Ischemia and Reperfusion Injury in Bone

3

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3.1 Introduction

Bone cells may be killed in a variety of ways, from radiation to poison. But the clinical phenomenon that has become most identified with the term "osteonecrosis" is associated with ischemia. Ischemic osteonecrosis (ION) is the preferred term, although clinicians traditionally use AVN (avascular necrosis) in spite of the fact that the absence of vessels has never been histologically confirmed (ischemic vessels are still vessels just as a dead body is still a body). It is generally agreed that ION results from two main causes of ischemia: (1) hypercoagulation that is associated with a wide range of diseases, leading to the name of this category as "idiopathic" ION, and (2) physical damage to bone blood vessels resulting from impact—high energy or compression—known as "traumatic" ION. This review is divided into three sections: (a) idiopathic ION, (b) traumatic ION and reperfusion, and (c) intravital microscopic investigation of both.

3.2 Idiopathic ION

Since the early 1990s a consensus has been building that the ischemia associated with idiopathic ION is caused by hypercoagulation [1–3]. Unfortunately, the number of pathophysiological roads to hypercoagulation is so great that one can find published data correlating a large fraction of them with clinical presentations [4].

Since ION of the femoral head is by far the highest in incidence [5, 6], it is logical to ask if there is a circulatory basis for such a bias. Why does ION not occur as frequently in other joints? For that matter, why does it not frequently occur elsewhere in the femur? Of course it occurs in any bone if the conditions stated in the first paragraph are met. But ION does not progress to bone collapse in other bones as often as it does in the hip.

Since pathology is a deviation from normal physiology, one cannot expect to understand circulatory pathophysiology without understanding normal circulatory physiology of the femur. The human femur is normally fed by two nutrient arteries from the femoral profunda that enter the upper diaphyseal cortex through foramina. They then branch just central to the endosteum into ascending and descending tributaries. These in turn send centrifugal branches into adjacent cortical bone and centripetal branches toward sinusoids in the medullary canal as the tributaries continue toward the metaphysis. There is a net centrifugal pressure drop across the diaphyseal cortex and a net movement of its interstitial fluid (bone fluid flow) toward the periosteum [7]. However, pressure changes in the medullary canal are not well translated into pressure changes within the cortex, suggesting that septi—anatomical or functional—exist interrupting any continuous medullary canal-to-periosteum fluid flow [7].

In most limbs the ascending nutrient artery branch eventually reaches metaphyseal cancellous bone where it appears to form anastomoses with metaphyseal arteries. Metaphyseal arteries often anastomose with epiphyseal arteries. Epiphyseal and metaphyseal bones typically have a more direct supply. Separate perforating arteries enter the subcondylar region at two or more foramina and after branching may anastomose.

Metaphyseal-epiphyseal blood supply to the proximal femur is different. Vessels enter at the femoral neck, superior to the greater trochanter and inferior to the capsule of the hip joint. There are two tributaries from the femoral profunda that circle the bone, the medial (MFCA) and lateral circumflex arteries. The lateral circumflex supplies the inferior bone at the level of the trochanter and the MFCA supplies the superior femoral head. There is variation between individuals. In some cases the inferior gluteal artery contribution dominates the MFCA [8, 9]. There is also evidence for anastomoses between the MFCA and inferior gluteal artery [10]. After penetrating the joint

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capsule, the MFCA runs along the posterosuperior aspect of the femoral neck sending subsynovial retinacular branches caudad [10]. One of the most interesting findings of Crock is the presence of sinusoids at the tips of these retinacular branches [11]. Other investigators do not seem to have been able to get their injected media to penetrate to vessels small enough to confirm or disprove this observation, although Figs 7 and 8 of Sevitt and Thompson's report suggest a similar result [12].

In adults some of the femoral head blood supply may come from the ligamentum teres artery [12, 13]. It enters the femoral head apex and its distal branches anastomose with the retinacular arteries [14].

