelialization compared to control. The effect was enhanced if administered prior to injury. Few BM-derived cells contributed to neoendothelium
Takamiya et al. [131]	1. G-CSF prior to balloon-mediated arterial injury in rats 2. GFP BM transfected mice subjected to balloon-mediated arterial injury	10G-CSF 5 placebo	$CD117^+KDR^+$	1. G-CSF enhanced EPC mobilization following vascular injury. Reendothelialization was accelerated and neointimal hyperplasia was reduced 2. GFP expressing BM derived cells contribute to neoendothelialization

BM bone marrow, *Dil-Ac-LDL* di acetylated low-density lipoprotein, *EPC* endothelial progenitor cell, *G-CSF* granulocyte colony-stimulating factor, *GFP* green fluorescent protein, *KDR* kinase domain receptor, *MCP-1* monocyte chemoattractant protein-1, *MNCs* mononuclear cells

Table 10.3 Preclinical studies of putative EPCs following vascular injury

Reference	Study design	Number of subjects	Putative EPCs studied	Effect of angioplasty
George et al. [132]	Retrospective Restenosis vs. no restenosis	16 ISR 11 no ISR	EC-CFU	No difference in EC-CFU in those presenting with or without angiographic restenosis, but EC-CFUs lower in those presenting with diffuse vs. focal restenosis and reduced adhesive capacity in restenosis
Schober et al. [133]	Prospective Elective PCI with no control group	17 PCI	$CD34^+$	Mobilization of $CD34^+$ following PCI predicts restenosis and correlates with late lumen loss
Bonello et al. [100]	Prospective Elective PCI with no control group	15 PCI	CEC $CD45^+CD34^+$ EC-CFU	CEC were increased sixfold at 6 h, $CD45^+CD34^+$ cells increased 2.6-fold at 6 h and EC-CFU increased 2.6-fold at 24 h
Matsuo et al. [134]	Retrospective Restenosis vs. no restenosis	16 restenosis 30 no restenosis	EC-CFU	EC-CFU twofold greater in patients without ISR and increased senescence in EC-CFU of patients with ISR
Banerjee et al. [102]	Prospective PCI in elective and ACS patients with diagnostic angiography control group	20 elective PCI 10 ACS PCI 8 controls	EC-CFU $CD34^+CD31^+$	No change in $CD34^+CD31^+$ with PCI in ACS or diagnostic angiography but 1.4-fold increase in EC-CFU with elective PCI
Lei et al. [135]	Retrospective Angiographic restenosis vs. no restenosis	15 Restenosis 17 no restenosis	EC-CFU $CD34^+VEGFR\text{-}2^+$	EC-CFU 3.5-fold greater in patients without ISR Reduced proliferative and migratory capacity in EC-CFU of patients without ISR but no difference in adhesive capacity
Inoue et al. [136]	Prospective Elective PCI with no control group	40 PCI	$CD34^+$	$CD34^+$ increased threefold following BMS at 7 days, but decreased by 30% following DES at 7 days
Marboeuf et al. [99]	Prospective Peripheral arterial intervention with no control group	14 angioplasty	EC-CFU	EC-CFU increased 2.5-fold at 24 h

(continued)

Table 10.3 (continued)

Reference	Study design	Number of subjects	Putative EPCs studied	Effect of angioplasty
Garg et al. [137]	PCI in NSTEMI with no control group	20 PCI	EC-CFU	EC-CFU increased 1.3-fold at 24 h
Thomas et al. [138]	Prospective Elective PCI with no control group	20 PCI	$CD34^+$, $CD34^+CD45^+$, $CD133^+$, $CD34^+CD133^+$, $CD34^+VEGFR\text{-}2^+$ $CD133^+VEGFR\text{-}2^+$	Transient fall in all populations at 6 h post PCI
Egan et al. [139]	Prospective Elective PCI vs. diagnostic angiography	10 PCI 13 controls	$CD34^+$, $VEGFR\text{-}2^+$, $CD133^+$, $CD117^+$, $CD34^+VEGFR\text{-}2^+$, $CD34^+CD117^+$, $CD34^+CD31^+$, $CXCR4^+$	Compared to angiography, $CD133^+$, $CD117^+$, $CD34^+CD117^+$, $CD34^+CD31^+$, and $CXCR4^+$ cells increased 6–12 h following PCI. No difference in $CD34^+$, $VEGFR\text{-}2^+$, or $CD34^+VEGFR\text{-}2^+$ cells. CXCR4 expression correlated with freedom from angina at 1 year
Lee et al. [140]	Prospective Elective PCI in diabetics No control group	8 patients	$CD34^+VEGFR\text{-}2^+$	Transient fall of ~50% in first 4 h following PCI
Mills et al. [11]	Prospective Elective PCI vs. diagnostic angiography	27 PCI 27 controls	EC-CFU $CD34^+VEGFR\text{-}2^+$	EC-CFU increased threefold 24 h following PCI but $CD34^+VEGFR\text{-}2^+$ cells were unchanged and neither was affected by diagnostic angiography alone
Arao et al. [141]	Prospective Elective PCI vs. controls	52 PCI 50 controls	$CD34^+CD133^+$	$CD34^+CD133^+$ concentration unaffected at 24 h following PCI.
Pelliccia et al. [142]	Prospective Elective PCI vs. control group Restenosis vs. atherosclerotic progression vs. no restenosis	30 ISR 103 no ISR 22 progressive CAD 20 controls	$CD34^+VEGFR\text{-}2^+$ $CD133^+VEGFR\text{-}2^+$ $CD14^+$	Increased $CD34^+VEGFR\text{-}2^+CD45^-$, $CD133^+VEGFR\text{-}2^+$ $CD45^-$ and $CD45^+CD14^+$ in patients developing ISR and progressive CAD compared to no ISR or controls

ISR in-stent restenosis, *PCI* percutaneous coronary intervention, *ACS* acute coronary syndrome, *NSTEMI* non-ST-segment elevation myocardial infarction, *CAD* coronary artery disease

infarction or discrete vascular injury, given the absence of a control group of patients not undergoing PCI.

