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REVIEW

Anti-cancer therapies targeting the tumor stroma

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Abstract For anti-tumor therapy different strategies have been employed, e.g., radiotherapy, chemotherapy, or immunotherapy. Notably, these approaches do not only address the tumor cells themselves, but also the tumor stroma cells, e.g., endothelial cells, fibroblasts, and macrophages. This is of advantage, since these cells actively contribute to the proliferative and invasive behavior of the tumor cells via secretion of growth factors, angiogenic factors, cytokines, and proteolytic enzymes. In addition, tumor stroma cells take part in immune evasion mechanisms of cancer. Thus, approaches targeting the tumor stroma attract increasing attention as anti-cancer therapy. Several molecules including growth factors (e.g., VEGF, CTGF), growth factor receptors (CD105, VEGFRs), adhesion molecules ($\alpha v\beta 3$ integrin), and enzymes (CAIX, FAPα, MMPs, PSMA, uPA) are induced or upregulated in the tumor microenvironment which are otherwise characterized by a restricted expression pattern in differentiated tissues. Consequently, these molecules can be targeted by inhibitors as well as by active and passive immunotherapy to treat cancer. Here we discuss the results of these approaches tested in preclinical models and clinical trials.

Keywords Angiogenesis · Endothelial cells · Extra-cellular matrix · Fibroblasts · Invasion · Therapy

Abbreviations

CAIX	Carbonic anhydrase IX
CAF	Cancer-associated fibroblast
CTGF	Connective tissue growth factor
DPPIV	Dipeptidyl peptidase IV
ECM	Extra-cellular matrix
FAPα	Fibroblast activation protein α
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
(N)SCL(C)	(Non) small cell lung (cancer)
PAGRIT	Pretargeted antibody guided radioimmunotherapy
PSMA	Prostate-specific membrane antigen
SIP	Small immunoprotein format
TAM	Tumor-associated macrophage
TEC	Tumor endothelial cell
TEM	Tumor endothelial marker
TIMP	Tissue inhibitor of metalloproteinases
uPA(R)	Urokinase plasminogen activator (receptor)
VEGF(R)	Vascular endohelial growth factor (receptor)

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Introduction

The development and progression of cancer depends on genetic and epigenetic alterations in the transformed cells. However, many steps in cancerogenesis, e.g., proliferation, invasion, angiogenesis, and metastasis are promoted by microenvironmental factors such as growth factors and proteolytic enzymes produced by stromal cells (Fig. 1). Indeed, the reciprocal interactions between tumor and tumor stroma cells, i.e., cancer-associated fibroblasts (CAFs), tumor endothelial cells (TECs), and tumor-associated macrophages (TAMs), result in tumor progression. CAFs are reactive fibroblasts with a distinctive phenotype as compared to quiescent fibroblasts in differentiated adult

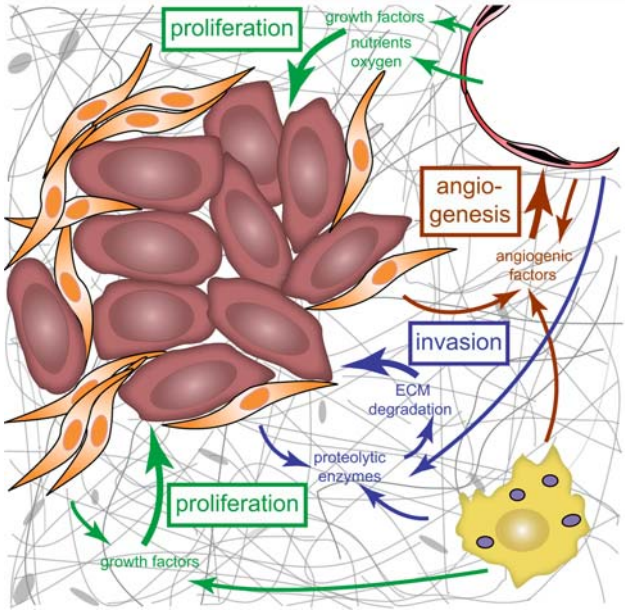

Fig. 1 Influence of the tumor microenvironment on tumor development. Tumor stromal cells, e.g., cancer associated fibroblasts (CAFs), tumor endothelial cells (TECs), and tumor-associated macrophages (TAMs) express growth factors sustaining tumor growth, angiogenic factors promoting angiogenesis, and proteolytic enzymes catalyzing the degradation of the ECM facilitating tumor cell invasion and finally metastasis. Tumor cells are depicted in *brown*, CAFs in *orange*, TECs in *red*, and TAMs in *yellow*

tissue. They are present in the close vicinity of tumor cells and enhance tumor growth by secreting growth factors such as transforming growth factor β (TGF-β), matrix degrading enzymes, e.g., matrix metalloproteinases (MMPs), and angiogenic factors, like vascular endothelial growth factor (VEGF). TECs also support the neoplastic cells by production of growth factors, but more importantly they are mandatory for tumor hem- and lymphangiogenesis. TAMs represent a major component of the leukocyte infiltrate in solid tumors. They secrete both growth factors and proteolytic enzymes, moreover, they generate an immune privileged state of the tumor microenvironment. Thus, both tumor stromal cells and their products are promising targets for cancer therapy.

Tumor stroma cells differ from their normal counterparts by upregulation or induction of various molecules (Table 1). Their upregulation is selective for the tumor microenvironment and occurs in a broad spectrum of solid tumors. Corresponding to the various interactions of tumor and stroma cells anti-stromal therapies fall into different categories. To date primarily molecules contributing to angiogenesis, e.g., vascular endothelial growth factor (VEGF) and its receptors have been targeted. Moreover, proteins involved in remodeling of the extra-cellular matrix (ECM) (e.g., MMPs, uPA/PAR system) have been silenced or functionally inhibited. However, due to redundancy of signals the inhibition of one factor or even a group of molecules is often not sufficient to significantly influence tumor growth and progression. Therefore, alternative strategies aim at modulating the tumor microenvironment by eliminating the stromal cells, e.g., by antibodies or by cytotoxic effector cells. Besides targeting stroma cells immunotherapeutically [41], new strategies are currently in preclinical or clinical testing. In the following sections we describe a selection of these approaches for the treatment of cancer (Table 2).

Strategies to prevent tumor angiogenesis

Angiogenesis, the development of new blood vessels, plays a central role in the pathogenesis of cancer. It is crucial for maintaining the supply of oxygen and nutrients and the removal of waste products to support tumor growth beyond a few mm^3 (Fig. 1). Consequently, TECs play a major role in tumor progression. Their growth is sustained by autocrine and paracrine secretion of growth factors by stroma and tumor cells, respectively. It should be further noted, that TECs are essential to maintain the oncogenic phenotype of tumor cells by production of several soluble factors. Consequently, growth and angiogenic factors, e.g., VEGF or TGF-β, and their receptors as well as TECs themselves have been targeted for cancer therapy (Fig. 2).