The diaphyseal periosteum of the femur is fed by periosteal arteries that branch from arteries in tendons connected to the cortex by Sharpey's fibers. These vessels supply not only the periosteum but up to 1/3 of the outer cortical bone under normal conditions [15]. When medullary nutrient artery tributaries are damaged as occurs when the canal is reamed, periosteal circulation increases its supply to the cortex [16]. There do not appear to be any studies of contributions of femoral neck periosteal blood supply to the neck cortex, although this bone is well vascularized [17]. This circulation may be a factor in healing of the osteonecrotic head.

When blood reaches the end of its retinacular artery, it flows into increasingly smaller-diameter branches regressing from terminal branches (down to 60 μm) to arterioles (down to 20 μm) to capillaries (down to 8 μm). All of these vessels are lined with endothelial cells (ECs). Precapillary vessels are surrounded with smooth muscle. As arterioles become capillaries, they are increasingly surrounded with pericytes that are not muscles but can change the vessel diameter by coiling actin of their intracellular matrix. Capillaries are one EC thick and surrounded with a laminin-plus-collagen IV basement membrane and an occasional pericyte. There are three general types of capillaries:

1. Continuous: with continuous basement membranes and ECs overlapping at junctions
2. Fenestrated: with continuous basement membranes and ECs with pores up to 1,000 Å wide at junctions
3. Discontinuous: with holes in basement membranes and clear fissures in EC junctions, sinusoids

In some tissues there are shunts called metarterioles from precapillary vessels to pre-venular capillaries. Should smaller capillaries become plugged, blood would divert across these shunts.

Systemic pathologies like disseminated intravascular coagulation (DIC) cannot by themselves explain a tendency for ischemia to occur in a femoral head [18]. Accordingly, one must determine if there is some basis for hypothesizing that head vasculature has an inherent tendency to be thrombogenic. Blood vessel function is essentially EC function, and if the microcirculation is to experience the hypercoagulation required for ION [3], ECs must be, logically, integral components of the thrombogenic process.

Table 3.1 Regulators of thrombosis and hemostasis in endothelium

EC Features	Antithrombotic	Prothrombotic
Coagulation protein binding sites	Glycosaminoglycans/ ATIII	Binding sites for fibrin, FIX, IXa, X, Xa, FXII, kallikrein
	TFPI	Tissue factor
	Thrombomodulin	Thrombin receptor
		Receptor for protein C/APC
Products produced and/or stored by platelets	PGI_2	vWF
	NO	PAF
	ADPase	Fibrinogen
		FV
		FXI
Fibrinolytic factors	t-PA production	PAI-1, PAI-2
	u-PA expression	PAI-3 (protein C Inhibitor)
	u-PAR	TAFI activation
	Plasminogen binding sites	
	Annexin II	
Vasomotor factors	NO	TxA_2
	PGI_2	Endothelin-1

From Cines et al. [42]
EC agents, binding sites, receptors, and actions that control coagulation in blood vessels
Abbreviations: *ATIII* antithrombin III, *PGI_2* prostacyclin, *TFPI* tissue factor pathway inhibitor, *APC* activated protein C, *PAF* platelet activating factor, *t-PA* tissue plasminogen activator, *u-PA* urokinase plasminogen activator, *u-PAR* urokinase plasminogen activator receptor, *PAI* plasminogen activator inhibitor, *TAFI* thrombin-activatable fibrinolysis inhibitor, *TxA_2* thromboxane A_2

ECs are not the same in all tissues. Those lining capillaries of different organs are arranged differently [19]. Consider those in brain with its blood–brain barrier vs. those in marrow sinusoids. The size of the vessel makes a difference (Kumar [20]). Endothelial cells from microcirculatory vessels express more MHC I and II as well as adhesion molecules than do those from larger vessels [21, 22]. In fact the surface proteins vary sufficiently to allow immunological differentiation of ECs by organ [22]. Pathologies that reflect this organ-to-organ variation are classified as regional endothelial dysfunctions or RED [18]. Such heterogeneity is not unique to humans. It is at least chordate phylum wide [23].