Mills et al. studied EC-CFU in patients undergoing diagnostic coronary angiography compared to PCI. EC-CFU were increased in patients who had follow on intervention, but importantly, were unchanged in those patients who underwent diagnostic angiography alone [11]. Mobilization of EC-CFUs within the first 24 h of angioplasty is a consistent finding, and suggests a role for these cells in the immediate response to vascular injury [11, 99, 100, 102, 137]. Few studies have addressed whether EC-CFU mobilization influences the development of ISR and the need for target vessel revascularization. In a retrospective study, George et al. demonstrated that in patients presenting with proliferative, as opposed to focal ISR, EC-CFUs were reduced both in number and in adhesive capacity, suggesting that a deficiency in EC-CFUs might predispose to aggressive neointimal hyperplasia [132]. This study did not detect a difference in the number of EC-CFUs between those patients with and without angiographic restenosis, however subsequently two other groups have reported a reduction in EC-CFUs in patients presenting with restenosis, and these patients had a reduced EC-CFU proliferative and migratory capacity as well as increased cellular senescence [134, 135].

A strong association exists between reduced numbers of $CD34^+VEGFR\text{-}2^+$ cells and those risk factors predictive of ISR (e.g. cigarette smoking [55], diabetes mellitus [56], and endothelial dysfunction [143]). Two small clinical studies demonstrated a fall in $CD34^+VEGFR\text{-}2^+$ cells within the first few hours of coronary angioplasty, although neither of these studies used a control group, and interpretation is therefore limited [138, 140]. It is likely that this "dip" in $CD34^+$ concentration is explained by the diurnal variation that is recognized to affect these populations [28, 144]. In a moderate-sized study of 102 patients, $CD34^+CD133^+$ concentration was significantly lower in patients undergoing PCI for stable anginal symptoms than in matched controls without angina. However, no change in the peripheral concentration had occurred at 24 h following PCI [141]. Egan et al. measured a variety of surface markers expressed on mononuclear cells, including CD34, CD133, VEGFR-2, CD117, CD31 and chemokine receptor type 4 ($CXCR4^+$) in patients undergoing PCI, diagnostic coronary angiography alone and healthy controls. In this small study, patients with CAD had lower resting levels of $CD34^+$, $CD133^+$, $CD117^+$, $CD34^+CD117^+$, $CD34^+CD31^+$ and $CXCR4^+$ cells compared to healthy controls. Following PCI, they observed an increase in those cells expressing CD133, CD117, CD34/CD31, CD34/CD117 and CXCR4 within 6–12 h [139]. As CXCR4 is thought to be integral to EPC homing and integration, it is of interest to note that reduced levels of CXCR4 correlated with the incidence of angina at 1 year. While underpowered to address clinical outcomes, this study suggests a role for CXCR4 in EPC homing and integration at the site of vascular injury following PCI.

Mobilization of ECFCs and $CD45^-CD34^+$ cells has been detected within hours of acute myocardial infarction in small preclinical and clinical studies. One may speculate that PCI would exert a similar effect, although no studies have specifically examined ECFC or $CD45^-CD34^+$ cells following PCI. Pelliccia et al. found that

higher concentrations of circulating $CD34^+VEGFR\text{-}2^+CD45^-$ and $CD133^+VEGFR\text{-}2^+CD45^-$ cells identify patients with an accelerated rate of atherosclerosis who are more likely to develop restenosis following PCI [142]. Similarly, a direct relationship exists between coronary atheroma burden and $CD34^+CD45^-$ cell-derived late outgrowth colonies [123]. $CD34^+CD45^-$ cells are therefore a measure of vascular injury; however, it is unclear whether or not they are derived from the bone marrow or possess reparatory function.

Several studies have documented an increase in $CD34^+CD45^+$ cells following PCI [100, 133, 136]. Importantly, there is an indication that this increase might be associated with an increased generation of neointimal hyperplasia. Schober et al. demonstrated that $CD34^+CD45^+$ mobilization following PCI was significantly correlated with, and independently predictive of, late lumen loss at 6 months [133]. Inoue et al. performed a similar study and combined coronary and peripheral venous sampling to detect a local inflammatory response within the coronary circulation in response to PCI [136]. The magnitude of inflammation within the coronary circulation correlated with $CD34^+CD45^+$ mobilization, and both were significantly greater in patients who subsequently developed ISR. Interestingly, the use of DESs in this study was associated with suppressed coronary inflammation and circulating $CD34^+CD45^+$ cells, suggesting that in addition to a local anti-proliferative action, DES may also reduce ISR by augmenting inflammatory signalling to the bone marrow. Inoue et al. demonstrated that circulating mononuclear cells of patients with ISR exhibited a propensity to develop a smooth muscle phenotype over that of an endothelial phenotype in culture. The potential role of circulating smooth muscle progenitor cells (SMPCs) in the development of ISR has been supported in other preclinical studies [145]. It is interesting to note that SMPC are also reported to express CD34 and VEGFR-2 [146]. Furthermore, both CD34 and CD133 are expressed at higher concentrations in the neointima of restenotic lesions compared to de novo lesions [147]. These populations may indeed potentiate neointimal hyperplasia if such a response is dictated by an inflammatory microenvironment in a given patient.