The potent pro-angiogenic growth factor VEGF and its tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) play a fundamental role in tumor vessel formation. VEGF is abundantly expressed in most tumors due to hypoxic conditions, acidic pH, hypoglycemia and several inducing cytokines, as well as genetic and epigenetic changes in tumor cells. Its expression, however, is not restricted to the tumor cells. VEGF is also expressed in CAFs, TECs, TAMs, and the inflammatory infiltrate (Table 1). Its expression is associated with increased angiogenesis, tumor growth, invasion, metastasis, and a greater risk of recurrence. Hypoxia and increased VEGF levels enhance expression of VEGFR-1 and VEGFR-2 on the vascular endothelium of the tumor, but both receptors are also expressed on tumor cells such as melanoma, thyroid, ovarian, breast carcinoma or cutaneous T cell lymphoma. In consequence, several VEGF- and VEGFR-antagonists have been developed and several of these inhibitors have already entered the clinic (Table 2; Fig. 2a).

In this regard the most advanced therapy consists of the recombinant humanized anti-VEGF mab Bevacizumab. It neutralizes VEGF, thereby blocking signal transduction mediated by VEGFR-1 and -2. Indeed, more then 200 clinical studies with Bevacizumab are currently being initiated or in progress (http://www.clinicaltrials.gov).

Table 1 Target molecules expressed by tumor stroma cells

Target	Expression		Function
	Tumors	Cells	
VEGF	Universal (e.g., breast, colorectal, renal cell, NSCL, hepatocellular cancer)	CAFs, TAMs, inflammatory infiltrate, tumor cells	Angiogenic factor
VEGFR-1 (Flt-1), VEGFR-2 (KDR, Flk-1)	Melanoma, thyroid, ovarian, breast carcinoma, cutaneous T cell lymphoma	TECs, tumor cells	VEGF receptors
CD105 (endoglin)	Breast, prostate, gastric, colorectal, renal cell, cervix, and endometrial carcinoma, melanoma, glioblastoma	TECs, activated monocytes, differentiated macrophages, early B cells, erythroid precursor cells, follicular DCs, fibroblasts, melanocytes, heart smooth muscle cells, trophoblasts	Accessory TGF-β superfamily receptor
PSMA	Prostate, breast, renal cell, bladder, NSCL, and rectal carcinoma, glioblastoma multiforme, melanoma, soft tissue sarcoma	TECs, tumor cells (prostate cancer), epithelia of prostate, urinary bladder, esophagus, stomach, large and small intestine, colon, kidney tubules, and liver	Glutamate carboxypeptidase
TEM8 (ATR)	Breast, esophagus, lung, bladder, and colorectal cancer	TECs, colorectal cancer cells	Adhesion molecule
CTGF (CCN2)	Glioblastoma, prostate, mammary carcinoma, pancreatic adenocarcinoma, lung adenocarcinoma, NSCL cancer, esophageal cancer, breast carcinoma, melanoma, chondrosarcoma, oral squamous cell cancer, acute lymphoblastic leukemia, rhabdomyosarcoma, hepatocellular carcinoma/hepatoma, colorectal cancer	CAFs, TECs, vascular smooth muscle cells, cancer cells, neural, and some epithelial cell types in diverse tissues, pancreatic stellate cells	Growth factor
$\alpha v\beta 3$ integrin	Breast, prostate, renal cell cancer, glioma, and melanoma	TECs	Adhesion molecule
MMPs	Breast, prostate, gastric, colorectal, pancreatic, NSCL, SCL, ovarian cancer, melanoma	CAFs, TECs, TAMs, tumor cells	Endopeptidases
uPa	Breast, gastrointestinal, renal, prostate, ovarian cancer	CAFs, TAMs, (TECs, tumor cells)	Serine protease
uPAR (CD87)	Breast, gastrointestinal, renal, prostate, ovarian cancer	Tumor cells, (TAMs)	uPa receptor
Tenascin-C	Breast, uterus, ovaries, lung, prostate, pancreas, colon, stomach, oral squamous cell, liver, and Merkel cell carcinoma, glioma, astrocytoma, lymphoma, sarcoma, melanoma	CAFs, tumor cells	Binds to cell surface receptors (integrins, proteoglycans, cell adhesion molecules of the immunoglobulin family) and ECM components (heparin, fibronectin, and collagen)
FAPα (seprase)	Breast, gastric, colon, colorectal, gastric, pancreas, prostate, bladder, renal, head and neck, ovarial carcinoma, NSCLC melanoma	CAFs, TECs, tumor cells	Serine protease, dipeptidyl peptidase
CAIX (MN, G250)	Renal cell, colorectal, cervix, NSCL, bladder, kidney cancer	CAFs, tumor cells, normal gastric epithelium	Carbonic anhydrase

Antigens expressed on tumor stroma cells, i.e., CAFs, TECs, and TAMs, are listed with their expression pattern and function

Table 2 Targeting the tumor stroma for anti cancer therapy

Substance	Target	Structure	Mechanism of action	State	References
VEGF/VEGFR					
Bevacizumab	VEGF	Recombinant humanized anti-VEGF mab	Neutralization of VEGF	Phase II, approved by the FDA for metastatic colorectal cancer	[42, 44–46, 107]
IMC-1C11	VEGFR-2	Antibody		Clinical (phase I)	[81]
Adsflt	VEGF	Soluble VEGFR-1 receptor	Interception of VEGF	Preclinical	[53]
ExFlk.6His	VEGF	Soluble VEGFR-2	Interception of VEGF	Preclinical	[61]
VEGF-Trap	VEGF	Decoy receptor based on VEGFR-1 and VEGFR-2 fused to an Fc segment of IgG1	Interception of VEGF	Preclinical	[21]
Flt1-intraceptors	VEGF	Transfection with plasmids encoding VEGFR-1 coupled with an endoplasmatic retention signal	Intracellular interception of VEGF (reduction of secretion)	Preclinical	[96]
RPI.4610 (angiozyme)	VEGFR-1	Anti-VEGFR-1 ribozyme	downregulation of expression	Clinical (phase II)	[49] NCT00021021
CD105 (endoglin)					
SN6j	CD105	Antibody		Preclinical	[99]
c-SN6j	CD105	Humanized SN6j antibody		Preclinical	[95]
scDb EDGCD3	CD105	CD3/CD105-bispecific single-chain diabody	Recruitment of CTL to TEC	Preclinical	[54]
PSMA					
MDX-070	PSMA	Fully human antibody		Clinical (phase II)	Medarex
MLN2704	PSMA	De-immunized antibody conjugated to maytansinoid 1 (DM1)	Depolymerization of mirotubuli	Clinical (phase I/II)	[37] Millennium Pharmaceuticals Inc., NCT00052000, NCT00070837, NCT00058409
A5-PE40	PSMA	Recombinant Pseudomonas exotoxin A (PE40) single chain antibody	ADP ribosylation of eukaryotic elongation factor 2	Preclinical	[103]
PSMA ADA	PSMA	Monomethylauristatin E conjugated to a human monoclonal antibody to PSMA	Inhibition of tubulin polymerization	Peclinical	[66] Progenics Pharmaceuticals, Inc
HuJ591	PSMA	Humanized antibody, partly labeled with radioactive isotopes (e.g., 177Lu-HuJ591)	ADCC radioimmunotherapy	Clinical (phase I and II)	[71] NCT00195039
	PSMA	Nanoparticle-aptamer bioconjugate	Targeting of docetaxel to PSMA expressing cells	Preclinical	[29]
TEM8 (ATR = the anthrax-toxin-receptor)					
LeTx	TEM8 positive cells	Anthrax toxin	Targeting anthrax toxin to tumor endothelial and tumor cells	Preclinical	[15]
	TEM8 positive cells	Anthrax toxin fusion proteins	Active toxin released by cleavage by MMPs	Preclinical	[62]
CTGF					
FG-3019	CTGF	Fully human antibody	Blocking antibody	Preclinical	[5, 27]

