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Antiangiogenic and anticancer molecules in cartilage

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Cartilage is one of the very few naturally occurring avascular tissues where lack of angiogenesis is the guiding principle for its structure and function. This has attracted investigators who have sought to understand the biochemical basis for its avascular nature, hypothesising that it could be used in designing therapies for treating cancer and related malignancies in humans through antiangiogenic applications. Cartilage encompasses primarily a specialised extracellular matrix synthesised by chondrocytes that is both complex and unique as a result of the myriad molecules of which it is composed. Of these components, a few such as thrombospondin-1, chondromodulin-1, the type XVIII-derived endostatin, SPARC (secreted protein acidic and rich in cysteine) and the type II collagen-derived N-terminal propeptide (PIIBNP) have demonstrated antiangiogenic or antitumour properties in vitro and in vivo preclinical trials that involve several complicated mechanisms that are not completely understood. Thrombospondin-1, endostatin and the shark-cartilage-derived Neovastat preparation have also been investigated in human clinical trials to treat several different kinds of cancers, where, despite the tremendous success seen in preclinical trials, these molecules are yet to show success as anticancer agents. This review summarises the current state-of-the-art antiangiogenic characterisation of these molecules, highlights their most promising aspects and evaluates the future of these molecules in antiangiogenic applications.

In designing strategies to counter cancer, restricting or eliminating angiogenic signals from the tumour is the working principle behind several therapeutic applications. An idea that was first seeded by Judah Folkman in the 1970s (Ref. 1) has matured into exponentially expanding treatment opportunities because of an ever-increasing knowledge base in angiogenesis pathways and molecular targets. The discovery of naturally occurring tissues that are antiangiogenic and where antiangiogenesis and lack of vasculature is a guiding principle dictating the developmental outcome has allowed for an increase in identification of antiangiogenic molecules. Chief among these is cartilage, a

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specialised form of extracellular matrix (ECM) synthesised by chondrocytes.

The characteristic that sets cartilage apart is that it is avascular, and this has piqued the interest of investigators who have sought to understand its avascular nature and to apply its principles to therapeutic applications. It stands to reason that the avascular nature of cartilage would be due to its biochemical composition that antagonises vascular invasion and there has been considerable interest in identifying these antiangiogenic components in cartilage. The primary components of cartilage are the two major macromolecules, type II collagen (Col II) and aggrecan (Fig. 1). Minor components include collagens types IX, XI, III, V, VI, X, XII, XIV as well as versican, perlecan, lubricin, biglycan, fibromodulin, thrombospondins (TSPs), chondromodulin-1 (ChM-1), endostatin and secreted protein acidic and rich in cysteine (SPARC), among others (reviewed in Ref. 2). Although in adults cartilage serves primarily to lubricate the joints, during embryonic development its chief function is to form the template for future skeletal development in mammals.

The process by which cartilage template is replaced by bone is called endochondral ossification, which occurs when the avascular privilege of cartilage is broken down, allowing for vascular invasion and endochondral bone formation. This topic has been well reviewed (Refs 3, 4), but to briefly summarise, the process begins with mesenchymal cell condensations at sites where the future skeletal elements will form; cells in these condensates differentiate to form chondrocytes that secrete a Col-II-rich avascular cartilage, with cells surrounding the chondrocytes forming the perichondrium. To allow for bone formation, the innermost chondrocytes differentiate to form hypertrophic chondrocytes that secrete a type-X-collagen-rich matrix that gets calcified, with the perichondral cells surrounding the hypertrophic chondrocytes differentiating into osteoblasts to form the bone collar. This allows for the vascular endothelial growth factor (VEGF)-dependent vascular invasion of the calcified cartilage matrix from the bone collar, which brings along with it chondroclasts and osteoblasts that degrade and replace the calcified matrix with bone and results in removal of the hypertrophic chondrocytes by apoptosis. This process thus allows for vascular invasion only of the calcified matrix, leaving the rest of cartilage matrix avascular. This is crucial to bone growth because the avascular cartilage continuously provides chondrocytes that differentiate to form hypertrophic chondrocytes that are removed during endochondral bone growth, thus allowing for continuous bone growth. With maturity, the centre of the avascular cartilage undergoes hypertrophic differentiation again to allow vascular invasion and the development of a secondary ossification centre. In adults, most of the cartilage is thus replaced by bone, with the exception of the articular cartilage at the bone ends, which remains avascular to maintain cartilage phenotype and functions to lubricate the joint. Breakdown of the antiangiogenic barrier during diseases such as osteoarthritis and rheumatoid arthritis results in undesirable vascular invasion of the articular cartilage and irreversible cartilage degeneration.

Tumours that exceed 1–2 mm in diameter require new capillary formation or angiogenesis that feeds their growth (Ref. 5). The recognition of this fundamental principle behind tumour growth has triggered the search for antiangiogenic molecules that would prevent tumour growth. Blessed with its avascular state, cartilage has since then become a prime target for investigation of its antiangiogenic properties (Refs 5, 6). Although primarily articular cartilage was tested, growth plate chondrocytes also demonstrated antiangiogenic effects (Ref. 7). These discoveries propelled an interest in the identification of cartilage components that produce these antiangiogenic and antitumour effect with the expectation that these cartilage-derived molecules could be used in therapeutic applications to treat tumours and related malignancies. This review summarises the status quo of some antiangiogenic molecules that are present in cartilage.

Neovastat and shark cartilage extract

Neovastat and shark cartilage extract (SCE) are preparations with antiangiogenesis activity derived from shark cartilage. Interest in shark cartilage as a source of anticancer agents grew because of its avascular state coupled with the misconception that sharks do not get cancer. The cartilaginous shark endoskeleton was thus thought to be an ideal source of large quantities of anticancer agents. Current literature finds researchers at extreme ends of the spectrum in

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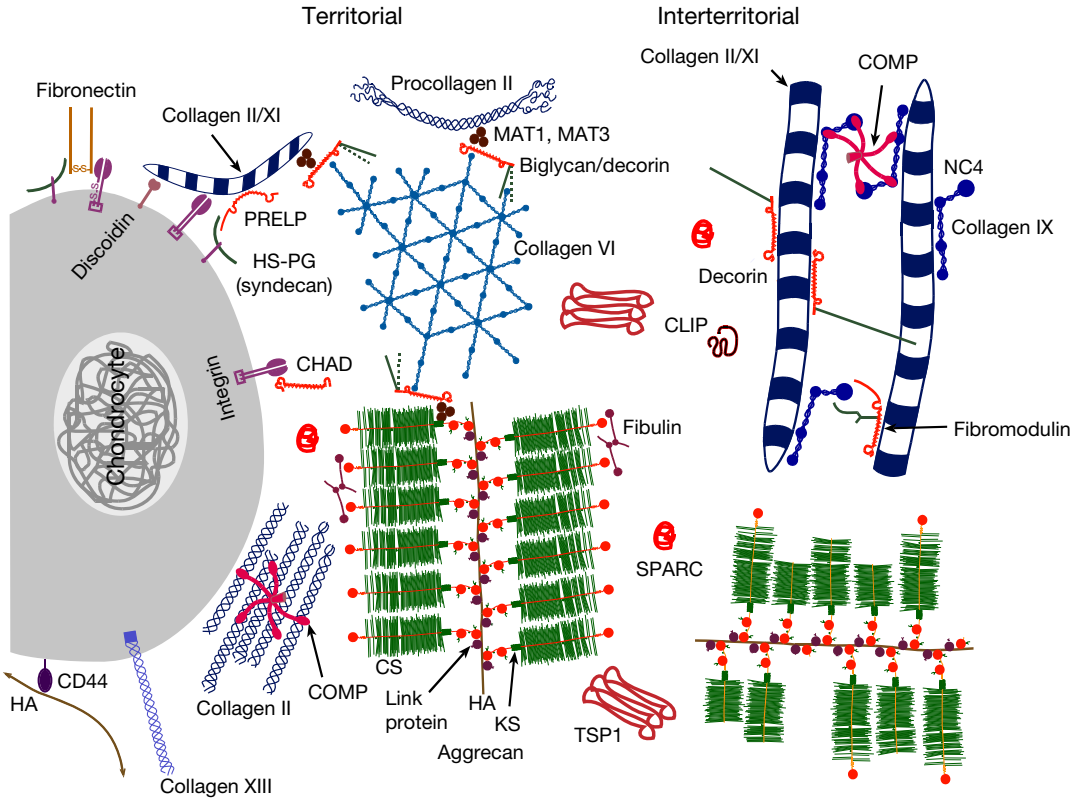