Therapeutic Use of EPCs in Discrete Vascular Injury

Current strategies to reduce the incidence of complications following percutaneous intervention are based on suppressing cellular proliferation rather than enhancing vascular repair. Drug-eluting stents have dramatically reduced the incidence of early ISR, but local anti-proliferative therapy may interfere with vascular healing and prevent formation of a functional endothelial layer [148]. Therapies designed to mobilize endothelial progenitors or to increase their ability to home to the site of stent implantation and facilitate vascular repair are attractive and have the potential to improve clinical outcomes following PCI. These approaches fall into three broad categories: pharmacological, stent-based and cellular therapies.

Pharmacological Mobilization of EPCs

Mobilization of precursors from the stem cell niche occurs via phosphatidylinositol 3-kinase/Akt/eNOS (PI3K/Akt/eNOS) activation. The PI3K/Akt/eNOS pathway is responsible for the regulation of cellular apoptosis, proliferation and migration in a variety of biological systems, including the cardiovascular system [149]. Activation of the PI3K/Akt/eNOS pathway by angiogenic factors such as VEGF, fibroblastic growth factor and angiopoietin stimulates nitric oxide (NO) synthesis by bone marrow stromal cells [111, 150]. Increased NO bioavailability leads to cleavage of intracellular adhesions between stem cells and stromal cells of the bone marrow by proteinases, such as elastase, cathepsin G and matrix metalloproteinases [151]. A high stromal cell-derived factor-1 (SDF-1) gradient across the bone marrow generated by bone marrow stromal cells acts through its cognate receptor CXCR-4 to force mobilized stem cells into the peripheral circulation such that they may home to regions of vascular injury where cell surface adhesion molecules such as CD18 mediate incorporation of EPCs into the vasculature [114, 152–154]. Mobilization of EPCs is heavily reliant on NO synthesis via PI3K/Akt/eNOS activation [155, 156]. Reduced NO bioavailability is evident in patients with cardiovascular risk factors and is integral to the development of atherosclerosis [157, 158]. Disordered function of eNOS is thought to be in part responsible for impaired mobilization of EPCs in these patients. Bone marrow cells treated with an eNOS transcription enhancer in order to increase eNOS activity exhibit enhanced migratory and neovascularization capacity, and when administered in a model of hind-limb ischaemia, can improve organ function. Furthermore, this beneficial effect is reversed by the use of an eNOS inhibitor, strongly implicating NO in the process of vascular repair and neovascularization [159]. Detailed mechanistic data regarding EPC mobilization in the context of discrete vascular injury however are limited, although there are numerous factors thought to induce EPC mobilization, including VEGF, fibroblastic growth factor, growth hormone, insulin-like growth factor, angiopoietin, SDF-1, erythropoietin, oestrogens, granulocyte colony-stimulating factor (G-CSF), statins, angiotensin receptor blockers, peroxisome proliferator-activated receptor (PPAR) antagonists and physical exercise. Several of these have been examined in the context of discrete vascular injury as potential therapeutic strategies for the mobilization of EPC.

Statins

3-Hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins) also mobilize $CD34^+$ and $VEGFR\text{-}2^+$ cells to the peripheral circulation via the PI3/Akt pathway in a dose-dependent manner [160–162]. Statins stimulate EC-CFU and ECFC formation and augment the quantity, migratory ability and proliferative capacity of a variety of putative EPCs in culture [124, 150, 155, 163, 164], and induce differentiation of $CD14^+$ and $CD34^+$ cells toward an endothelial phenotype [111]. Statins reduce telomere shortening [165] and TNF-alpha-induced apoptosis in EPCs [166],

thereby increasing EPC longevity. The reduced rate of ISR following PCI in patients treated with statins may in part be explained by enhanced mobilization and function of EPCs [167]. Walter et al. demonstrated an accelerated rate of reendothelialization and a reduction in neointimal hyperplasia in rats subjected to balloon-mediated arterial injury treated with simvastatin. Treatment with simvastatin was associated with an increase in quantity and adhesive capacity of Ac-LDL$^+$BS-1-Lectin$^+$ cells in culture. Although Ac-LDL$^+$BS-1-Lectin$^+$ expression is not specific for endothelial cells, the neoendothelium in statin-treated animals was confirmed to be of bone marrow origin by the use of a Tie2/LacZ bone marrow transplant model [124]. Similar results were produced by Werner et al. using rosuvastatin [125]. In a study by Fuduka et al. administration of fluvastatin ameliorated impaired reendothelialization caused by sirolimus-coated stents [89]. This effect, however, did not appear to be mediated by mobilization of bone marrow-derived progenitors, but rather through enhancing the traditionally recognized mechanism of reendothelialization: proliferation of mature endothelial cells adjacent to the stented segment. The stimulatory effects of statins may not be sustained. Hristov et al. demonstrated a reduction of both EC-CFU and CD34$^+$VEGFR-2$^+$ cells in patients with CAD on chronic statin therapy [168]. The authors speculate over the possible exhaustion of EPC reserves, desensitization of the bone marrow to the effects of statins, or possible enhanced recruitment of circulating EPCs to peripheral sites of endothelial disrepair. While another small study concurred with the finding of reduced colony formation (similar to EC-CFU) on chronic statin therapy, ECFCs were increased [164]. Stimulation of EC-CFUs may therefore represent an initial haematopoietic effect of statin therapy, as opposed to the long-term angiogenic effects of chronic therapy reflected by the increased quantity of ECFCs. Statin therapy is, of course, already well established as a treatment for primary and secondary prevention for atherosclerotic events. The novel effects of statins on enhanced EPC mobilization and function may support an argument for intensive statin therapy prior to PCI.