Table 2 continued

Substance	Target	Structure	Mechanism of action	State	References
DN-9693	CTGF	Inhibitor	Prevents stabilization of CTGF mRNA	Preclinical	[51]
αvβ3 integrin					
Cilengitide (EMD 121974)	αv integrins	Cyclic RGD peptide	Inhibition	Clinical phase II	[6] Merck KgaA, NCT00112866, NCT00085254, NCT00121238, NCT00103337, NCT00077155
EMD270179 + SU5416	αv integrins	Cyclic RGD peptide + VEGFR2 antagonist	Inhibition of αv integrin and VEGFR2 signaling	Preclincial	[98]
S247	αv-integrins	Peptidomimetic	Antagonist	Preclincial	[1, 86]
HPMA-RGD4C, HPMA-RGDfK	αvβ3 integrin positive cells	^{111}In, ^{99m}Tc or ^{90}Y-labeled peptide HPMA polymer	Radiotherapy	Preclinical	[72, 73]
	αvβ3 integrin positive cells	RGD peptide coupled to immunoliposome	Delivery of combretastatin	Preclinical	[79]
αv-siRNA	αv integrins	Liposome encapsuled siRNA	Downregulation of expression	Preclinical	[10]
tTF-RGD	αv integrins positive cells	Truncated tissue factor fused to GRGDSP	Induction of thrombosis in the tumor	Preclinical	[47]
RGD-4C AAVP-HSVtk	αv integrins positive cells	Adeno-associated virus bacteriophage hybrid vector encoding HSVtk (herpex simplex virus thymidin kinase)	Targeting of HSVtk to αv-integrin positive cells, activation of prodrug GCV	Preclinical	[36]
Vitaxin MEDI-523	αvβ3 integrin	Humanized antibody	Blocking antibody	Clinical (phase I/II)	[35,78] MedImmune, Inc
Abergrin MEDI-522	αvβ3 integrin	Humanized antibody	Blocking antibody	Clinical (phase II)	[69] NCT00049712, NCT00111696, NCT00066196, NCT00284817, NCT00072930, MedImmune, Inc
MMPs					
Marimastat (BB-2516)	Broad spectrum (MMP-1, -2, -3, -7, -9, and -12)	Collagen-based, peptidomimetic hydroxamate	Inhibition of activity (mimics the structure of collagen and reversibly chelates the zinc atom in the MMP's active site)	Clinical (phase III)	[16, 17, 94, 97]
Tanomastat (BAY 12-9566)	MMP-2, -3, and -9	Non-peptidic biphenyl MMP inhibitor	Inhibition of activity	Clinical (phase III)	[39, 74, 75]
Rebimastat (BMS-275291)	Broad spectrum	Non-peptidic MMP inhibitor	Inhibition of activity	Clinical (phase III)	[59, 70, 88]
Prinomastat (AG3340)	MMP-2, -3, -9, -13, -14 collagenase III	Non-peptidic MMP inhibitor	Inhibition of activity	Clinical (phase III)	[11] Agouron Pharmaceuticals Inc
Metastat (CMT-3, Col-3)	MMP-1, -2, -8, -9, and -13	Tetracycline	Inhibition of activity	Clinical (phase II)	[26] NCT00020683
TIMPs	MMPs	Adenoviral expression of TIMPs	Inhibition of activity	preclinical	[4, 18]
Ro-28-2653	MMP-2, -9 and -14	Pyrimidine-trione derivative	Inhibition of activity	Preclinical	[2, 67]

Table 2 continued

Substance	Target	Structure	Mechanism of action	State	References
SB-3CT	MMP-2, -9	Mechanism-based inhibitor	Inhibition of activity	Preclinical	[56]
uPA/uPAR					
PAI-2	uPA	213Bi labeled PAI-2	Local radiotherapy	Preclinical	[83]
	uPA or uPAR	siRNA constructs	Downregulation of expression	Preclinical	[52, 82]
mhATF-BPTI	uPAR	Receptor binding part of uPA linked to the plasmin inhibitor BPTI (aprotinin)	Inhibition of plasmin activity	Preclinical	[58]
uPA-UT1	uPAR	uPAR binding part linked to urinary trypsin inhibitor (UT1)	Inhibition of plasmin activity	Preclinical	[50]
DTAT	uPAR positive cells	Catalytic portion of diphtheria toxin (DT) fused to amino-terminal (AT) fragment of uPA	Diphteria toxin binding to uPAR	Preclincial	[101]
	uPA/uPAR positive cells	TNFα prodrugs comprise either an uPA-selective or a dual uPA-MMP-2-specific linker	TNFα activation by uPA	Preclinical	[32]
PrAg-U2 + FP59 or PrAg-U2-R200A + PrAg-L1-I210A + FP59	uPA/MMP expressing cells	Anthrax toxin lethal factor	Toxin activation by uPA or uPA and MMP	Preclinical	[63, 90]
Å6	uPA/uPAR interaction	uPA-derived peptide	Noncompetitive antagonist	Clinical (phase II)	[9] Ångstrom Pharmaceuticals, NCT00083928
Tenascin-C					
81C6	Tenascin-C	Murine ^{131}I-labeled antibody	Radioimmunotherapy	Clinical (phase I, phase II)	[85, 89] NCT00002753, NCT00002752
ST2146 and/or ST2485	Tenascin-C	^{125}I-labeled biotinylated antibody	PAGRIT (Pretargeted Antibody Guided Radioimmunotherapy)	Preclinical	[80]
TTA1	Tenascin-C	Aptamer		Preclinical	[38]
SIP (F16)	Tenascin-C	125I labeled human SIP antibody		Preclinical	[14]
ATN-RNA	Tenascin-C	siRNA	Inhibition of expression	Clinical	[108]
FAPα (seprase)					
PT-100 (Val-boro-Pro)	FAPα, DPPIV	Small molecule inhibitor (amino boronic dipeptide)	Inhibition of activity	Clinical (phase I)	[3, 76]
Gly-Pro^{P}-$(OPh)_2$, Tyr-Pro^{P}-$(OPh)_2$	FAPα, DPPIV	Small molecule inhibitors	Inhibition of activity	Preclinical	[33]
Ac-Gly-BoroPro	FAPα	Small molecule inhibitor	Inhibition of activity	Preclinical	[28]
	FAPα	Antibody	Inhibition of activity	Preclinical	[24]
Sibrotuzumab (BIBH 1)	FAPα	Antibody (humanized F19 antibody)		Clinical (phase II)	[40, 93]
	FAPα	FAPα-CD3-bispecific single chain antibody	Recruitment of CTLs to the tumor stroma	Preclinical	[104]
	FAPα	Anti-FAPα-TNFα fusion protein	Binding of the soluble construct to the cell surface mimicks membrane-integrated TNFα signaling	Preclinical	[8]