Is the heterogeneity that leads to RED reflected in thrombogenicity? Table 3.1 summarizes various EC components that influence the clotting process. Each component can induce or inhibit thrombosis by secreting or activating various agents that control clotting. Tissue factor, PAI-1, vWF, and protease-activated receptors vary in circulatory

Table 3.2 Extracellular signals that regulate procoagulant or anticoagulant mRNA expression in endothelial cells

Source of signal	Effect
TNFα	Decreases expression of thrombomodulin
	Increases expression of plasminogen-activator inhibitor type 1 and tissue factor
IL-1	Decreases expression of thrombomodulin
TGF-β	Decreases expression of thrombomodulin
	Increases expression of plasminogen-activator inhibitor type 1
VEGF	Increases expression of thrombomodulin, plasminogen-activator inhibitor type 1, and tissue-type plasminogen activator
PDGF	Increases expression of von Willebrand factor
Shear stress	Increases expression of thrombomodulin, tissue-type plasminogen activator, tissue factor, and nitric oxide synthase
Hypoxia	Increases expression of plasminogen-activator inhibitor type 1
	Decreases expression of tissue-type plasminogen activator

From Rosenberg et al. [24]

beds from organ to organ [24, 25]. Tissue factor expression is heterogeneous over the body [26]. Thrombomodulin and endothelial protein C receptors are expressed heterogeneously in different vascular beds [27]. There is circulatory heterogeneity as well. Venular valves are more frequent in bone than in skeletal muscle vessels [28]. It has been postulated that individuals with poor circulation to femoral head retinaculars are at high risk for thrombi and EC apoptosis resulting from reduced fluid shear in their microvasculature [1]. Signals that affect thrombogenesis are summarized in Table 3.2.

Does the heterogeneity in EC MHCII antigens contribute directly to RED and thrombogenicity? ECs present MHCII antigens to $CD4^+$ T cells—more in micro- than macrovasculature [21, 22]—and some express CD40 (in dendritic cells this marker interacts with T cells), CD80, and CD86 (both T cell costimulatory) suggesting presentation to $CD8^+$ T cells as well [28]. There are conflicting data on the extent to which these interactions anergize the T cells [29, 30]. But there is support for the conclusion that $CD8^+$ T cells can activate and induce proinflammatory changes in ECs [31, 32].

Dendritic cells (DCs) may have a role in multifoci ION. Their role in DIC begins with systemic challenge to the innate immune system. In experiments with lipopolysaccharide (LPS), the challenge begins with detection of the immunogen by DC TLR4. In the lymph nodes LPS activates DC TLR4 that in turn upregulates its protease-activated receptor 1 (PAR1). Through an internal cascade, PAR1 activates sphingosine-phosphate 3 receptor (S1P3R) that initiates secretion of TF and IL-1β. In lymph circulation this TF has little effect. But the continuous LPS signal eventually stimulates migration of DCs from the lymph nodes to lymph vessels that carry them to ducts that drain into the blood circulation. As the DCs circulate they secrete TF stimulating receptive ECs to secrete thrombin and form local thrombi. Thrombin directly stimulates DC PAR1 to complete the autocrine cycle, further amplifying DIC [33].

Local thrombosis may be stimulated by LPS in vessels like the retinacular microcirculation as well. Endothelial cells have TLR4s [34], S1P3R [35], Waeber [36] and PAR1 [37]. This effect has apparently been demonstrated by Okazaki et al. [38] who challenged mice with LPS and were able to produce femoral ION and cytokines associated with TLR4 stimulation.

3.3 Traumatic ION

Thrombogenesis is not necessary for ION if vessels are simply destroyed as is the case for traumatic ION. But bone not directly made ischemic via traumatic destruction of feeder vessels may become ischemic as the result of reperfusion injury. Compartment syndrome would likely produce the necessary ischemia, as would a crush injury of limited force.

Endothelial cells normally control both upstream and downstream vessel diameters by converting L-arginine to NO that is delivered countercurrently or downstream to sphincters. They also normally oxidize their purines using xanthine oxidase to produce superoxide anions, which at physiologic pH dismutates (via superoxide dismutases) to H_2O_2 and in the presence of ferrous ions and peroxidases to H_2O and ferric ions.