Renin–Angiotensin–Aldosterone Antagonism

Activation of the renin–angiotensin–aldosterone system (RAAS) occurs in a variety of cardiovascular disorders, with associated deleterious long-term consequences. Antagonism of the RAAS is recognized to be beneficial in the setting of conditions such as heart failure, myocardial infarction, atherosclerosis and hypertension. The mechanism of action is thought to be partly via amelioration of the endothelial dysfunction that characterizes these conditions. Hypothetically, such benefits may be in part mediated via regulation of EPCs and enhanced endogenous vascular repair. Treatment with ACE inhibitors augments the mobilization of bone marrow-derived progenitors and enhances the proliferative and migratory capacity of CACs in vitro [169]. Similarly, treatment with angiotensin receptor blockers causes circulating EC-CFU to increase [170], probably through a reduction in cellular senescence by reducing oxidative stress caused by angiotensin II [171]. Paradoxically, angiotensin

II has also been shown to stimulate VEGF receptor expression in a dose-dependent manner, and increase proliferation of EC-CFU and tube formation in Matrigel when co-treated with VEGF [172]. Aldosterone itself has an inhibitory effect on the progenitor cell maturation in bone marrow cells ex-vivo in a PI3K/Akt-dependent manner [173] and treatment with both spironolactone [174] and eplerenone [175] increase circulating EPC with an associated increase in capillary density in animal models of ischaemia [175]. The effect of antagonizing the RAAS on EPC mobilization and function remains poorly understood; however, the behaviour of putative EPC populations in response to RAAS antagonism in small experiments has largely mirrored the beneficial effects observed in clinical practice.

Granulocyte Colony-Stimulating Factor

Granulocyte colony-stimulating factor (G-CSF) has no established role in the treatment of cardiovascular disease but is routinely used to mobilize progenitor cells in the context of bone marrow transplantation. G-CSF induces release of elastase and cathepsin G from neutrophils, which allows release of progenitors from the stem cell niche into the blood stream. G-CSF mobilizes a variety of EPC phenotypes, including CACs [176], EC-CFU [177, 178], $CD34^+CD133^+$ and $CD133^+VEGFR\text{-}2^+$ [178], $c\text{-}Kit^+VEGFR\text{-}2^+$ [131], $CD34^+VEGFR\text{-}2^+$ [130] and $CD34^+CD133^+VEGFR\text{-}2^+$ [177]. Several studies have demonstrated a positive effect of G-CSF on reendothelialization [128, 130, 131, 179, 180]. Kong et al. treated rats with G-CSF and demonstrated accelerated reendothelialization and reduced neointimal hyperplasia following endovascular balloon injury, associated with increased expression of CD34, eNOS, VEGFR-2, stem cell factor receptor-c-kit, and E-selectin on circulating MNCs [128]. Takamiya et al. showed similar effects of G-CSF on arterial repair associated with the mobilization of $c\text{-}Kit^+VEGFR\text{-}2^+$ cells and enhanced endothelial coverage [131]. A green fluorescent protein bone marrow transplant model was used to confirm that a significant proportion of the neoendothelium was derived from the bone marrow. This contrasts with the findings of Yoshioka et al. who found that although G-CSF mobilized $CD34^+VEGFR\text{-}2^+$ cells and accelerated reendothelialization, very few bone marrow-derived EPCs contributed to reendothelialization [130]. The mechanism through which G-CSF reduces neointimal hyperplasia is therefore unclear. Yoshioka et al. speculate that G-CSF may increase proliferation and migration of adjacent endothelial cells either directly or through stimulation by attaching EPCs. Even a marginal increase in attaching EPCs might be enough to increase the secretion of angiogenic factors [181, 182]. Shi et al. used G-CSF to accelerate endothelialization of Dacron grafts implanted in the aortas of dogs, but despite enhanced endothelialization, animals treated with G-CSF had considerably more neointimal formation than controls [180]. Concordant with this preclinical study, intracoronary G-CSF administered to patients with myocardial infarction was associated with an increased incidence of ISR, despite improvements in left ventricular ejection fraction (LVEF%) [183]. This effect may have been mediated

through G-CSF's non-specific proinflammatory actions and the mobilization of smooth muscle progenitors from the bone marrow. The non-specific nature of G-CSF's effects hampers its translation into a targeted therapy for vascular repair. Ongoing studies using G-CSF in combination with Sitagliptin, an anti-diabetic dipeptidylpeptidase IV–inhibitor known to cleave SDF-1 and augment progenitor cell mobilization, will address whether the effects of G-CSF may be honed to make it clinically useful in the context of cardiovascular disease [184].

Peroxisome Proliferator-Activated Receptor Agonists

PPAR agonists inhibit vascular smooth muscle proliferation and migration, improve endothelial function and accelerate reendothelialization and can therefore attenuate neointimal following experimental vascular injury. PPARs regulation of vascular smooth muscle cell proliferation, migration and reendothelialization can attenuate neointimal formation in mice subjected to femoral angioplasty. Wang et al. demonstrated that PPAR agonist rosiglitazone can drive pluripotent bone marrow-derived vascular progenitors away from a smooth muscle phenotype toward an endothelial phenotype and this may explain such favourable effects on vascular repair [185]. PPAR agonists may therefore be useful in attenuating a maladaptive response to iatrogenic vascular injury, although concerns remain regarding the safety profile of these drugs in patients with ischaemic heart disease [186].