Table 2 continued

Substance	Target	Structure	Mechanism of action	State	References
	FAPα	Human tissue factor fused with a single chain anti-FAPα antibody	Coagulation	Preclinical	[87]
sc40-FasL	FAPα	FAPα single chain antibody FasL fusion protein	Induced apoptosis of FAPα positive cells	Preclinical	[91]
CAIX (MN, G250)	CAIX (and CAXII)	Sulfonamides	Inhibition of activity	Preclinical	[7, 22, 77, 100]
Rencarex WX-G250	CAIX	Antibody	ADCC	Clinical (phase III)	[12, 13] NCT00209183, NCT00087022
cG250-131I, cG250-In111, cG250-Lu177	CAIX	Radioactive labeled anti CAIX antibody	Radioimmunotherapy	Clinical (phase I/II)	[19, 20] NCT00003102, NCT00142415
	CAIX expressing cells	Adoptive transfer of autologous T lymphocytes expressing a single-chain antibody directed to CAIX	Recruitment of T cells to CAIX expressing cells	Clinical (phase I)	[57]

A selection of cancer therapeutica targeting the tumor stroma in clinical or preclinical development is listed with their names, the substance class, their mechanism of action, and their current state of development

Bevacizumab significantly inhibits growth of different tumor types (e.g., colorectal, renal cell, NSCL cancer) already as single-agent therapy, which can be additively or synergistically enhanced by chemo- or radiotherapy [42, 107]. Hence, a combined regimen has been approved by the FDA [46]. Alternatively VEGF signaling may also be inhibited by receptor-specific antibodies. Anti-VEGFR-2 antibodies act synergistically with chemo- or radiation therapy [48, 55]. The growth of established human tumor xenografts of different origin was inhibited due to decreased angiogenesis, reduced tumor cell proliferation and enhanced tumor cell apoptosis by treatment with antibodies. The VEGFR-2 antibody IMC-1C11 is currently being tested in clinical studies [81]. Soluble receptors intercept the growth factor before it can bind to the cell surface receptor. To this end, an adenoviral vector encoding the extra-cellular domain of VEGFR-1 showed promising activity in different tumor models and a reduction of vascular density was also reached by soluble VEGFR-2 [53, 61]. A soluble receptor comprising portions of VEGFR-1 and -2 demonstrated an even higher efficacy [21]. Another approach to reduce VEGF level in vivo has been tested in corneal neovascularization but may be used in anti-tumor therapy as well; transfection with plasmids encoding VEGFR-1 coupled with an endoplasmatic retention signal reduced injury-induced VEGF secretion [96]. Other approaches investigated include VEGF antisense oligonucleotides or ribozymes. In this regard, VEGFR-1 ribozymes were well tolerated in patients and objective tumor responses were reached in combination with carboplatin and paclitaxel [49].

An endothelial cell surface molecule of particular interest for vascular targeting is CD105 (endoglin), a 95 kDa cell surface protein expressed as a homodimer. CD105 functions as an accessory protein for kinase receptor complexes of the TGF-β superfamily and modulates TGF-β signaling, i.e., it antagonizes the inhibitory effects of TGF-β1, e.g., the suppression of growth, migration and capillary tube formation. In addition, CD105 has anti-apoptotic effects under hypoxic conditions. CD105 expression is not restricted to TECs (Table 1). However, only on TECs CD105 is expressed in significant amounts. CD105 is upregulated by TGF-β2 and hypoxia. Its expression has been described for a multitude of solid tumors (Table 1). Expression of CD105 is correlated with vascular density and poor prognosis. These properties render CD105 an attractive target for therapeutic interventions (Table 2; Fig. 2a). In preclinical studies, CD105-specific antibodies—either alone or conjugated with different effector molecules—react only weakly or not at all with quiescent endothelium but specifically bind to proliferating endothelial cells during tumor angiogenesis [31]. Correspondingly, side effects were low [68]. To test

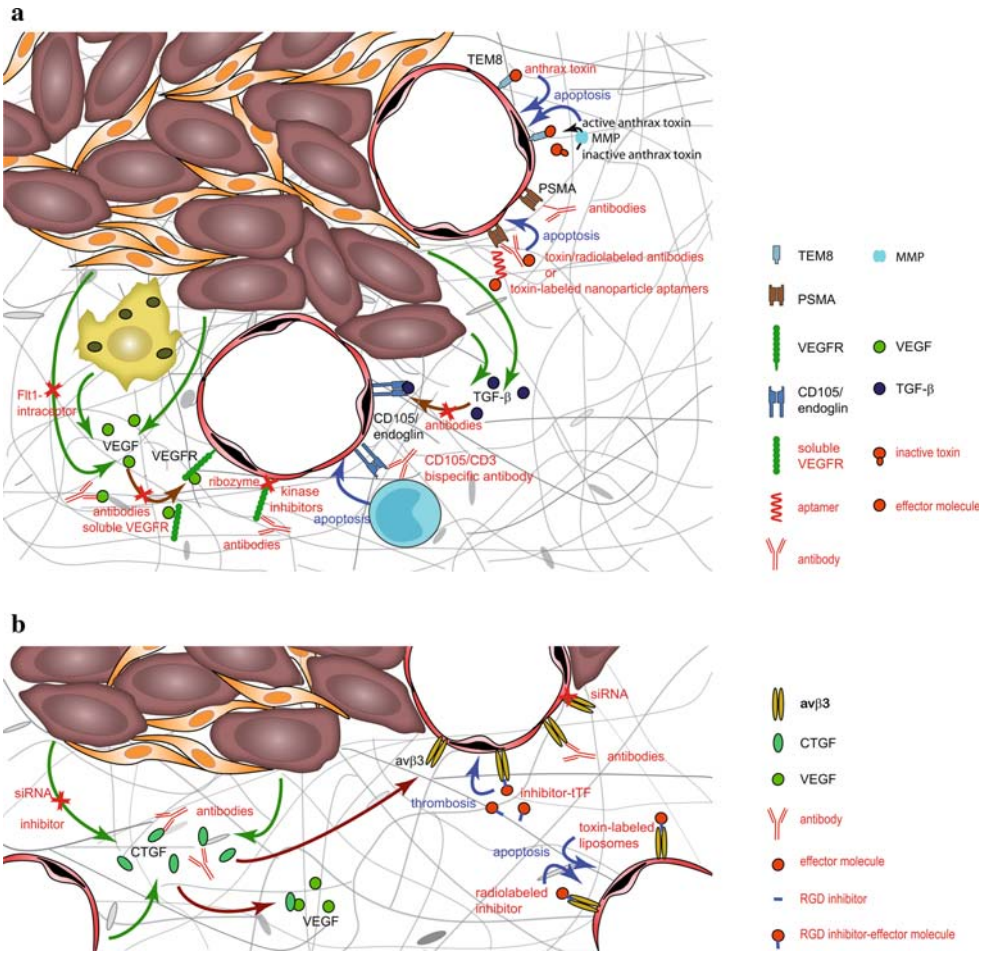

Fig. 2 Targeting endothelial cells for anti cancer therapy. A variety of proteins are targeted to prevent angiogenesis. **a** The function of molecules expressed on endothelial cells, e.g., VEGFRs, PSMA, TEM8, and CD105 is inhibited either directly by inhibitors and antibodies or alternatively their expression is prevented by ribozymes. In addition, they are exploited to eliminate tumor endothelial cells, e.g., by bispecific antibodies or specific expression or accumulation of toxins at endothelial cells. **b** CTGF is targeted by siRNA, inhibitors or antibodies. $\alpha v\beta 3$ integrin expression is prevented by siRNA, its function is inhibited by antibodies, and effector molecules are targeted to $\alpha v\beta 3$ integrin positive cells using inhibitors binding to $\alpha v\beta 3$ integrin. Expression/secretion of target molecules is indicated by *green arrows*, binding to receptors by *brown arrows*, and their effects by *black arrows*. Therapeutical substances are depicted in red and their effects by *blue arrows*