Schematic representation of the cartilage matrix showing many of the molecules present in cartilage and some of their interactions

Figure 1. Schematic representation of the cartilage matrix showing many of the molecules present in cartilage and some of their interactions. The matrix closest to the chondrocytes is indicated as territorial and between the chondrocytes is interterritorial. The cartilage extracellular matrix (ECM) is made of molecules that are synthesised by the chondrocyte. It has two major macromolecules, collagen type II and the large proteoglycan, aggrecan. The aggrecan protein consists of a globular core protein to which are attached chains of chondroitin sulfate (CS) and keratan sulfate (KS). The figure shows aggrecan monomers attached to hyaluronic acid (HA), which is stabilised by the link protein, to form a larger proteoglycan aggregate in interaction with the smaller proteoglycan, fibulin. In addition to these, the cartilage ECM is also composed of minor amounts of other collagens such as collagen types IX, XI, III, V, VI, X, XII, XIV and XVIII (from which endostatin is derived). Collagen type IX has four noncollagenous (NC) domains and has the ability to form heterotypic fibrils with collagen II/XI (shown in the interterritorial matrix) that is coated with smaller proteoglycans such as decorin and fibromodulin. The cartilage intermediate layer protein (CILP) is found primarily in the interterritorial matrix. Other smaller proteoglycans include biglycan, decorin and chondromodulin-1. The other major cartilage proteins are the thrombospondins (TSPs), which consists of TSP1, TSP2 and COMP (cartilage oligomeric matrix protein also known as TSP5). Matrilins 1 and 3 (MAT1, MAT3) are the primary members of the multimeric matrilin family of proteins found in the cartilage. PRELP (proline arginine-rich end leucine-rich repeat protein) and CHAD (chondroadherin) are small leucine-rich repeat proteins (LRRs) that bind to fibre forming collagens with high affinity due to the LRR domain. Syndecan is a heparan sulfate proteoglycan (HSPG) found on the chondrocyte cell surface where it acts as receptors for matrix components such as fibronectin. A number of matricellular proteins such as SPARC (secreted protein acidic and rich in cysteine) are also seen in the cartilage ECM. The cartilage matrix and its composition are reviewed in depth elsewhere (Ref. 2). This figure is modified from Ref. 2 with permission from Lippincott, Williams & Wilkins (Philadelphia).

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their faith in shark-cartilage-derived products. The discovery of cancer in sharks (Ref. 8) and the lack of promising results from the most recent clinical trial with Neovastat (see below) were serious setbacks to its use in cancer treatment.

Neovastat (or AE-941) is not a single molecule but a mix of water-soluble components derived from shark cartilage prepared by a proprietary manufacturing process developed by Aeterna Laboratories (Quebec, Canada) (Ref. 9). Homogenisation of the shark cartilage in water followed by sequential extraction to remove water-insoluble and inactive molecules results in a concentrated biological derivative with a mixture of components less than approximately 500 kDa. To date, the identity of all the individual components of Neovastat is not known, but extensive characterisation has attributed it with antiangiogenic properties. Neovastat induced a concentration-dependent inhibition of cell proliferation in human umbilical vein endothelial cells (HUVECs) and bovine endothelial cells; inhibited the formation of blood vessels induced by basic fibroblast growth factor (FGF) in the chicken chorioallantoic membrane model; severely inhibited in vivo the vascular invasion of bFGF-containing Matrigel implanted in C57BL6 mice fed orally with Neovastat; and inhibited lung metastases in the murine Lewis lung carcinoma model (Ref. 10). Neovastat combined with cisplatin, a conventional anticancer agent, exhibited greater anticancer activity than cisplatin alone. These data indicated the successful extraction of the antiangiogenic and antitumour agents from shark cartilage into Neovastat, and surprisingly, its success as an orally active biological compound.

Antiangiogenic features of Neovastat

Mechanistically, Neovastat induced apoptosis specifically in endothelial cells that involved the activation of caspase-3, caspase-8 and caspase-9 (Ref. 11). Neovastat interferes with VEGF binding to VEGFR-2 (Flk-1/KDR), which is required to make new blood vessels (Ref. 12), thereby inhibiting several mitogenic, chemotactic and antiapoptotic responses in endothelial cells (Ref. 13). Neovastat also inhibited the activity of matrix metalloproteinases (MMPs), primarily MMP2, but also of MMP1, MMP7, MMP9, MMP12 and MMP13 (Ref. 14), limiting the metastatic potential of tumour cells (Ref. 15). Neovastat-treated ovalbumin-sensitised mice exhibited reduced airway hyper-responsiveness and inflammation when challenged (Ref. 16); these mice demonstrated significantly reduced levels of MMP9 activity in their bronchoalveolar lavage fluid and reduced VEGF and hypoxia-inducible factor (HIF)-2α expression in their lung tissue, suggesting that the anti-inflammatory effects of Neovastat are linked to inhibition of VEGF and HIF-2α (Ref. 17).

Neovastat can activate the Jun amino terminal kinase (JNK) and nuclear factor-kappaB (NF-κB) signalling pathways as demonstrated by its ability to modulate the plasmin–plasminogen-activator (PA) systems. Plasmin–PA systems are vital components of the proteolytic machinery that degrade the ECM along with MMPs during angiogenesis (Refs 18, 19). Tissue PA (tPA) and urokinase PA (uPA) are the two major types of PA that convert plasminogen into plasmin (Ref. 20). Binding of uPA to its cell surface receptor is thought to have a role in neovascularisation and therefore tumour angiogenesis (Refs 21, 22). However, tPA is synthesised primarily by endothelial cells and has a role in cleavage of fibrin-bound PA to plasmin, leading to fibrin degradation (Ref. 20). Neovastat stimulates tPA-mediated plasmin generation (but only marginally inhibits uPA); BIAcore surface plasmon resonance spectroscopy demonstrated direct physical interaction of Neovastat with plasminogen that increased its catalytic efficiency (Ref. 23). Neovastat increased tPA production by endothelial cells, increased the phosphorylation and activity of JNK1 and JNK2, and the phosphorylation of IκB (an inhibitor of NF-κB) thereby inducing NF-κB signalling (Ref. 24). Administration of SCE (a generic preparation of Neovastat) to mouse glioma models resulted in tPA activity stimulation that is thought to direct the antiangiogenic property of SCE because tumours derived from glioma cells that overexpressed PAI-1, an inhibitor of tPA, did not exhibit decreased blood vessel formation (Ref. 25). Mice orally treated with SCE demonstrated a reduction in glioblastoma infiltration into the brain, with very small tumours, in contrast to mice that did not get SCE treatment. These studies indicated beneficial aspects of orally administered Neovastat across the blood–brain barrier and beneficial aspects of SCE in immune-compromised athymic mice with glioma.

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Clinical trials with Neovastat

Multi-targeting properties of Neovastat propelled its use as a multimodal, anticancer drug in humans. Phase I/II clinical trials for Neovastat in patients with renal cell carcinoma (RCC) (Ref. 26) and non-small cell lung cancer (NSCLC) (Ref. 27) were conducted. In both RCC and NSCLC patients, Neovastat was well tolerated, with no dose-limiting toxicity, with oral administrations of up to 240 ml/day showing significantly longer median survival than those receiving 60 ml/day. However, neither of these studies reported any response within the tumour [in contrast to the tumour necrosis observed in mouse models on Neovastat administration (Ref. 25)]. In the NSCLC study, about half the number of patients did not complete standard imaging assessments because of progressive disease as per clinical symptoms. A randomised, double-blinded, placebo-controlled Phase III clinical trial was conducted to determine Neovastat efficacy in treating Stage III NSCLC patients (Ref. 28). NSCLC patients who had previously received chemoradiotherapy were treated further with chemoradiotherapy plus Neovastat (or placebo). Although no tumour responses were observed with Neovastat administration, no statistically significant difference in patient survival was observed between those receiving Neovastat plus chemoradiotherapy and those receiving chemoradiotherapy alone. To rationalise the failure of Neovastat, the authors noted that as Neovastat is a complex, natural product as opposed to a well-defined chemical molecule, it lacks pharmacokinetic/pharmacodynamic (PK/PC) assays, which makes it difficult to control the product for quality (Ref. 28). Furthermore, no validated biomarker is available to follow the antiangiogenic effect in humans. Although the authors conclude that their study does not support the use of shark-cartilage-derived products as a therapy, the study design limits the conclusion and leaves behind an open question regarding their value in clinical applications (Ref. 29).