Erythropoietin

Erythropoietin (EPO) treatment also accelerates reendothelialization following discrete vascular injury. Serum EPO concentration correlates tightly with progenitor cells expressing $CD34^+VEGFR\text{-}2^+$, $CD34^+CD133^+$ and $CD34^+Lin^+$, as well as cultured EPC of a similar phenotype to CAC in patients with CAD [187]. EPO therapy causes acute mobilization of $CD34^+VEGFR\text{-}2^+$ [188] and increases the proliferative and adhesive capacity of cultured EPC in a PI3K/Akt-dependent manner [189]. EPO treatment enhanced proliferation of resident endothelial cells and reduced apoptosis of the injured artery, in association with eNOS-dependent mobilization of $Sca\text{-}1^+VEGFR\text{-}2^+$ cells [190]. In patients with congestive cardiac failure, however, chronic EPO therapy had no effect on circulating $CD34^+$, $CD34^+CD45^+$, $CD34^+CD133^+$, $CD34^+VEGFR\text{-}2^+$ or $CD34^+CD133^+VEGFR\text{-}2^+$ progenitor cells. The acute effects of EPO may therefore be attenuated over time, although the bone marrow suppression known to be associated with congestive cardiac failure may explain these differences [191]. EPO has also been associated with an increased incidence of neointimal proliferation despite adequate reendothelialization in animal studies, possibly as a result of the non-specific mobilization of smooth muscle progenitors and proinflammatory effects [192]. Preliminary data from

the HEBE-III trial suggest that EPO therapy has a favourable impact on clinical outcomes following myocardial infarction. Although the mechanism is uncertain, this effect appears to be independent of improvement in left ventricular (LV) function [193]. Further data from the HEBE-III [194] and REVEAL [195] trials may address whether erythropoietin will have a favourable effect on vascular remodelling.

Estrogens

Estrogens have been shown to enhance vascular repair in models of arterial injury, in part through eNOS-dependent mobilization and proliferation of bone marrow-derived EPCs, which exhibited enhance migratory and mitogenic activity [196], and reduced apoptotic signalling through the actions of caspase-8 [197].

Physical Exercise

Physical exercise is a known stimulus of EC-CFU [198], and using running wheels has been shown to enhance vascular repair in mice subjected to vascular injury in association with enhanced progenitor cell mobilization [199].

Stent-Based Therapy

Technological advances and the evolution of intracoronary stents provide a potential vehicle to deliver novel therapies directly to the site of vascular injury. Attempts to coat intracoronary stents with endothelial mitogens, such as VEGF, have not been encouraging in terms of reendothelialization [200]. However, gene-eluting stents directly delivering naked plasmid DNA encoding for VEGF-2 can accelerate reendothelialization and reduce lumen loss in animal models [201]. The Genous Bioengineered R-stent is coated with monoclonal antibodies directed against CD34 and is designed to attract EPCs and encourage reendothelialization. Genous stents have already progressed to Phase II and III clinical trials and have been deployed in more than 5,000 patients. Preliminary data from small registries reported major adverse cardiovascular event (MACE) rates ranging from 7.9 to 13% [202–206]. The e-HEALING registry, with 4,939 patients, reports target vessel failure (TVF) rate of 8.4% with a target lesion revascularization rate of 5.7% and a MACE rate of 7.9%, with a low incidence of stent thrombosis (1.1%) [207]. The HEALING II registry reported that patients with normal $CD34^{+}VEGFR\text{-}2^{+}$ titers had lower rates of ISR compared to those patients with reduced circulating EPCs (late luminal loss 0.53 ± 0.06 mm vs. 1.01 ± 0.07 mm). Furthermore, a subgroup of 30 patients in this

non-randomized study underwent serial evaluation using intravascular ultrasound, and regression of neointimal volume was observed in patients with higher concentrations of EPC [203]. The first randomized controlled trial in a small cohort of patients with ST-segment elevation myocardial infarction reported a trend toward increased restenosis with the Genous stent when compared to a standard chromium-cobalt stent [208]. The Tri-stent Adjudication Study (TRIAS) compared the Genous stent with tacrolimus-eluting Taxus stents in 193 patients with lesions carrying a high risk of restenosis (i.e. chronic total occlusions, small caliber vessels (<2.8 mm), long lesions (>23 mm) or diabetic patients). At 1 year there was a highly significant increase in restenosis in the Genous stent group. TVF was also increased (17.3% vs. 10.5%), although this was not statistically significant, presumably due to the relatively small number of patients in the trial. The Genous stent does, however, have a favourable safety profile with respect to stent thrombosis; no stent thromboses occurred in the Genous stent group compared to four stent thromboses in the Taxus stent group, and this was despite a significantly lower use of clopidogrel in patients randomized to receive the Genous stent [209]. It is difficult, however, to envisage a role for the Genous stent, as the early discontinuation of clopidogrel it allows, which would be favourable in patients at higher risk of developing bleeding complications, is similarly provided by BMS. Yet, BMS may have a more favourable profile with respect to ISR. Restenosis may be increased with CD34 capture stents for a variety of reasons. As discussed previously, CD34 is not specific for EPCs, being common to a number of progenitors including SMPCs [146]. Circulating SMPCs are poorly characterized, but are known to contribute to neointimal hyperplasia [145]. Both CD34 and CD133 are detected at increased levels in the neointima of restenotic lesions compared to de novo lesions [147]. The effect of indiscriminate binding of $CD34^+$ cells to intracoronary stents could theoretically exaggerate the restenotic effect due to proliferation of smooth muscle progenitors, particularly if exposed to the proinflammatory microenvironment of patients susceptible to ISR, such as those with diabetes mellitus. As our understanding of EPC biology improves, so too will our ability to use intracoronary stents to modulate the cellular response to vascular injury and directly enhance reendothelialization following iatrogenic vascular injury through gene and drug delivery or progenitor cell capture.