the effect of CD105-specific antibodies on human blood vessels, chimeric vasculature of human and murine origin was generated by transplantation of human foreskin onto immunodeficient mice: The growth of tumors induced in these tissues is reduced by anti-CD105 antibodies [99]. Recently, the safety of a humanized anti-CD105-antibody has been demonstrated in a primate model [95]. Lysis of endothelial cells is also achieved by retargeting CTL to CD105-positive cells by means of CD3/CD105-bispecific single-chain diabodies in vitro [54].

Prostate-specific membrane antigen (PSMA) is a 110 kDa glycoprotein with glutamate carboxypeptidase activity. PSMA is the prototype cell surface marker of prostate cancer as it is expressed on malignant prostate cancer cells. In addition, PSMA is expressed on some normal tissues (Table 1). Nevertheless, it has been widely used as therapeutic target in preclinical prostate cancer models [29, 37, 66]. PSMA is also abundantly expressed on TECs of many solid tumors (Table 1). Thus, targeting PSMA seems to be a feasible therapeutic option for different kinds of tumors apart from prostate carcinoma (Table 2; Fig. 2a). Currently, the fully human anti-PSMA antibody MDX-070 is tested in phase II trial for prostate cancer (Medarex).[1] While PSMA-specific antibodies alone often did not inhibit tumor growth, immunotoxins significantly delayed tumor progression in preclinical models [37]. A single-chain antibody fused to Pseudomonas exotoxin A specifically binds to PSMA-positive prostate cancer cells and reduces their viability [103]. Auristatin conjugated to a human monoclonal antibody to PSMA enhanced survival in a murine xenograft prostate model [66]. Furthermore, in clinical studies PSMA-specific antibodies labeled with radioactive isotopes induced objective responses [71]. Similarly, docetaxel is targeted to PSMA expressing cells by nanoparticle-aptamer bioconjugates which exhibited anticancer efficiency in a xenograft model [29].

TEM8 (ATR = the anthrax-toxin-receptor) is a transmembrane receptor belonging to the tumor endothelial markers (TEMs). It binds to the collagen subunit $\alpha 3$ (VI) but its cellular function is still unknown. TEM8 is strongly

[1] http://library.corporate-ir.net/library/63/639/63952/items/176589/6_MEDX120905_Nichol1.pdf.

expressed on vascular endothelial cells during embryo- and carcinogenesis. Its expression on endothelial cells is associated with enhanced cell–matrix-interaction and migration. TEM8 protein has been found on TECs of different cancer types but also on cancer cells themselves (Table 1). Recently, TEM8 mRNA has been detected in a cell population, coexpressing DC and endothelial markers, capable of generating functional blood vessels; thus, indicating that TEM8 has a function during vasculogenesis [25]. Since TEM8 functions as a docking protein for the Bacillus anthracis toxin, its upregulation on tumor endothelial cells has been exploited to target anthrax toxin to tumor endothelial and tumor cells [15] (Table 2). Fusion proteins that have been designed to allow the active toxin to be released by cleavage by metalloproteinases which are present in the tumor microenvironment, have demonstrated therapeutic activity, thereby illustrating the feasibility of an anti-TEM8 therapy (Fig. 2a) [62].

The connective tissue growth factor (CTGF, CCN2) is a 38 kDa extra-cellular matricellular protein that belongs to the CCN cysteine-rich family of proteins. CTGF has multiple functions. It interacts with integrin receptors, including $\alpha v\beta 3$, and several growth factors, e.g., TGF-β. In addition, it serves as biostore for angiogenic factors such as VEGF. The VEGF signaling can be restored by cleavage of CTGF by MMP. CTGF is upregulated by estrogen, TGF-β, EGF (epidermal growth factor), PDGF (platelet-derived growth factor), VEGF and hypoxia, for example. CTGF modulates cell adhesion, migration, proliferation, chemotaxis, apoptosis, ECM deposition, and angiogenesis and is important in reproduction, embryonic development, wound repair, inflammation, fibrosis disorders, and tumorigenesis. CTGF expression levels are elevated in a multitude of cancers where it is produced by several types of stromal cells including TECs, vascular smooth muscle cells, and CAFs, and can also be expressed in cancer cells (Table 1). This over-expression correlates with tumor stage and/or prognosis [105, 106]. Moreover, addition of CTGF to the medium increases proliferation and invasiveness of pancreatic cancer cells in vitro [5]. Nevertheless in some cancers such as esophageal, NSCL, and colorectal carcinoma high CTGF expression has been associated with improved survival, reduced angiogenesis, and metastasis. To this end, transfection of lung adenocarcinoma cell lines with CTGF-over-expressing vectors reduced VEGF expression, microvessel density, tumor growth, and metastasis in xenograft models [23]. As CTGF frequently is upregulated in fibrosis, CTGF mainly has been targeted in fibrotic diseases [60]. However, these approaches may also be effective in cancer. A CTGF-specific antibody, FG-3019, is currently being tested in a preclinical pancreatic cancer model (FibroGen, Fig. 2b), where it decreases tumor growth, metastasis, as well as angiogenesis and enhances the therapeutic effect of gemcitabine [5, 27]. Alternatively, stabilization of CTGF mRNA induced by VEGF can be inhibited by the angiogenesis inhibitor DN-9693 (Table 2) [51].