Chondromodulin-1

ChM-1 is an extensively studied molecule identified in cartilage with demonstrated antiangiogenic properties in vitro. ChM-1 (also known as the leukocyte cell-derived chemotaxin 1 encoded by the *LECT1* gene in humans) is a 25 kDa glycoprotein first identified in fetal bovine epiphyseal cartilage extracts (Ref. 30) with ability to stimulate DNA synthesis in cultured rabbit growth plate chondrocytes in the presence of FGF. The mature ChM-1 protein is the C-terminal portion of a larger precursor protein with one N-linked and two O-linked potential glycosylation sites. Interest in ChM-1 grew when it was discovered that it could inhibit bovine carotid artery endothelial cell growth and prevent a tube-like cellular network in vitro (Ref. 31). This was considered a prognostication of its ability to inhibit proliferation and tube morphogenesis of invading endothelial cells during angiogenesis in vivo. In situ hybridisation analyses in bovine embryonic growth plate (Ref. 31) and developing mice (Ref. 32) detected ChM-1 mRNA in the proliferating and pre-hypertrophic cartilage zones (which are avascular) with expression missing from the mature hypertrophic zone (where vascular invasion during endochondral bone growth takes place) and vascular bone. The detection of ChM-1 protein in the cartilage inter-territorial matrix in the same avascular zones as its mRNA strengthened the notion of ChM-1 as an antiangiogenesis molecule in cartilage. Once considered specific to cartilage, ChM-1 has since then been identified in other avascular tissues, such as the eye, thymus (Ref. 33) and cardiac valves (Ref. 34).

Antiangiogenic features of ChM-1 and skeletal homeostasis

In humans, ChM-1 protein was detected in the ECM surrounding mature hypertrophic chondrocytes in neonatal vertebrae, at the junction with bone (Ref. 35). This is not consistent with results in mice or bovine growth plate, nor can this be deemed favourable for vascular invasion during endochondral bone growth if it is antiangiogenic in vivo. The results from mouse models cast doubt on the ability of ChM-1 to regulate angiogenesis in vivo. Based on its distribution in the bovine and murine growth plate, it was predicted that ChM-1 allows the cartilage to remain avascular and its reduced expression in the hypertrophic cartilage allows for vascular invasion during endochondral bone development. However, ChM-1-null mice demonstrated normal cartilage

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and bone development, with no reported abnormal cartilage vascularisation (Ref. 36). Even though recombinant human ChM-1 demonstrated inhibition of human retinal endothelial cell tube morphogenesis in vitro (Ref. 37), suggesting that it might be required to maintain normal avascular state of the retina, mice lacking ChM-1 did not show any abnormal vascularisation of the retina or any other abnormalities in eye development. It was thought that loss of ChM-1 in mice was compensated by its close homologue, tenomodulin (also called tendin) (Ref. 33). Like ChM-1, tenomodulin inhibited the growth of HUVECs and tube morphogenesis in vitro (Ref. 38). However, mice that lack both ChM-1 and tenomodulin also did not show any abnormalities in eye development, nor were any abnormalities in cartilage and endochondral bone development reported (Ref. 39).

Despite these contradictions, the notion of ChM-1 being an antiangiogenic factor in vivo cannot be overruled. During endochondral ossification in mice, chondrocyte hypertrophic differentiation results in loss of ChM-1 expression prior to matrix calcification in the cartilage anlagen (Ref. 32). Hypertrophic chondrocyte differentiation and osteoblast differentiation is governed among other factors, by the transcription factor Runx2 (Cbfa1) (Refs 40, 41, 42, 43). Runx2-null mice lack hypertrophic differentiation and osteoblast development and show no endochondral bone formation. Interestingly, this was accompanied by an increase in ChM-1 in the cartilage of these mice (Ref. 43). However, rescue of Runx2-null mice by a transgene that expressed Runx2 in chondrocytes reduced ChM-1 expression in the cartilage and allowed for vascular invasion. This indicated that Runx2 regulates ChM-1 expression in chondrocytes and that ChM-1 is required to maintain cartilage avascular state. Likewise, in experimental osteoarthritis in rats, ChM-1 levels decreased in all cartilage zones with advancing osteoarthritis with a concomitant increase in VEGF expression (Ref. 44). However, inhibition of VEGF by administration of antibodies against VEGF improved articular cartilage repair in an osteochondral defect model with parallel increases in ChM-1 (Ref. 45).

ChM-1-null mice exhibited delayed union of the fractured bone in a tibial fracture repair model (Ref. 46). Fracture repair usually mimics endochondral bone development where a cartilaginous callus is initially formed enveloping the fracture site that is replaced by bone. ChM-1-null mice exhibited severely reduced external cartilaginous callus formation restricted only to the fracture site, with reduced gene expression for the cartilage-promoting factor Sox9 and for type X collagen. ChM-1-null mice exhibited a bony callus instead in the periosteal region and an enhanced internal fracture callus. ChM-1 might also be responsible for the good-quality cartilaginous repair tissue formed when femoral chondral lesions in miniature pig knee joint were treated with matrix-associated autologous chondrocyte transplantation (MACT) (Ref. 47). In the absence of MACT, these chondral lesions were filled with excessive bony tissue and vascular invasion from the subchondral bone plate with overlying fibrocartilage repair tissue of poor quality that lacked ChM-1 expression. With MACT treatment, the cartilage repair tissue formed was rich in proteoglycan and Col II, and positive for ChM-1, with calcification of repair tissue and subchondral bone overgrowth inhibited. ChM-1 was also detected in normal healthy noncalcified articular pig cartilage.

In the same porcine model, chondroprogenitor cells overexpressing recombinant human ChM-1 when transplanted into the chondral lesions stimulated chondrogenic differentiation that produced a superior hyaline-like matrix than those in the absence of recombinant human ChM-1, by inhibiting chondrocyte hypertrophy, preventing vascularity and therefore endochondral ossification, thus stabilising the chondrocyte phenotype (Ref. 48). In an immobilisation-induced cartilage degeneration model (Ref. 49), rats with plaster-cast-immobilised ankle joints demonstrated thinning of the ankle joint cartilage with reduced ChM-1, in contrast to rats with continuous passive motion. Immobilised ankle joints also exhibited increased VEGF and HIF-1α expression, with vascular invasion of the calcified cartilage zone from the subchondral bone plates. ChM-1 is also abundantly expressed in other avascular tissues such as cardiac valves in mice (Ref. 34). Young ChM-1-null mice exhibited normal cardiac valves; but as these mice aged, in strong contrast to wild-type mice, they exhibited bulky aortic valves that showed VEGF expression, enhanced angiogenesis of the aortic valves, increased calcium deposits and consequent aortic stenosis

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characterised by turbulent blood flow. In human heart diseases such as in congenital bicuspid aortic valves stenosis, cardiac valves exhibited ChM-1 downregulation in regions of neovascularisation, calcification and VEGF-A expression, with aggressive macrophage infiltration and inflammation, indicating that ChM-1 is important for normal valvular function (Ref. 50).

Modes of ChM-1 antiangiogenic inhibition

Application of ChM-1 in clinical trials as an antitumour agent has not been reported. However, ChM-1 is able to suppress tumourigenesis in mouse tumour models. The application of recombinant human ChM-1 to OUMS-27 (that do not express ChM-1) xenograft tumour model in mice (Ref. 51) or to HepG2-induced tumours in athymic mice (Ref. 52) suppressed tumour growth and prevented blood vessel invasion into the tumour. Interestingly, ChM-1 can act directly on tumour cells and not just endothelial cells. ChM-1 inhibited DNA synthesis and reduced growth of human tumour cells such as HepG2, PC-3 and NOS-1, presumably by suppressing the STAT signalling pathway (Ref. 52). ChM-1 also downregulated cell cycle proteins such as cyclin D1, cyclin D3 and cdk6 and upregulated the cell cycle inhibitor protein $p21^{cip1}$, although no apoptosis was observed. These data suggested that ChM-1 mediates its cytotoxic effect on human tumour cells primarily by causing cell cycle arrest. However, in HUVECs, ChM-1 inhibited their migration through disruption of actin reorganisation and suppression of Rac1/Cdc42 GTPase activity (Ref. 53). In a mouse model for rheumatoid arthritis, recombinant human ChM-1 suppressed proliferation of mouse splenic T cells and production of interleukin (IL)-2 from $CD4^+$ T cells (Ref. 54). It also suppressed the development of antigen-induced arthritis and the proliferation of synovial cells from joints of rheumatoid arthritis patients. How ChM-1 manifests these diverse inhibitory physiological responses in different cell types remains to be investigated. In addition to its antiangiogenic activity, ChM-1 might have additional attributes (Ref. 48) that remain to be identified. These data suggest that ChM-1 has a strong potential to be used as a therapeutic agent for treatment of localised tumours as well as for suppressing the severity of symptoms in rheumatoid arthritis patients.