During ischemia blood flow in microvasculature stops and neutrophils that have become trapped are no longer stimulated by blood flow shear to remain in the vessel lumen. They upregulate their L-selectin and seek to extravasate through the nearest endothelium. Meanwhile, ECs, in the absence of flow and O_2, are increasingly exposed to an anaerobic and decreasing pH environment. As a result NO production and peroxidase activity stops, allowing accumulation of superoxide anion. Accumulation of these free radicals damages ECs, primarily by lipid peroxidation [39].

As tissue adjacent to that made necrotic directly by the trauma proceeds through a wound healing inflammatory phase, constricted vessels upstream begin to relax and blood is shunted to the patent ischemic vasculature. When the highly oxygenated reperfusing blood meets a lumen with neutrophils lining the walls and free radicals and inflammatory cytokines (some from adjacent mast cells) in high concentration, incoming platelets are activated and, in the absence of NO, thrombosis and vessel constriction condemn the flow to stasis.

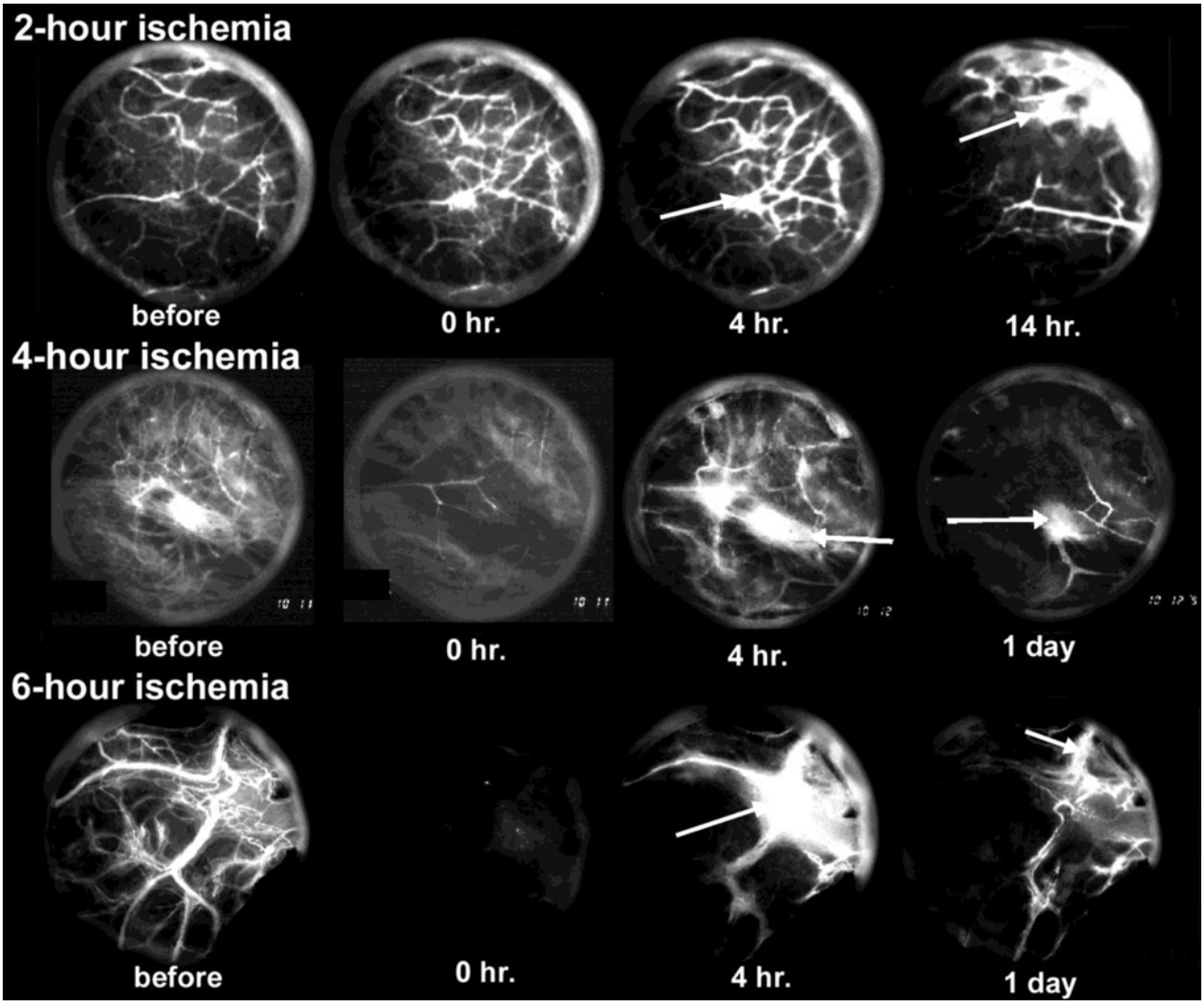

Fig. 3.1 Examples of reperfusion phase vascularity for three ischemia durations. Each circle has a diameter of about 2 mm (disregarding the outer ring of reflected light). Visible vessels show circulating FITC-D70 which normally leaks only through so-called large pores (according to the two-pore permeability model; few in number in tissue at equilibrium). Baseline vascularity is shown at far left. Reperfusion at zero hours (0 H) is photographed 10 min after cuff deflation and shows a pattern suggesting an inverse relationship between duration and initial reperfusion. At 4 h vessels exhibit abnormal leakage which appears to increase with ischemia duration. At 14 per day, reperfusion has decreased from its 4-h value for all three treatments. Also, leakage at this time is less for the higher durations, probably because so little plasma is circulating (From Hsieh et al. [40] with permission)

Selection of vessels that undergo reperfusion injury first is probably not as important for the progress of ION as is the ischemia length of time. We have found significant effects at 2 h [40] as can be seen in Fig. 3.1. These events probably occur at different points and times in the femoral head, depending on local conditions. Diffusion through the extracellular matrix of cytokines and other agents—from mast cells in particular—will spread signals to adjoining tissue. Of particular interest is the source of macrophages, ECs, and fibroblasts that will form the repair tissue of early healing. Unlike idiopathic ION wherein the pathological agent often persists, the trauma source is usually gone when repair begins.