On TECs several integrins, e.g., $\alpha v\beta 3$ and $\alpha 5\beta 1$, are upregulated. These cell surface adhesion molecules are involved in cell growth, migration, tumor invasion, proliferation, metastasis, angiogenesis, and survival. The integrin $\alpha v\beta 3$, is minimally expressed in mature vasculature and found on new blood vessels as well as the surface of many solid tumors (Table 1). Its expression is associated with an aggressive tumor phenotype. In consequence, it represents a target for anti-cancer therapy (Table 2; Fig. 2b). Several antagonists, including antibodies, Arg–Gly–Asp (RGD) peptides, and recombinant proteins induce tumor regression by reduction of angiogenesis and enhanced apoptosis of TEC [6, 84]. The RGD peptide cilengitide, for example, is tested in several clinical phase II studies for the treatment of glioblastoma (NCT00112866, NCT00085254) and prostate cancer (NCT00121238, NCT00103337) alone or in combination with radiation therapy. Similarly, the RGD peptidomimetic S247 and the cyclic peptides RGD4C and RGDfK decrease angiogenesis, tumor growth, and metastasis and improve survival in mice [86, 98]. In addition, radiosensitivity of endothelial cells is synergistically enhanced [1]. Recently, efforts were made to improve the affinity and pharmacological properties of these antagonists, e.g., by binding them to HPMA [*N*-2-hydroxypropyl) methacrylamide] polymers thereby enhancing tumor to background ratios of the antagonist's distribution in the body [72, 73]. The RGD4C peptide has also been chosen to target αv integrin positive cells by integration of this peptide into virus capsids or liposomes. An adeno-associated virus bacteriophage hybrid vector encoding the herpex simplex thymidine kinase that activates the prodrug ganciclovir proved effective in preclinical models [36]. Alternatively, combretastatin has been targeted to tumors by liposomes linked to RGD peptides which delayed tumor growth in a melanoma model [79]. Such liposomes have also been used to deliver siRNAs resulting in reduced prostate cancer bone metastases [10]. Fusion of truncated tissue factor to an αv-targeting peptide induced thrombosis in tumor vessels, thereby inhibiting growth or inducing even regression of tumors in adenocarcinoma, melanoma and fibrosarcoma models [47].

In murine models, antibodies to $\alpha v\beta 3$ integrin block angiogenesis, tumor formation, and metastasis. In clinical studies the $\alpha v\beta 3$ inegrin-specific antibody Vitaxin (MEDI-523) proved to be safe, however, only limited efficacy was observed, e.g., disease stabilization in a leiomyosarcoma patient [35, 78]. Vitaxin is now being investigated in phase II trials in melanoma and prostate cancer (MedImmune, Inc.). The antibody Abergrin (MEDI-522), that has a

greater affinity to $\alpha v\beta 3$ integrin, was similarly well tolerated in patients and is currently being tested in prostate (NCT00072930) and colorectal cancer (NCT00284817) as well as metastatic melanoma alone or in combination therapy (NCT00111696, NCT00066196, MedImmune, Inc.) [69].

Interference with the remodeling of the extra-cellular matrix

Malignant progression of tumors is a complex process in which cells need to gain the ability to invade into surrounding tissues. For this purpose, cancer cells need to attach to, degrade and invade the ECM. These events are followed by invasion of the wall of blood or lymphatic vessels; after transport within the blood or lymph circulation, extravasation of cancer cells again involves degradation of the basement membrane and the ECM. For all these steps structural changes of the ECM are essential. Several proteases are involved in these processes including the MMPs and the urokinase plasminogen activator (uPA).

MMPs are a family of membrane-anchored and secreted zinc-dependent endopeptidases. Collectively, they are capable of degrading all ECM and basement membrane components. Their activity is important for matrix remodeling in biological processes such as embryonic development, tissue regeneration, and wound healing. In the tumor micromilieu MMPs are frequently upregulated in response to growth factors, cytokines and membrane-anchored molecules. They are produced by both tumor and tumor stroma cells, predominantly at the invasive front of the tumor (Table 1). Indeed, MMP expression correlates with an invasive phenotype of tumor cells. MMPs have been implicated in tumor growth, invasion, metastasis, angiogenesis, and cancer cell survival/apoptosis as well as the clinical course in a variety of cancer types. Thus, several approaches to inhibit MMP expression and function have been tested for cancer therapy (Table 2; Fig. 3a). A wide variety of substances has been used to block MMP activity, e.g., hydroxamates, bisphosphonates, tetracyclines, and tissue inhibitors of metalloproteinases (TIMPs). The therapeutic efficacy of synthetic MMP inhibitors has been demonstrated in vitro and in preclinical studies. Moreover, several drugs have proceeded into the clinic and even advanced to phase III clinical trials [102]. Pseudopeptidic hydroxamate inhibitors, for example, bind the zinc atom in the catalytic domain of MMPs and have broad specificities. Batimastat (BB-94), iIlomastat (Galardin, GM-6001) and the orally bioavailable derivative marimastat (BB-2516) inhibit the activity of many MMPs. Marimastat clinically improved progression free and overall survival of patients with advanced gastric cancer [16]. However, neither progression free nor overall survival was prolonged in metastatic breast cancer or small cell lung (SCL) cancer patients after first line chemotherapy or in pancreatic carcinoma patients in combination with gemcitabine [17, 94, 97]. The dose-limiting side effect of marimastat treatment is musculoskeletal pain. The non-peptidic biphenyl MMP inhibitor tanomastat (BAY 12-9566) has higher specificity towards MMP-2, -3, and -9. It demonstrated anti-invasive, anti-metastatic, and anti-angiogenic activity in a variety of tumor models and has also been evaluated in clinical studies. Although clinical effects were seen in combination with chemotherapy toxicity limits its usage [74]. Moreover, phase III trials revealed that tanomastat had a negative impact on progression free and overall survival in ovarian, pancreatic, and SCL cancer [39, 75]. A small molecule, broad spectrum inhibitor that lacks musculoskeletal side effects is rebimastat (BMS-275291) [88]. However, it could not demonstrate any beneficial effect if used as monotherapy for treatment of patients with breast and colorectal cancer or in combination with paclitaxel and carboplatin in non small cell lung (NSCL) cancer [59, 70, 88]. A nonpeptidic inhibitor that has also been tested in phase III clinical studies is prinomastat (AG3340). It was examined in prostate and NSCL cancer in combination with chemotherapy, but it also lacked efficacy [11]. Another group of MMP inhibitors are chemically modified tetracyclines, like metastat (CMT-3, Col-3) which inhibits several MMPs, e.g., MMP-1, -2, -8, -9, and -13. In rodent models, it inhibits malignant cell invason and angiogenesis [65]. Metastat has been examined in a phase II clinical trial in patients with Kaposi sarcoma where it reduced MMP-2 and MMP-9 plasma levels [26]. Apart from these synthetic inhibitors endogenous MMP inhibitors have also been evaluated. Under physiological conditions these TIMPs are essential for the regulation of MMP activity. TIMPs have antitumoral function since they can inhibit cell invasion, tumor growth, metastasis and angiogenesis. This notion has been confirmed by adenoviral expression of TIMPs in tumor tissues [4, 18]. However, TIMPs do not act selectively on the MMPs promoting tumor growth. Furthermore, TIMPs are associated with the upregulation of the antiapoptotic protein Bcl-X_L [43].