Thrombospondins

TSPs are a family of five cartilage matrix proteins of which only TSP1 and TSP2 have antiangiogenic properties and are of interest in clinical applications as anticancer agents. Although TSP1 and TSP2 are detected in cartilage and have important roles in maintaining growth plate homeostasis (Refs 55, 56, 57), their distribution is not limited to cartilage and their antiangiogenic properties discovered in other systems have been well reviewed (Refs 58, 59). In addition to their antiangiogenic properties, TSP1 and TSP2 also have angiogenic functions (reviewed in Ref. 60).

Antiangiogenic features of TSP1 and TSP2

The antiangiogenic properties of TSP1 and TSP2 are complex and may arise from several mechanisms. In the presence of the CD36 receptor, TSP1 mediates its antiangiogenic effect on endothelial cells in vitro, and inhibition of neovascularisation in vivo, through apoptosis by sequential upregulation of the Src family kinase p59fyn, caspase-3 like proteases and the p38 mitogen-activated protein kinase (MAPK)-mediated signalling and activation of Fas ligand (FasL) (Refs 61, 62), or by tumour necrosis factor (TNF)-receptor 1 and TNFα in the absence of FasL (Ref. 63). TSP1 also inhibits lymphangiogenesis (formation of lymphatic vessels) through CD36 ligation on monocytic cells in a mechanism that inhibits transforming growth factor β (TGFβ)-mediated expression of VEGF-C (Ref. 64), providing an added anticancer benefit because lymphangiogenesis facilitates cancer metastases to distant lymph nodes and organs (Refs 65, 66). TSP2 also mediates apoptosis through CD36 interaction (Refs 67, 68). However in HUVECs, in the absence of CD36, TSP1 induced upregulation of $p21^{cip/waf}$, p53 phosphorylation and Rb dephosphorylation resulting in cell cycle arrest (Ref. 69). Ligation of the VLDL receptor by TSP1 or TSP2 also inhibited cell division in endothelial cells by a similar nonapoptotic pathway with cell cycle arrest (Ref. 70). A third mechanism is the binding of TSP1 and TSP2 to heparan sulfate proteoglycans (HSPGs) through its N-terminal heparin-binding domain (Ref. 71). HSPGs are co-receptors for the low-density lipoprotein receptor-related protein (LRP1). LRP1 is a scavenger receptor with endocytic and signal transmission behaviour that is required

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for chondrocyte differentiation (Ref. 72). LRP1 functions to clear complexes of TSP1 and TSP2 with MMP2, MMP9 and VEGF (Refs 73, 74, 75) resulting in decreased activity of these enzymes to inhibit vascular invasion.

TSP1- and TSP2-deficient mouse models and skeletal homeostasis

It is not known as to whether the mechanisms enumerated above are used in cartilage, but antiangiogenic benefits from TSP1 and TSP2 can be surmised from the following. Mice deficient in TSP1 or TSP2 show only mild chondrocyte growth plate disorganisation, but no abnormal vascularisation of the cartilage was reported (Ref. 57), although TSP2-deficient mice showed enhanced cortical bone formation (Ref. 76). However, loss of TSP1 in mice exhibited increased retinal vascular density, a reduced rate of endothelial cell apoptosis, and excessive capillary formation of heart and skeletal muscles (Refs 77, 78). In a collagen-induced arthritis model in rats, recombinant murine TSP1 delivered intraarticularly by adenoviral vectors (AdTSP1) into the ankle joints of collagen-sensitised rats, reduced the pathogenesis of arthritis with lower levels of VEGF, IL-1β, blood vessels and synovial hypertrophy (Ref. 79). Absence of TSP2 altered the dynamics of osteogenic and chondrogenic differentiation in the callus in a tibial fracture model such that the invading mesenchymal cells showed decreased chondrogenic differentiation with less cartilage formation and increased bone formation in the callus characterised by increased blood vessel density (Ref. 80).

In cartilage repair models, as seen with ChM-1, femoral chondral lesions in miniature pig knee joint treated with MACT developed superior cartilage that stained positively for TSP1 (Ref. 47). Application of recombinant human TSP1 to microfracture lesions discouraged ingrowing mesenchymal bone marrow stromal cells from terminal differentiation thus preventing chondrocyte hypertrophy and bone outgrowths from the subchondral plate, though this did not induce chondrogenesis and the lesions did not repair (Ref. 81). But application of recombinant human TSP1 along with osteogenic protein-1, which can induce chondrogenesis, but cannot prevent chondrocyte differentiation and endochondral ossification, resulted in the lesions repairing favourably with production of cartilage that resisted hypertrophic differentiation and bone formation. RT-PCR analysis of human chondrocytes treated with TSP1 showed downregulation of GADD45β, which is required for chondrocyte hypertrophy, although no increase in $p21^{cip/waf}$ was observed (Ref. 81). In a rat model of osteoarthritis with anterior cruciate ligament transection (ACLT), intraarticular injection of AdTSP1 in the ACLT-treated knee joint resulted in angiogenesis inhibition, with reduced microvessel formation, macrophage infiltration, IL-1β and MMP13 levels, and reduced synovial tissue hyperplasia that suppressed osteoarthritis progression (Ref. 82). How TSP1 modulates these effects is not completely understood but TSP1 induced TGFβ in this study, which is known to aid cartilage repair and chondrogenesis (Refs 83, 84).

Clinical applications with TSP1 and TSP2

The benefits from TSP1 and TSP2 in preclinical applications in treating cancer are considerable and have been well reviewed (Refs 68, 85, 86, 87, 88). TSP1 applications have gained further credibility as a result of some recent noteworthy observations. Treatment of prostate tumour cells with quinoline-3-carboxamide tasquinimod (chemotherapy for metastatic prostate cancer) induced upregulation of TSP1 that was paralleled by reduced VEGF expression in the tumour tissue (Ref. 89). TSP1 was tested for its ability to treat cerebral arteriovenous malformation (AVM) as a noninvasive therapy. Cerebral AVM endothelial cells have different angiogenic characteristics than endothelial cells from normal control brain; TSP1 was able to normalise the rate of proliferation and migration of AVM endothelial cells (Ref. 90). The contradictory absence of angiogenesis in hypoxic tissues of patients suffering from systemic sclerosis could be explained by the aberrant expression of TSP1 in these tissues (Ref. 91). A fusion protein of TSP2 with IgG-Fc1 could inhibit both primary tumour growth of MDA-MB-435 tumour cells in vivo and lymph node and lung metastases from the mammary gland in athymic nude mice (Ref. 92).

Clinical applications with TSP1-derived ABT-510 and ABT-526

Several logistical considerations precluded the use of the whole molecule for clinical development and

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application in humans. These include the large size of TSP1, cost-prohibitive large-scale production and the possibility of unwanted side effects that could arise from multiple biological complications because of the multiple TSP1 receptors and target cells, including increased angiogenesis with TSP1 overexpression (reviewed in Ref. 68). A possible solution has been the use of TSP1-derived peptide mimetics. Successful peptides were based on the antiangiogenic sequence GVITRIR derived from the second type I repeat of TSP1 (Ref. 93), later modified for better half-life and PK/PC profiles, through the incorporation of non-natural amino acids, resulting in ABT-510 and ABT-526 (Ref. 94). ABT-510 and ABT-526 demonstrated both safety and efficacy in treatment of dogs with cancer (Refs 95, 96), in mice with malignant glioma and in inducing apoptosis of human brain endothelial cells in vitro (Ref. 97), and in combination with valproic acid to inhibit neuroblastoma in vivo (Ref. 98). In an orthotopic syngeneic mouse model of epithelial ovarian cancer, treatment of mice with cisplatin or paclitaxel was more effective in combination with ABT-510, primarily because ABT-510 normalised the tumour vasculature by direct apoptotic effect on immature blood vessel endothelial cells, allowing for better uptake of cisplatin or paclitaxel, thereby significantly reducing tumour size (Ref. 99). ABT-510 was tested successfully for its ability to reduce tumours in mouse models of head and neck squamous cell cancer (Ref. 100) and epithelial ovarian cancer (Ref. 101) that merited clinical trials in humans.