Infusion of EPCs in Acute Vascular Injury

As an alternative to pharmacological mobilization, direct intravenous, and also intra-arterial infusions of “EPCs” have also been used in an attempt to accelerate vascular healing in the context of discrete vascular injury. In animal models of endovascular injury, transfusion of mononuclear cells cultured in angiogenic medium to produce endothelial-like cells have successfully accelerated reendothelialization and attenuated neointimal hyperplasia [126, 181, 210, 211]. Generally these studies have used unselected cell populations of “EPC”; therefore, the mechanism through which favourable effects may have occurred is uncertain. Beneficial effects may

have been mediated through the paracrine influence of angiogenic monocyte and lymphocyte populations. Wassmann et al. suggest that restoration of endothelial function is best imparted by the synergy that exists between unselected mononuclear cell subpopulations [212].

Concerns that infusion of mononuclear cells may increase inflammatory signalling and smooth muscle proliferation are well founded theoretically, and there are some reports of an increased incidence of coronary events, including restenosis and thrombosis, following such treatment. Circulating progenitor cells have also been implicated in the progression of atherosclerotic plaques in the context of ischaemia [213]. Mononuclear cells cultured in endothelial growth medium and genetically engineered to overexpress eNOS are effective at accelerating reendothelialization, with an associated reduction of neointimal hyperplasia and thrombosis [214]. No clinical studies using infused progenitor cells specifically to influence restenosis have been reported to date. Data regarding the transfusion of progenitor cells in human studies are derived largely from studies examining the effect of mononuclear cell infusion on left ventricular dysfunction and myocardial ischaemia in the context of acute myocardial infarction.

Cell Therapy in Myocardial Infarction and Left Ventricular Dysfunction

Putative EPC populations mobilized in the context of discrete vascular injury are similarly increased following acute myocardial infarction. Bone marrow-derived haemangioblastic precursors are capable of inducing vascular proliferation within infarcted myocardium with demonstrable improvements in myocardial perfusion and LV function. [117, 182, 215–217] Circulating EPCs may therefore have a role following acute myocardial infarction; reendothelializing atherosclerotic plaques, intracoronary stents or in the neovascularization of ischaemic myocardium. Clinical trials studying the effects of cell therapy in the context of myocardial infarction have predominantly observed the effects of unselected, autologous bone marrow-mononuclear cells (BMNCs) administered by means of intracoronary transfusion on indices of LV function, and most of these studies have been performed in the context of PCI.

Cell Therapy in Acute Myocardial Infarction

In one of the earliest trials of cell therapy, Strauer et al. used intracoronary autologous BMNC transfusion in ten patients approximately 1 week following successful PCI for acute myocardial infarction. At 3 months, infarct size was significantly smaller compared to a matched control group, and wall motion velocity of the

infarcted region was significantly higher**;** however, LVEF% was no different between the groups [218]. Huang et al. randomized 30 patients in an open-label fashion to intracoronary autologous BMNC vs. saline immediately following PCI for acute inferior ST-segment elevation myocardial infarction (STEMI). At 6 months LVEF% had significantly improved in both groups, but to a marginally greater extent in those randomized to cell therapy. Infarct size was also significantly smaller in the BMNC group [219]. In similarly conducted small studies, intracoronary BMNC transfusion has been associated with significant improvements in myocardial perfusion post-acute myocardial infarction, suggesting that accelerated revascularization may be a potential mechanism underlying the improvement in LV function observed in these studies [220, 221]. Meluzin et al. randomized 66 patients with acute myocardial infarction undergoing PCI to intracoronary transfusion of either 1×10^7, or 1×10^8 autologous BMNC 5–9 days post-acute myocardial infarction or optimal medical therapy. At 3 months, LVEF% had improved by 2% in the control group but was significantly greater by 3 and 5% in the low- and high-dose groups respectively, indicating the presence of a dose-dependent response to cell therapy in the short term [222]. However, in a well-conducted, double-blind trial in which all patients underwent bone marrow aspiration, Janssens et al. randomized 67 patients to BMNC therapy or saline placebo within 24 h of acute myocardial infarction. LVEF% improved in both groups at 4 months, but there was no significant difference between the two therapies. There was, however, a significant reduction in infarct size, and regional LV systolic function as determined by MRI in those receiving cell therapy [223]. The BONAMI trial randomized 101 patients to BMNC therapy or control and similarly demonstrated modest improvements in LVEF% in both groups at 6 months Although some trends toward improved myocardial viability were associated with cell therapy, there was no difference in LVEF% between the two groups [224]. Karpov et al. were unable to attribute any benefit to BMNC following acute myocardial infarction in an open-label study of 44 patients [225], and a trial by Penicka et al. was discontinued early due to an increased incidence of adverse events associated with stem cell therapy [226]. It is unlikely however that the complications were related to cell therapy; ventricular septal rupture in late presentation myocardial infarction, stent thrombosis in a complicated PCI, biliary cancer and distal vessel occlusion. LV function improved to a similar extent in both groups.

Meta-analyses of these and other similar trials indicate that cell therapy is very well tolerated with very few adverse events associated with BM aspiration and cell administration [227, 228]. These analyses indicate that cell therapy confers a small but significant improvement in LVEF% of approximately 3% through a reduction in infarct size and end-systolic volumes. Benefit appears greater in association with greater quantity of cells, and delayed delivery of therapy to beyond 7 days post-acute myocardial infarction. However, the studies included in these meta-analyses are small and have inconsistent reporting of randomization procedures and power calculations, in addition to a very short follow-up, generally between 3 and 6 months. Therefore, definitive conclusions cannot be drawn on the effect of BMNC therapy on LV function purely on the basis of these studies [218–228].