As described above, despite some clinical effects of MMP inhibitors, the results of clinical trials have been rather disappointing. In most studies the MMP inhibitors had no beneficial and sometimes even adverse effects [11, 17, 94, 97]. This may partly be explained by the usage of broad spectrum inhibitors and the treatment of patients without knowledge about the expression of the respective MMPs in the individual tumors. MMPs are over-expressed in most tumors, however, their expression pattern varies in each tumor type and even in metastases of the same tumor

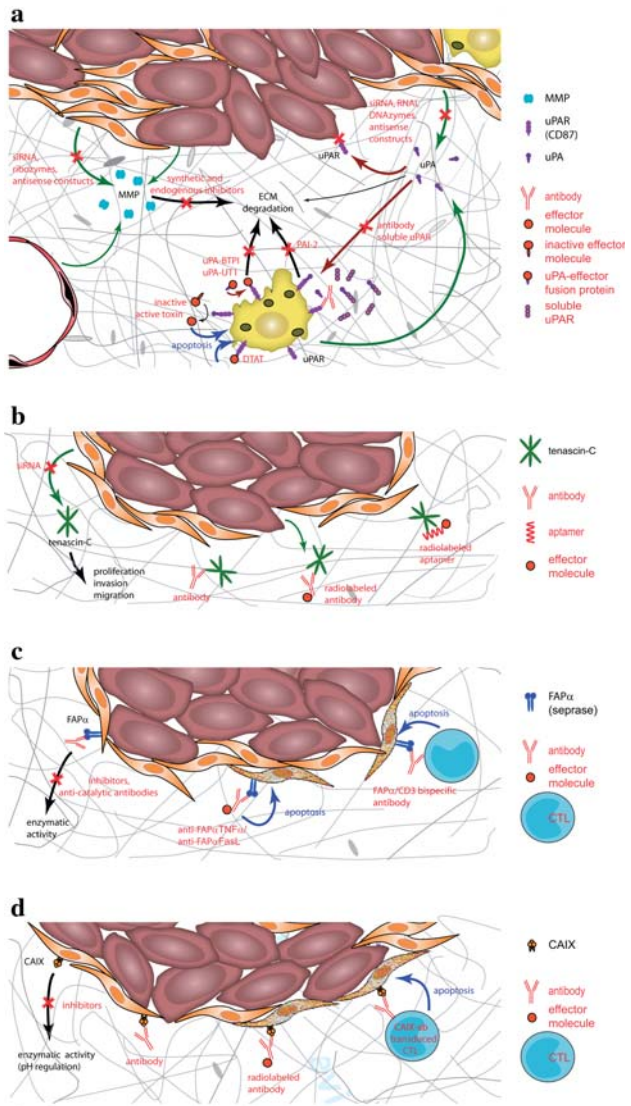

Fig. 3 Therapy approaches influencing the ECM degradation and targeting CAFs. **a** MMPs and the uPA/uPAR system influence the ECM degradation in the tumor microenvironment and promote invasion and metastasis of tumors. They are targeted by inhibiting their expression, e.g., by siRNA, antisense constructs, ribozymes or DNAzymes or their function by inhibitors, antibodies or soluble receptors, for example. In addition the uPA/uPAR system is used to induce apoptosis by recruitment or activation of toxins. **b** Tenascin expression is downregulated by siRNA approaches or targeted by antibodies or aptamers labeled with radioisotopes. Molecules expressed on the cell surface of CAFs, e.g., **c** FAPα and **d** CAIX, are also used to directly destroy these stroma cells mediated by antibodies (labeled with radioactive isotopes or different effector molecules) or cellular immune responses. Expression/secretion of target molecules is indicated by green arrows, binding to receptors by *brown arrows*, and their effects by *black arrows*. Therapeutical substances are depicted in *red* and their effects by *blue arrows*

to different organs. As broad spectrum inhibitors may have both inhibitory and promoting effects on tumor development and progression, more selective inhibitors for tumor-progression-associated MMPs have been developed. Ro-28-2653 inhibitis MMP-2, -9 and -14 and shows high anti-invasive activity in vitro. In preclinical models, it inhibits growth of MMP producing tumor cells as well as the tumor growth promoting effect of stromal cells [67]. The anti tumor effect is enhanced by combination with chemotherapy [2]. SB-3CT, a mechanism-based inhibitor that is formed only within the active site of the targeted enzymes, i.e., MMP-2 and MMP-9, inhibits liver metastases and increases survival in an aggressive murine model of T-cell lymphoma [56].

Several approaches to selectively inhibit the expression of a specific MMP have been examined, e.g., antisense constructs, RNA interference, and antisense ribozymes. However, even if single MMPs are targeted, the results are not always predictable as some MMPs may have a dual effect on tumor progression. Notably, MMPs cleave a diverse variety of substrates, resulting in opposing effects on tumor growth [30]. Indeed, activation of growth and angiogenic factors, and cleavage of FasL promote tumor development whereas the generation of angiogenesis inhibitors, e.g., angiostatin, arrestin, canstatin, endostatin, tumstatin, and ADAMTSs may inhibit tumor progression.

The serine protease urokinase plasminogen activator (uPA) converts plasminogen to plasmin which is able to degrade many ECM proteins such as collagen IV, laminin, and fibronectin either directly or through activation of other proteases. uPA, its inhibitor plasminogen activator inhibitor-1 (PAI-1), and its receptor uPAR (CD87) are involved in cell migration, tissue degradation, and angiogenesis under normal and pathological conditions. They do not only regulate ECM degradation but also cell adhesion and migration mediated by the interaction between uPAR and integrins, as well as ECM components, such as vitronectin. Over-expression of uPA and uPAR is a characteristic of various human cancers (Table 1). Whereas uPAR is particularly detected on tumor cells, uPA is mainly expressed by TAMs and CAFs, and to lesser extent on TECs. Studies in uPA knock out mice have confirmed the role of stromal uPA expression for tumor progression. Consequently, the uPA system has been addressed for anti-cancer therapy. Several approaches to interfere with the uPA system, ranging from neutralizing antibodies, soluble receptors, catalytically inactive uPA fragments, and synthetic peptides/peptidomimetics to antisense approaches, RNAi vectors, and DNAzymes have been tested to date (Table 2; Fig. 3a). The inhibitory effects of these substances have been conclusively demonstrated in preclinical models. Treatment with 213Bi labeled PAI-2 inhibits tumor growth in a pancreatic cancer xenograft model [83]. In murine tumor models intratumoral injection of small interfering RNA constructs for uPA and uPAR abrogated growth of established tumors [52, 82]. Adenoviral delivery of a chimeric protein composed of the receptor binding part of

uPA linked to the plasmin inhibitor BPTI (aprotinin) or the treatment with hybrid proteins consisting of the uPAR binding part and urinary trypsin inhibitor (UT1) also reduced tumor growth and metastases in tumor models [50, 58]. In addition, toxins or bioactive molecules binding to components of the uPA system or being activated after cleavage by uPA in the tumor microenvironment have been developed [32, 63, 90, 101]. In all these systems the selective targeting of cells expressing uPA/uPAR, was not associated with major side effects. Recently, an uPA-derived peptide, Å6, which in animal models reduced tumor growth, metastasis, and angiogenesis alone or in combination with other therapies was evaluated in a phase I clinical study in patients with gynaecologic, especially ovarian, cancer. This study demonstrated the safety of Å6. Moreover, this trial suggested some clinical potential [9]. Thus, Å6 is currently being tested in a phase II study (NCT00083928, Ångstrom Pharmaceuticals).