Although ABT-526 has remained largely preclinical, ABT-510 has been tested in clinical trials. The impact of ABT-510 in human clinical trials, however, is not impressive. Phase I/II clinical trials to determine safety, toxicity and PK/PC responses with ABT-510 in patients with a variety of advanced cancers (Refs 102, 103), metastatic melanoma (Ref. 104), advanced RCC (Ref. 105) and advanced soft tissue sarcoma (Ref. 106) demonstrated that ABT-510 was well tolerated with negligible adverse effects at the doses studied (although the maximum tolerated dose was never defined). These studies, however, failed to demonstrate any significant favourable clinical benefit to patients on ABT-510 treatment. This led to Phase I/II trials for ABT-510 in combination with gemcitabine and cisplatin for treatment of solid tumours (Ref. 107), or 5-fluorouracil and leucovorin (Ref. 108), or in combination with chemoradiation for treatment of glioblastoma (Ref. 109). Although these combinations were deemed safe, the efficacy of ABT-510 in treatment of human cancers remains inconclusive because the number of patients showing improvement due to ABT-510 treatment was not impressive. Randomised Phase III trials with ABT-510 remain to be reported.

Endostatin

Endostatin is a 20 kDa fragment derived from the C-terminus of collagen XVIII by the activity of several proteinases, chiefly MMP9 (Refs 110, 111). Endostatin, similar to other inhibitors of angiogenesis, inhibits endothelial cell proliferation and migration and induces apoptosis. It is expressed in cartilage, but is not specific to cartilage and has been detected in both fetal epiphyseal cartilage and adult articular cartilage in humans, rats and mice, where its spatio-temporal distribution might provide an antiangiogenic benefit against cartilage vascularisation (Refs 112, 113). In the meniscal fibrocartilage, its concentration is highest in the completely avascular central portion as compared with the outer region that has some vascular elements.

Antiangiogenic features of endostatin

The mechanism by which endostatin mediates its antiangiogenic effect is complex and is not completely understood because of the myriad pathways that it affects. To highlight some major pathways and recent advances, endostatin can suppress VEGF expression and induce expression of the antiangiogenic pigment epithelium-derived factor (Refs 114, 115, 116); it can also upregulate the antiangiogenic VEGF165b isoform through inhibition of specificity protein 1 (Ref. 117). Endostatin can directly bind the VEGF receptor KDR/Flk-1 (VEGFR-2) on endothelial cells and therefore inhibit VEGF-induced activation of p38 MAPK (Ref. 118). It can downregulate TNFα and vascular cell adhesion molecule-1 (VCAM-1) (Ref. 115), which induces chemotaxis and angiogenesis in quiescent endothelial cells. Endostatin also binds to other receptors on endothelial cells such as glypicans (Ref. 119), but most of its effect is manifested by its binding to

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its high-affinity receptor, nucleolin. Nucleolin is tissue specific and present only on angiogenic, but not mature, blood vessels; antinucleolin antibodies can inhibit the antiangiogenic and antitumour activities of endostatin in vivo (Ref. 120). Endostatin binding to nucleolin results in the internalisation of endostatin and transportation to the nucleus, an event that requires integrin α_5. Endostatin then inhibits the phosphorylation of nucleolin in the nucleus that is inhibitory to nucleolin activity and retards endothelial cell proliferation. Mechanistically, how endostatin is internalised and translocated to the nucleus is not known, but the heparin-binding motif in its N-terminus is a key structural motif required for nucleolin interaction (Ref. 121); mutation of six arginine residues (individually or in combinations) to alanine in this motif eliminates endostatin–nucleolin interactions and its antiangiogenic and antitumour activity. Endostatin–nucleolin interaction also inhibits lymphangiogenesis by inhibiting lymph endothelial cells in active lymphangiogenic vessels, and prevents liver metastasis in a mouse breast cancer model (Ref. 122).

Endostatin can negatively impact on endothelial cell migration and adhesion in several ways. It binds to $\alpha_5\beta_1$ integrins on endothelial cells, resulting in the activation of Src kinase and downregulation of RhoA GTPase in a caveolin-1- and heparin-sulfate-dependent manner resulting in actin cytoskeleton disorganisation (Refs 123, 124). It inhibits MT1-MMP-mediated activation of proMMP2 protease (Ref. 125) and directly inhibits MMP2 activity by binding to its catalytic site (Ref. 126), thereby eliminating their activities vital to endothelial cell migration. Endostatin downregulates uPA and PAI-1 and removes uPA from focal adhesion molecules to effect actin cytoskeletal disorganisation (Ref. 127). Endostatin downregulates antiapoptotic members Bcl-2 and Bcl-x_L (with no effect on the pro-apoptotic Bax) to facilitate apoptosis (Ref. 128). It inhibits the Wnt-signalling pathway through the glypican receptors to target β-catenin for degradation (Ref. 129), which eventually suppresses Myc and cyclin D1 (Ref. 130). Endostatin can also upregulate levels of Beclin-1, a physiological partner of Bcl-2 and Bcl-x_L; this upregulation distorts the Bcl-2 (or Bcl-x_L) to Beclin 1 ratio, which can modulate endothelial cell apoptosis (Ref. 131). It can also induce apoptosis in endothelial cells by inducing tyrosine phosphorylation of the Shb adaptor protein by binding to HSPG (Ref. 132).

In animal models of ulcerative colitis, increase in VEGF in the colonic tissue was paralleled by a concomitant increase in endostatin as a defense mechanism; the larger the colonic lesion, the greater the increase in VEGF and therefore endostatin (Ref. 133). Induction of ulcerative colitis in MMP9-deficient mice resulted in less endostatin than in wild-type mice pointing to the in vivo role of MMP9 in generating endostatin from collagen XVIII in lesions. The levels of VEGF and endostatin are often linked, and although the molecular mechanism is not completely understood, VEGF might positively influence endostatin levels through the activation of MMP9 (Ref. 134). Finally, the large number of genes that are up- or downregulated by endostatin in a genome-wide microarray analysis (Ref. 135) suggests that a lot remains to be understood about endostatin's role in antiangiogenesis.

Derivatives of endostatin with antiangiogenic properties

Several studies have explored structural modifications of endostatin to improve function, routes of endostatin administration and combinatorial therapeutic applications as an antitumour agent in preclinical and clinical studies. Structural modifications include a single amino acid substitution (P125A endostatin) that was more antiangiogenic than wild-type endostatin (Ref. 136). Zinc has an important role in both structure and biological functions of endostatin with the amino acid residues H1, H3, H11 and D76 coordinating with a single zinc ion (Refs 137, 138, 139). Impaired zinc binding in recombinant endostatin expressed in *Pichia pastoris* showing N-terminal truncations demonstrated different biological activity depending on the extent of the truncations. However, addition of an extra zinc binding motif (ZBP) to its N-terminal region (ZBP-endostatin) showed more potent antiendothelial and antitumour activity than wild-type endostatin (Ref. 140). Addition of the integrin-targeting Arg-Gly-Asp (RGD) moiety to its N-terminus improved targeted localisation of endostatin to the tumour vasculature, which overexpresses $\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\alpha_V\beta_1$ integrins

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compared with normal tissues, and improved its antiangiogenic activity (Ref. 141). Addition of the IgG Fc fragment increased endostatin half-life in systemic circulation (Ref. 142); the addition of both RGD and Fc effectively inhibited tumour-cell-induced angiogenesis, and in combination with Bevacizumab (monoclonal antibody to VEGF-A), additively inhibited ovarian cancer growth in vivo (Ref. 142). A novel therapeutic fusion protein, EndoCD, which links endostatin to cytosine deaminase and uracil phosphoribosyl transferase (CD) (the latter converts 5-fluorocytosine to the antitumour drug 5-fluorouracil) suppressed tumour growth and metastasis in human breast and colorectal animal models (Ref. 143). The P125A endostatin fusion protein with anti-Her2 antibody (αHer2-IgG3-huEndo-P125A) showed better half-life in serum and improved activity in breast cancer xenograft models (Ref. 144). Combining endostatin with another angiostatic protein, the angiostatin–endostatin hybrid, provided a potent synergistic effect (Ref. 145).