3.4 Intravital Microscopy for Assessing Bone Microcirculatory Pathology

If one wishes to directly view microcirculatory events in vivo and in situ in an intact animal, it is usually necessary to expose the organ and place it in a special microscope. The observing instrument is called an intravital microscope (IVM). P.I. Brånemark developed it for use in bone by adding a window implant in 1964 [41]. In order to give direct visual access to bone cortex, medullary canal, and their vasculature, Albrektsson modified the window into a surgically implanted optical bone chamber. The images in Fig. 3.1 were obtained with a horizontal modification of the original

vertical IVM. The horizontal IVM allows the animal being observed to be upright and free of clamping pressure on the bone being observed.

The bone chamber used for intravital microscopy is usually implanted in the tibial medial cortex immediately distal of the medial collateral ligament insertion. It is permanently exposed and is managed for infection prevention by daily peroxide lavage. It has been designed to perforate both cortices so that transmitted light may pass into the lateral end and illuminate bone that is growing and has grown into a 100 μm deep × 2 mm wide discoid chamber. Vessels are illuminated by fluorescence. Fluorescent objects may range from molecules small enough to trace nutrient exchange through capillary walls to microspheres large enough to observe as discrete points, but small enough to not significantly alter vessel fluid mechanics.

If transmitted light is not required, the chamber may be redesigned to extend no further than the nearest medullary canal. Such a short chamber may be implanted nearer the joint and possibly serve in a model for ION of the knee. Unless an endoscopic form of the chamber were developed, its implantation near the femoral head would be problematic because of musculature surrounding the femur. The chamber is designed for chronic studies (18 months has been achieved). Thus, it would be suitable for experiments testing hypotheses about the effects of long-term exposure to an agent on microcirculation. Fluorescent dyes for bone could be added to evaluate its growth and viability.

The reader is encouraged to "google" "intravital microscopy" to gain an appreciation of the current use of this technique in microcirculation research, particularly in the area of cancer.

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