Several studies have also been conducted with a longer duration of follow-up. In the ASTAMI trial, 100 patients undergoing PCI to the LAD for anterior STEMI were randomized to receive intracoronary injection of BMNC or optimal medical therapy. In both groups, a steady improvement occurred in LV longitudinal strain in the LAD territory, LV filling pressures and diastolic function; however, there was no difference between the two therapies over 3 years [229]. The BOOST study randomized in an un-blinded fashion 60 patients undergoing PCI following acute myocardial infarction to intracoronary infusion of autologous BMNC or optimal medical therapy [230]. Although there was an initial improvement in LVEF at 6 months compared to optimal medical therapy, similar to the findings of ASTAMI, LVEF% was no different between the groups at 18 months or at 5 years. REPAIR-AMI enrolled 204 patients into a double-blind, placebo-controlled, multicenter trial. All patients underwent BM aspiration and were randomized to receive intracoronary infusion of BMNC or saline placebo 3–7 days following successful PCI. At 4 months there was an improvement in LVEF% [231], although this was not sustained at 2 years [232]. Bone marrow (BM) cell therapy was associated with a significant reduction in the cumulative end point of death, myocardial infarction, or revascularization compared with placebo (HR, 0.58; $P = 0.025$); however as the authors recognize, this study was not powered to detect differences in clinical outcomes [232]. Therefore, although numerous studies have suggested that recovery of LV function may be accelerated by cell therapy, relatively larger studies with longer follow-up have failed to show that intracoronary BM cell transfusion provides a sustained benefit in LV function following acute myocardial infarction.

Cell Therapy in Chronic Ischaemic Cardiomyopathy

Cell therapy has also been tested in chronic ischaemic cardiomyopathy. Strauer et al. performed intracoronary transfusion of autologous BMNC in 18 patients with chronic myocardial infarction (>5 months) and compared them to a matched control group. In this non-blinded study, infarct size was reduced, and LVEF% increased after 3 months. Patients also exhibited demonstrable improvements in functional capacity [233]. The STAR-heart study followed, allocating 391 patients with depressed LV function to intracoronary BMNC (191 patients) or optimal medical therapy (200 patients). Over 5 years of follow-up, BMNC therapy was associated with a significant 6% increase in LVEF%, with demonstrable improvements in exercise capacity, oxygen uptake, LV contractility and arrhythmogenicity. Similar improvements were not observed in the group allocated to optimal medical therapy. Although not powered to address clinical outcomes, all-cause mortality was significantly reduced following cell therapy compared with the control group at 5 years. However, it is of importance to note that patients were allocated to treatment on the basis of individual preference, with those unprepared to receive cell therapy comprising the control arm. The median time from the index infarction for

the whole cohort was 8.9 years, however whether the individual groups differed in this respect was not reported. While intriguing, the results of this trial must be interpreted with caution, given these methodological considerations that may have introduced significant bias in favour of cell therapy.

Cytokine Facilitated Cell Therapy

Although feasible on a small scale, the need for bone marrow aspiration in the context of acute myocardial infarction clearly creates additional practical difficulties and patient discomfort. The capacity of G-CSF to mobilize BM resident stem cells to the peripheral blood provides a potential means of avoiding BM aspiration. Suárez de Lezo et al. randomized patients to intracoronary BMNC infusion, G-CSF or optimal medical therapy in a small study of 30 patients undergoing PCI for acute myocardial infarction. LV systolic function improved compared to baseline regardless of randomization, although only significantly so in those patients receiving cell therapy, indicating a lack of efficacy of G-CSF on its own [234]. In a study by Li et al. peripheral blood mononuclear cells (PB-MNCs) were harvested following treatment with G-CSF and delivered by intracoronary transfusion following successful PCI for acute myocardial infarction. LV function was reassessed at 6 months in 35 patients receiving cell therapy and in 23 matched controls. Compared to baseline LVEF%, regional wall motion and both end systolic and diastolic volumes were improved following cell therapy, an effect not observed in the control group, indicating that peripheral harvesting of stem cells facilitated by G-CSF is a potentially feasible means of facilitating this form of therapy [235]. In the MAGIC study, G-CSF-facilitated intracoronary cell therapy led to improvements in exercise capacity, myocardial perfusion and systolic function; however, an increased incidence of ISR in the cell therapy group led to premature discontinuation of the study [236]. The follow-up, MAGIC Cell-3-DES study examined the effects of intracoronary G-CSF-mobilized MNCs in patients with acute vs. chronic myocardial infarction. In patients with acute myocardial infarction there was a small but significant improvement in LVEF% compared to controls; however, no improvement in LVEF% was seen in those patients with chronic myocardial infarction despite improvements in coronary flow reserve. In this study, however, in which DESs were used, ISR associated with G-CSF was not encountered [237]. PB-MNC harvested without the use of G-CSF has also been shown to have comparable effects to BMNC on LV function following acute myocardial infarction in the TOPCARE-AMI in which LVEF% improved at 4 months following both PB-MNC and BMNC, with similar improvements in coronary flow reserve and PET-derived myocardial viability [238]. LV function remained similar in the two groups at 1 year, indicating comparable efficacy of the two methods. However, the lack of a control group in this analysis limits interpretation of the source of benefit in this trial [239]. The TOPCARE-CHD study compared BM and PB-MNC in chronic ischaemic myocardial infarction.