Clinical applications of endostatin

Recent years have seen an increased interest in applications of endostatin through different vectors, and combinatorial therapies, for various tumour models. Application routes tested include adenoviral vectors (Ref. 146), adeno-associated vectors (Ref. 147), the oncolytic herpes simplex virus (Ref. 148) and mesenchymal stem cells (Ref. 149), all of which deliver endostatin; the Lister vaccine vaccinia viral strain, which delivers an endostatin–angiostatin fusion protein (Ref. 150) or endostatin plus carboxylesterases (which converts a latent drug into an active drug) (Ref. 151); and NIH/3T3 cells expressing murine endostatin from retroviral vectors to provide sustained levels of endostatin and a long-term antiangiogenic effect (Ref. 152). Recent attempts at combinatorial therapies in preclinical models included endostatin plus oxaliplatin (Ref. 153) or paclitaxel (Ref. 154), which decreased tumour blood vessel density and VEGF expression, and normalised the tumour blood vasculature, allowing for increased drug uptake. Combined with radiotherapy, endostatin inhibited tumour growth and induced tumour regression (Ref. 155), or reduced VEGF levels in combination with gemcitabine (Ref. 156); ZBP-endostatin potentiated the effects of chemo-radiotherapy (Ref. 157) or the antitumour effect of dexamethasone (Ref. 158); it disrupted the actin cytoskeleton in lymph endothelial cells by inhibiting the fibronectin alternative extra domain A and integrin α9 (which facilitates lymphangiogenesis of colorectal tumours) expression and interactions (Ref. 159). In cartilage repair studies, collagen scaffolds supplemented with chondrocytes and endostatin-expressing mesenchymal stem cells allowed the formation of a cartilaginous implant suffused with endostatin, which promoted the anabolic activity of the chondrocytes to produce more sulfated glycosaminoglycans indicating better cartilage production (Refs 160, 161). It is hoped that endostatin-infused scaffolds when implanted into a cartilage fissure in vivo, such as during osteoarthritis, would allow for better cartilage repair.

Clinical trials with endostatin and its derivatives

Phase I/II trials with endostatin that showed any benefit were largely conducted with ZBP-endostatin referred to as Endostar (trade name) or YH-16 (in Chinese publications that have been reviewed (Ref. 162)); however, those conducted with recombinant human endostatin showed no benefit (Refs 163, 164, 165, 166, 167). The better response to Endostar was due to the ZBP domain that supported the vital nature of the N-terminus in endostatin functions. More recently, Phase I/II trials were conducted with Endostar in patients with solid cancers (Ref. 168), in patients with extensive-stage small-cell lung cancer combined with cisplatin and etoposide (Ref. 169), or recombinant human endostatin in combination with gemcitabine/cisplatin for NSCLC (Ref. 170), demonstrated that endostatin was safe for human administration with minimal adverse reactions and that patient responses were better in combination therapies compared with the use of endostatin alone. However, a recent, Phase II, multicentre randomised double-blind placebo-controlled study in NSCLC patients receiving Endostar plus paclitaxel-carboplatin showed that although this combination had a good safety profile, overall increased patient survival was not significant (Ref. 171). No Phase III trial has been reported in an English language publication.

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Secreted protein acidic and rich in cysteine

SPARC is a matricellular glycoprotein that is not only multifunctional, but is perhaps the only molecule here that has extensive reviews devoted to both its angiogenic (or tumour-promoting) and antiangiogenic (or tumour-inhibiting) roles (Refs 172, 173, 174). Also known as osteonectin/BM40 and known initially as a bone protein (Ref. 175), SPARC is present in cartilage (Refs 176, 177), where it is thought to have a function in secretion of the ECM components (Ref. 178). SPARC has been tested extensively in vitro and in preclinical tumour models that demonstrated its antiangiogenic ability to induce endothelial cell apoptosis, inhibit endothelial cell migration (Ref. 179), directly induce apoptosis of ovarian cancer cells (Ref. 180), and inhibit breast cancer cells (Ref. 181) and neuroblastoma (Ref. 182). As with other molecules discussed above, the exact mechanism of SPARC antiangiogenesis behaviour remains unclear, but it can inhibit the mitogenic activity of bFGF (Ref. 183), and by binding directly to platelet-derived growth factor (Ref. 184) and VEGF (Ref. 185) can suppress their binding to their respective receptors. No abnormal cartilage vascularisation in SPARC-null mice has been reported, but these mice exhibit cataract formation in the eye lens (Ref. 186) that is not associated with angiogenesis. There are no reported clinical trials of SPARC. Of late, SPARC has been used primarily in disease prognosis, where its expression is associated with better prognosis for colon cancer (Refs 187, 188), although high SPARC expression is associated with enhanced tumour and poor prognosis of gastric carcinoma (Ref. 189) or oesophageal carcinoma (Ref. 190).

The N-terminal propeptide of the type IIB collagen protein

The trimeric fibrillar type II collagen protein (Col II) has only recently been attributed as a source of antiangiogenic activity in cartilage. It is synthesised as a procollagen with N- and C-terminal propeptides that are removed by proteinases from the collagen trimers prior to its incorporation into the ECM (Ref. 191). The Col II protein exists in two splice forms, type IIA and type IIB (Ref. 192). The type IIB form (Col IIB) is distinguished from type IIA (Col IIA) by the absence of exon-2-coded sequences and is chondrocyte specific, as opposed to the chondroprogenitor-derived Col IIA. The N-terminal propeptide is not removed from the Col IIA procollagen and is deposited intact into the ECM, where it binds growth factors such as BMP-2 and TGFβ through the exon-2-coded sequences (Ref. 193). The removal of the N-terminal propeptide (PIIBNP) from the Col IIB procollagen results in a free peptide whose molecular function is not understood. The presence of two RGD sequences in tandem (RGDRGD) that is well conserved across mammals in PIIBNP suggests its interaction with integrin family members.

Recent work indicated that PIIBNP has strong antiangiogenic and antitumour activities (Ref. 194) that might have a role in keeping the cartilage avascular and could be exploited to kill tumours in vivo. The interaction of recombinant human PIIBNP (GST-PIIBNP) with hCh-1 cells (human chondrosarcoma cell line) was dependent on $\alpha_V\beta_5$ and $\alpha_V\beta_3$ integrins and took place in an RGDRGD-dependent manner in vitro. Notably, PIIBNP was able to directly induce cell death in hCh-1 cells, in HeLa cells (cervical carcinoma cell line) and in MDA-MB231 (breast cancer cell line) in a dose-dependent manner. Disruption of the RGD-integrin interaction by mutation of the RGDRGD motif in PIIBNP or downregulation of integrin α_V in the cell line using siRNA technology significantly reduced cell death. The significance of these in vitro observations hit home when it was realised that PIIBNP exists in cartilage and that normal chondrocytes do not express, or have very low levels of α_V, β_5 and β_3 integrins, and presumably therefore are not killed by PIIBNP in vivo. GST-PIIBNP induced necrosis as opposed to apoptotic cell death and was also inhibitory to tumour cell migration. When tested in vivo, GST-PIIBNP suppressed MDA-MB231 induced tumours in nude mice (Fig. 2) demonstrating a 75% reduction in tumour growth over untreated tumours. Several cell types such as endothelial cells, osteoclasts and tumour cells express $\alpha_V\beta_5$ and $\alpha_V\beta_3$ integrins, suggesting that PIIBNP might function in vivo to keep the cartilage avascular and free from bone invasion. Indeed, PIIBNP was able to induce apoptotic death in osteoclasts (but not osteoblasts and macrophages) in an RGD- and integrin-dependent manner both in vitro and in vivo (Ref. 195). These data indicate that PIIBNP