Tenascin-C is an extra-cellular hexameric glycoprotein expressed during embryonic development and adult tissue remodeling. Alternative splicing results in monomers of different sizes and the large isoform is virtually undetectable in differentiated tissues but is abundantly expressed in the stroma of most solid tumors (Table 1). Tenascin-C is expressed by both cancer cells and CAFs, especially at the invasive front. Tenascin-C is upregulated by hypoxia, mechanical stress, and various cytokines including TGF-β and CTGF. Tenascin-C promotes tumor growth by several mechanisms including enhanced proliferation, invasion, and migration, as well as escape from immune surveillance, and it is associated with a poor prognosis. Antibodies to tenascin-C delay tumor growth and induce apparent cures in xenograft models (Table 2; Fig. 3b). Treatment of patients with recurrent brain tumors, i.e., glioma and astrocytoma, with the I131 labeled antibody 81C6 demonstrated limited toxicity and induced a prolonged survival [85]. This antibody has also induced clinical responses in patients with non-Hodgkin lymphoma [89]. Additive tumor targeting was obtained by combining two different antitenascin antibodies [80]. In another approach, fluorescence and radiolabeled aptamers to tenascin-C were used in xenograft models for tumor imaging, suggesting that labeling of aptamer conjugates may be used to deliver radioisotopes and chemotherapeutics [38]. Affinity matured human antibodies to tenascin-C in a small immunoprotein format (scFv disulfide linked homodimer) have been generated and their specific accumulation in glioblastoma of a murine model has been demonstrated [14]. In glioblastoma multiforme and astrocytoma patients following brain resection tenascin-C specific RNA interference was applied to suppress tumor growth [108].

Fibroblast activation protein α (FAPα, seprase) is another enzyme participating in ECM degradation. FAPα is a serine protease with dual function, i.e., gelatinase/collagenase and dipeptidyl peptidase (N-terminal, post-prolyl amino peptidase) activity. Its natural substrate has not yet been identified. FAPα is expressed during embryonic development and wound healing. While it is hardly present in differentiated adult tissues, it is selectively induced on reactive stromal fibroblasts of more than 90% of common solid tumors. Besides its primary localization in fibroblasts, FAPα mRNA has also been detected in endothelial cells undergoing reorganization and capillary morphogenesis. Endothelial expression of FAPα protein in complexes with dipeptidyl peptidase IV (DPPIV) has been described in capillary-like blood vessels in breast ductal carcinoma. The biological function of FAPα in the tumor microenvironment still remains elusive. It has been suggested that FAPα functions via degradation of the ECM and/or processing of soluble factors (such as chemokines, hormones or bioactive peptides). FAPα over-expression is associated with reduced dependency on exogenous growth factors, enhanced tumor growth, invasion, angiogenesis, and metastasis. However, the role of FAPα in cancerogenesis is still controversial as in some studies a beneficial effect of FAPα expression has been demonstrated: (i) expression of FAPα in a mouse melanoma model decreased tumorigenicity, restored contact inhibition, induced cell cycle arrest and growth factor dependence; (ii) in human breast carcinoma expression of FAPα in fibroblasts is associated with longer overall and disease-free survival. Nevertheless, the differential expression of FAPα in cancer versus normal tissues makes it a promising therapeutic target (Fig. 3c). PT-100, an inhibitor of FAPα and DPPIV dipeptidyl peptidase activity, upregulates cytokine and chemokine production by stroma cells, and thereby augments the anti-tumor immune response, and it was well tolerated in a phase I clinical study [3, 76]. Other peptide inhibitors are also in preclinical development. Dipeptide proline diphenyl phosphonates exert an anti-invasive effect on the FAPα positive melanoma cell line LOX [33]. Recently, an FAPα-specific small molecule inhibitor has been developed which specifically targets FAPα but not DPPIV [28]. FAPα-directed anti-catalytic antibodies have been demonstrated to reduce the growth of FAP$^+$ tumor cells [24]. However, some of the biological effects of FAPα are mediated independently from its catalytic function as catalytic mutants still influence tumor growth. In consequence, several antibody constructs have been tested not only to inhibit the enzymatic activity of FAPα but also to mediate the destruction of FAPα^+ cells. The humanized version of the mab F19 to FAPα, Sibrotuzumab (BIBH 1) has been demonstrated to be safe and well tolerated in clinical studies [40, 93]. The ^{131}I-labeled BIBH 1 was used to characterize its biodistribution in humans, demonstrating that it selectively accumulated in primary and metastatic colorectal as well as

NSCL cancer [93]. However, the FAPα-specific antibody alone lacked therapeutic efficacy. Thus, FAPα-specific antibodies have been used to target bioactive molecules to the tumor stroma (reviewed in [92]): (i) An FAPα-CD3-bispecific single chain antibody was used to recruit CTLs to the tumor stroma [104]; (ii) TNFα was directed to FAPα expressing cells by an anti-FAPα-TNFα fusion protein; binding of the construct mimicks membrane-integrated TNFα signaling and leads to apoptosis and tissue factor production in vitro and reduced tumor growth in vivo [8]; (iii) the function of tissue factor in the coagulation cascade was also used in another approach where the human tissue factor was fused with a single chain anti-FAPα antibody; after binding to the cell surface coagulation was triggered in vitro [87]; finally (iv) a FasL fusion protein regained full activity upon cell-surface binding and induced apoptosis of FAPα positive cells in vitro and prevented growth of FAPα-positive tumor cells without systemic toxicity in vivo [91].

For the selective destruction of CAFs antigens not directly implicated in the ECM degradation may also be considered (Fig. 3d). The carbonic anhydrase IX (CAIX, MN, G250) is a marker for tumor hypoxia and is important for pH regulation. CAIX is over-expressed in CAFs of renal cell, colorectal, cervix, NSCL, bladder, and kidney cancer as well as on some malignant cells themselves (Table 1). High CAIX expression in tumors is associated with an unfavorable prognosis. It should be noted, however, that CAIX is also expressed in normal gastric epithelium. Efforts are in progress to design carbonic anhydrase inhibitors specifically binding to CAIX [77, 100]. Sulfonamide derivatives, for example show efficacy at inhibiting hypoxia-induced acidosis in vitro [7, 22]. With respect to passive immune therapy, the CAIX-specific antibody WX-G250 (Rencarex) efficiently mediated ADCC in vitro [64]. Moreover, treatment of renal cell carcinoma patients with this antibody resulted in clinical responses [12, 13]. Conjugates of this antibody with radioactive isotopes improved survival in preclinical studies, and currently are being tested in the clinic [19, 20]. Spontaneous T cell responses against CAIX are only rarely detected in cancer patients even if TIL are used for analysis [34]. Thus, in a recently initiated clinical trial the adoptive transfer of autologous T lymphocytes transduced to express a single-chain antibody to CAIX is being investigated [57].

Conclusion

Stroma cells influence tumor initiation and progression. They allow vasculo- and angiogenesis, as well as recruitment of additional stromal cells; secrete growth factors and proteolytic enzymes, and modify the ECM to make it more suitable for the tumor cells. A variety of methods to inhibit these tumor-promoting interactions of tumor and tumor stroma cells are currently being tested in preclinical and clinical studies. These approaches target one or multiple molecules to inhibit their expression, to interfere with their function or to destroy the tumor stroma cells. Several of these strategies achieved promising results in early clinical trials demonstrating that they may become an effective tool to treat cancer. In consequence, efforts are in progress to advance tumor stroma-directed therapies both to be applied by themselves or to complement conventional treatments.

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