Seventy-five patients were randomized to PBMNC, BMNC or control. Patients were at least 3 months, and on average 80 months, post-myocardial infarction. Compared to the other groups, BMNC therapy was associated with an improvement in New York Heart Association (NYHA) functional class and a modest, yet statistically significant, 3.7% improvement in LVEF% at 3 months. A subsequent crossover phase was performed in which patients randomized to each cell therapy underwent further treatment with the alternate cell type, and those initially randomized to control were randomized again to PBMNC or BMNC. Evaluation was repeated an additional 3 months later. The results from crossover phase mirrored that of the initial randomization, with improvements in LVEF% following BMNC regardless of initial therapy. This result indicates that the positive effect of cell therapy was specific to BM-derived cells; however, the follow-up of this study was very short, and the mechanisms underlying the small functional improvement in LVEF following BMNC therapy in this study are unknown.

Cell Therapy Using Specific Subpopulations

The majority of cell therapy studies have employed unselected cell preparations. The use of specific cell types with targeted function theoretically may provide a concentrated beneficial effect. Both intramyocardial [240] and intracoronary [241] $CD133^+$ cells have been used in small clinical studies in patients undergoing revascularization, with some promise. Bartunek et al. detected an improvement in LVEF% following intracoronary $CD133^+$ infusion, although there was a concerning significant increase in coronary events, including ISR, vessel occlusion and de novo atherosclerotic lesion formation associated with cell infusion [242]. The double-blind, randomized, placebo-controlled trial, COMPARE-AMI, will evaluate the safety and efficacy of intracoronary $CD133^+$ infusion in patients with left ventricular dysfunction undergoing PCI for acute myocardial infarction [243]. In the REGENT study, 200 patients were randomized to optimal medical therapy or intracoronary infusion of BMNC, either unselected or enriched for $CD34^+CXCR\text{-}4^+$, a cell population known to be mobilized in response to myocardial ischaemia [244]. At 6 months, 46 and 51 patients randomized to unselected or $CD34^+CXCR\text{-}4^+$ cell therapy respectively, and 20 patients from the control group, underwent MRI assessment of LV function. No difference was apparent between the groups, however compared directly to baseline there were significant improvements in those patients randomized collectively to cell therapy. Within-group comparisons, however, indicated that the benefit was only significant for those receiving unselected cells. This may have been due to a dosing effect, as the unselected group received ~100-fold more cells than the selected group. There was no significant change of LVEF in the notably smaller control group. The benefit derived from BMNC therapy appeared to be restricted to those with greater degrees of LV systolic dysfunction [245].

Limitations of Studies in Cell Therapy

While meta-analyses have indicated favourable effects on LV perfusion and systolic function following stem cell therapy in LVSD, none of the studies thus far performed have been sufficiently powered to detect differences in clinical outcomes. The absence of sham procedures in most of the studies increases the potential of influence of the placebo effect. Furthermore, it is well recognized that small, negative studies more frequently fail to be published, and such a publication bias may tip the balance in favour of cell therapy, despite the negative outcomes observed in the relatively larger studies. This effect is very difficult to control for. Longitudinal studies of patients following acute myocardial infarction indicate that in as many as 72% LV function will improve over the first year following acute myocardial infarction [246]. Not surprisingly therefore, most studies of stem cell therapy have observed an improvement in LV function regardless of randomization to treatment or placebo. In small studies such as those discussed, the normal improvement in LVEF% may go undetected, and clinical benefit may therefore be erroneously attributed to cell therapy. One would expect this effect to be diminished if the baseline assessment were to be performed after a longer period of time following myocardial infarction, although the benefits of cell therapy appear to be greater in those studies in which cell therapy was delivered later.

Autologous cell therapy for acute myocardial infarction may be limited by the inherent cellular dysfunction that is present in patients with atherosclerosis and risk factors for myocardial infarction that, by definition, might benefit from such treatment. The ENACT-AMI trial will attempt to circumvent this problem by delivering intracoronary MNC preparations that have first been modified by angiogenic culture and genetic manipulation to over express endothelial nitric oxide in order to enhance their vasoregenerative function [247]. Such modifications have shown promise in enhancing the regenerative capacity of cell preparations in preclinical studies [214]. Cell therapy may also be limited by the mode of cell delivery. It is apparent that only a small proportion of the transfused cells remain in the heart. Following intracoronary transfusion as little as 7.8% of cells are detectable in the myocardium at 30 min, falling to 3.2% by 24 h [225]. It is questionable therefore whether such a small proportion of cells could confer meaningful benefit. Optimal delivery of cells has not yet been established, and further studies are required if direct administration of cell populations is to develop as a means of therapy.

Conclusions

Endothelial cell regeneration is a key component of an effective response to cardiovascular injury. Preclinical studies have produced encouraging results in support of a role for circulating progenitor cells in vascular repair and some progress has been made in determining a variety of populations involved in the cellular response to

vascular injury. However, considerable uncertainty exists over which populations have clinical relevance. There is still no clear definition of EPC or how they might best be harnessed. Perhaps as a result of this clinical studies have failed to replicate the findings of preclinical studies. A better understanding of the mechanisms governing EPC behaviour is therefore required in order to develop therapies targeting the acceleration of vascular healing. While circulating progenitors contribute to vascular healing, it is evident that their function is dependent on both the local microenvironment and a synergy among other populations mobilized in response to vascular insult. Efforts should be focused on determining not only the identity of the EPC, but also how best to augment the local microenvironment. Determining the optimal method of isolation and preparation of autologous cells, the timing and mode of delivery, and any adjunctive therapy that might optimize EPC function, remain important obstacles to be overcome.

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