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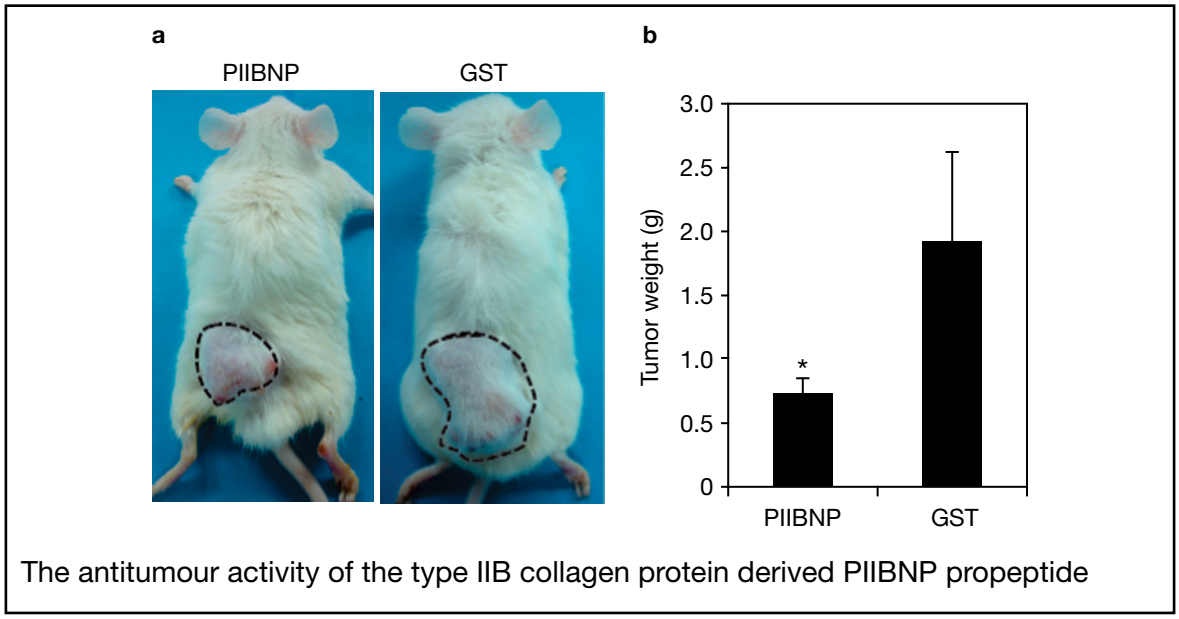

Figure 2. The antitumour activity of the type IIB collagen protein derived PIIBNP propeptide. (a) An in vivo tumour assay showing the antitumour activity of PIIBNP peptide. NOD/SCID mice were injected into the dorsal surface with MDA-MB231 breast cancer cells to form solid tumours. When the tumours grew to 0.5 cm in diameter, the mice were divided into two groups with three mice in each. Mice received either 6 nmol of PIIBNP or GST per day by subcutaneous injection into the tumour for 2 weeks. The dashed line shows the boundary of the tumour. (b) When the largest tumour size in the control (GST) group reached 2 cm in diameter, the mice were sacrificed and tumours removed and weighed. The graph shows tumour weights in grams (g) represented as mean ± s.d. (*$P < 0.01$ compared with GST control, $n = 6$) derived from these mice after treatment with PIIBNP or GST. PIIBNP suppressed tumour growth in mice. Reproduced from Ref. 194, with permission. © The American Society for Biochemistry and Molecular Biology, 2010.

might have antiangiogenic role in cartilage and could be used as an anticancer agent.

Future prospects for antiangiogenic research for cartilage-derived molecules

Interest in antiangiogenic molecules has also triggered an interest in their application as anticancer agents in the clinic. The current antiangiogenic modality approved to treat different forms of cancer in the clinic uses monoclonal antibodies against VEGF or to its receptor. Bevacizumab, an anti-VEGF humanised antibody is used as a treatment for pancreatic cancer, colorectal cancer, nonsmall lung cancer, metastatic renal cancer, carcinoma, glioblastoma and metastatic breast cancer, often in combination with chemotherapy. It is also under investigation for treatment of solid tumours. Despite the tremendous interest generated by the applications of anti-VEGF therapy, it is beleaguered with several drawbacks, primarily its sporadic success in clinical treatments and an inability to provide significant survival benefit (Refs 196, 197). In fact Avastin, a commercially marketed bevacizumab preparation, was banned recently by the FDA for breast cancer treatment, although it continues to be used for lung and kidney cancers. Furthermore, some tumours demonstrate initial sensitivity to anti-VEGF therapy, but acquire resistance later as a result of the upregulation of bFGF within the tumour after treatment with an anti-VEGFR2 antibody (Ref. 198). Some tumours also have a natural resistance to anti-VEGF therapy because of $CD11b^{+}Gr1^{+}$ myeloid cells (Ref. 199). The high cost of these therapies and their lack of efficacy, coupled with significant toxicity in the form of hypertension (Ref. 200), suggest a need for designing additional antiangiogenic agents. Cartilage as a naturally occurring avascular tissue was considered a good source of antiangiogenic molecules that might fill the gaps from anticancer therapies resulting from

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targeting VEGF alone. Indeed, studies on these cartilage-derived antiangiogenic molecules have demonstrated the versatile and complex nature of their antiangiogenic, and in some cases, direct anticancer (ChM-1 and PIIBNP) effect, that is not restricted to targeting VEGF (see Table 1 and Fig. 3).

However, none of these cartilage-derived molecules have so far had much success in clinical trials. Their in vitro assessments and highly successful preclinical trials have had little bearing on their success in humans. Although their in vivo antiangiogenic benefit was largely visible only under certain experimental conditions, such as in a fracture model or cartilage lesion model, deficiency of these molecules in mouse models did not create any abnormal pathology. Perhaps redundancies in functions are responsible for this effect. Discovery of these molecules has not helped to explain how cartilage remains avascular either. Furthermore, Col IIB, ChM-1 and endostatin levels in cartilage drop drastically with maturity and yet the cartilage remains avascular in a healthy individual. Thus, cartilage might derive its antiangiogenic benefit, not from a single molecule, but from the sum total from several individual antiangiogenic molecules, or all cartilage components might need to be present together for it to be antiangiogenic. This suggests that it would be improbable to expect total comprehensive benefit based on administration of a single antiangiogenic molecule in clinical trials. Combinations of molecules, such as TSP1 plus endostatin, in trials might provide better benefit. In preparations of Neovastat, the individual molecules remain largely unidentified. It needs to be ascertained whether Neovastat extract by any chance contains the water-soluble fractions of all the single molecules discussed above. The only identified molecule in Neovastat is the kappa light chain (which had the ability to stimulate tPA activity; see Ref. 201), which cannot be considered cartilage specific.

Attempts to identify individual shark cartilage components that demonstrate antiangiogenic properties showed sequence similarity to alpha parvalbumin family (SCP1 protein) (Ref. 202) or novel proteins (Refs 203, 204), but not to the molecules discussed above. Although Col IIB has recently been recognised, molecules in cartilage identified as antiangiogenic so far are largely the minor cartilage components. Not many reports on articular cartilage aggrecan, the other major macromolecule, as an antiangiogenic compound exist, although intervertebral disc aggrecan has been demonstrated to be inhibitory to endothelial cell adhesion and migration (Ref. 205), and a polysaccharide isolated from porcine cartilage, an aggrecan-derived component, has demonstrated apoptosis of tumour cells by activation of caspase-3 and caspase-9 (Ref. 206). Recently, articular cartilage glycosaminoglycan has been demonstrated to inhibit endothelial cell adhesion (Ref. 207). Other cartilage components such as Troponin 1 (Ref. 208), which interferes with bFGF function by binding to bFGF receptor (Ref. 209), metastatin complex, which disrupts endothelial cell proliferation by binding to hyaluronan (Ref. 210), the 16 kDa N-terminal fragment of prolactin with ability to inhibit bFGF (Ref. 211), or the tissue inhibitors of metalloproteinases that are often upregulated by drugs that inhibit angiogenesis (Ref. 212), have not been pursued much beyond their initial recognition of their antiangiogenic properties.

There is no simple explanation for the overall disappointment with these molecules. In the case of Neovastat, lack of PK/PC data was considered a hindrance to analyse its failure. But PK/PC values are available for TSP1 and endostatin, but have not helped in alleviating their failures. Peptide mimetics of TSP2 have not been reported and considering the lack of significant benefit from TSP1, antiangiogenic properties of TSP2 might need further evaluation. The mechanisms of inhibitory processes for most of these molecules are only marginally understood. Understanding the complete antiangiogenic mechanism might better prepare us for clinical trials. This can be exemplified for endostatin where a lack of understanding of the ZBP domain in the N-terminus led to the use of *P. pastoris* expressed, N-terminally truncated, recombinant human endostatin in clinical trials, which did not demonstrate any therapeutic efficacy. The genetic heterogeneity of the human population could also be a hindrance to translating the success from the preclinical, syngeneic mouse models. Formulating a new strategy or criteria for testing antiangiogenic molecules might be necessary before any further investments in clinical trials.

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Table 1. Summary of cartilage-derived antiangiogenic molecules, their modes of inhibition in endothelial cells and outcomes in clinical trials

	Receptor(s)	Mode(s) of inhibition	Clinical trials	Outcome of clinical trials
Neovastat	Not known	Activates caspase-3, -8, -9 (Ref. 11) Interferes with VEGF binding to its receptor (VEGFR-2) (Ref. 13) Inhibits MMPs (Ref. 14) Activates JNK and NF-κB signalling pathways (Ref. 24) Stimulates tPA plasminogen system (Ref. 24)	Phase I (Ref. 27) Phase II (Ref. 26) Phase III (Ref. 28)	No toxicity in Phase I/II trials; No efficacy and no survival benefit in Phase III trials
ChM-1	Not known	Disrupts actin organisation (Ref. 53) Suppresses Rac1/Cdc42 GTPase (Ref. 53) In tumour cells: Inhibits DNA synthesis (Ref. 52) Suppresses STAT signalling (Ref. 52) Downregulates cyclin D1/D3 and cdk6 and upregulates cell cycle inhibitor $p21^{cip1}$ (cell cycle arrest) (Ref. 52)	None	
TSP1	CD36 (Refs 61, 62, 64) VLDL receptor (Ref. 70) HSPGs/LRP1 (Ref. 71)	Activates caspases (Ref. 61) Activates FasL (Ref. 62) Upregulates $p21^{cip}$/cell cycle arrest (Ref. 69) Reduces MMP/VEGF activity by forming complexes with them that are targeted for clearing (Refs 73, 74, 75)	Phase I (Refs 102, 103, 107, 108, 109) Phase II (Refs 104, 105, 106)	Phase I/II trials demonstrated safety and good PC and PK profiles, but patient survival was not impressive
Endostatin	VEGFR-2 (Ref. 118) Glypican (Ref. 119) Nucleolin (Refs 120, 121) $\alpha_5\beta_1$ integrins (Ref. 123)	Suppresses VEGF and induces PEDF (Refs 114, 115, 116) Upregulates VEGF165b (Ref. 117) Inhibits nucleolin phosphorylation (Ref. 121) Downregulates Rho-GTPase/uPA to disorganise actin cytoskeleton (Ref. 123) Prevents MMP activation (Refs 125, 126) Downregulates antiapoptotic Bcl-2 and Bcl-x_L (Ref. 128) Inhibits Wnt signalling (Ref. 129) Upregulates Beclin-1, thereby altering Bcl-2 (or Bcl-x_L)/Beclin ratios (Ref. 131) Tyrosine phosphorylation of Shb adaptor protein (Ref. 132)	Phase I (Refs 163, 164, 165, 166, 167, 168) Phase II (Refs 169, 170, 171) Phase III (?)	Phasel/II with recombinant human endostatin showed no benefit. Phase I/II with Endostar (ZBP-endostatin) showed benefit but primarily in combination with therapeutic drugs, but no benefit was seen in a Phase II randomised double-blind placebo trial
PIIBNP (Ref. 194)	$\alpha_V\beta_5$ and $\alpha_V\beta_3$ integrins	In tumour cells: Induction of necrosis in an integrin- and RGD-dependent manner	None	

Question mark indicates lack of publication in an English language publication and therefore inability to review (see Further Reading section).

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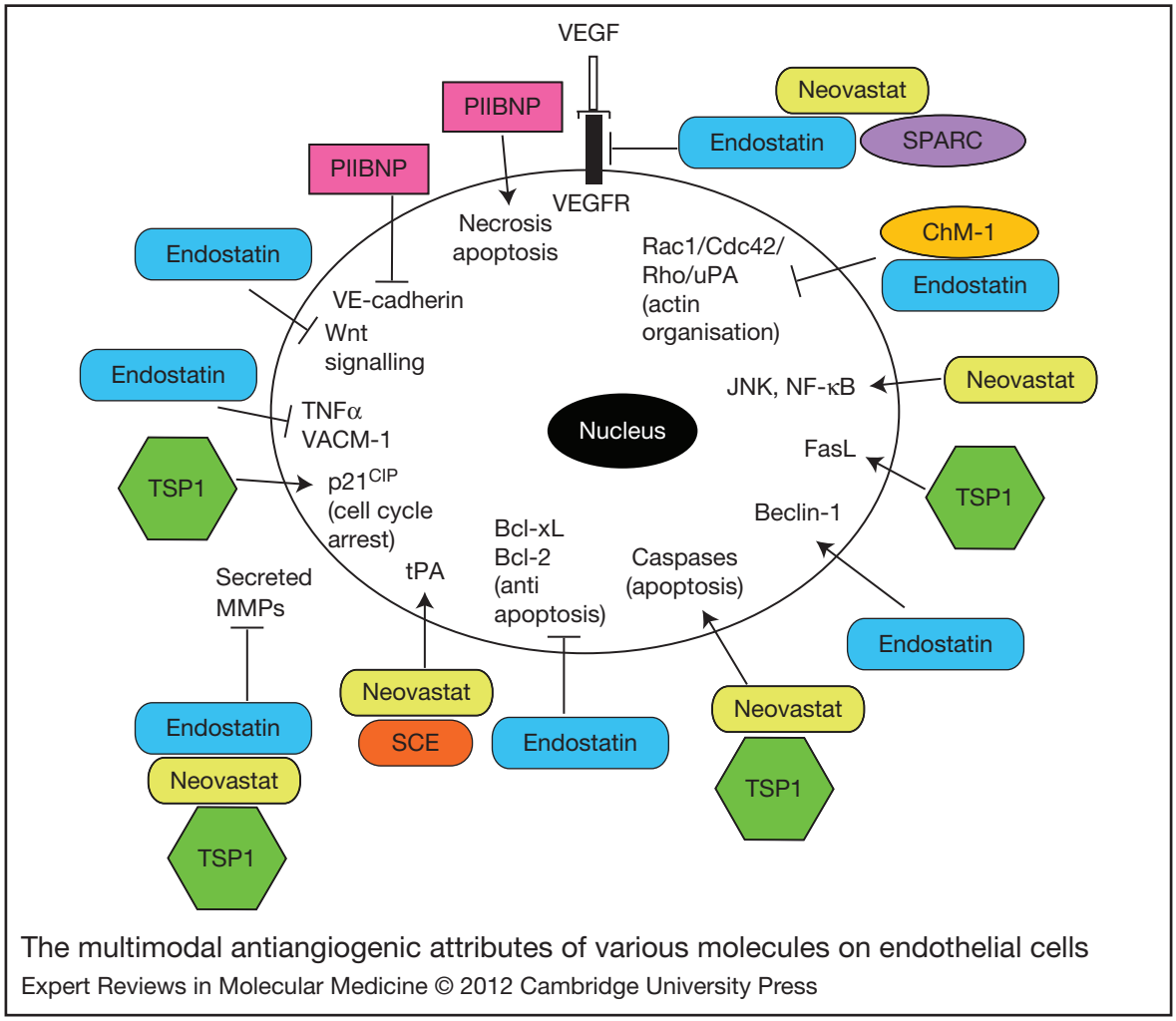

Figure 3. The multimodal antiangiogenic attributes of various molecules on endothelial cells. Representation of the effects of Neovastat, chondromodulin-1 (ChM-1), thrombospondin-1 (TSP1), endostatin, SPARC (secreted protein acidic and rich in cysteine) and the type II collagen-derived N-terminal propeptide (PIIBNP) on endothelial cells (see also Table 1 and text for further details).

Designing effective administration, improving half-life, or identification and modification of peptide derivatives are being tested to tackle these shortcomings. For example, attachment of polyethylene glycol to the N-terminus of Endostar to reduce proteolytic susceptibility (Ref. 213), peptide derivatives of endostatin further modified by the addition of RGD sequence for improved activity (Refs 214, 215), addition of the tumour-penetrating peptide internalisation sequence (iRGD) to endostatin to facilitate penetration into extravascular tumour tissue that demonstrated stronger antiangiogenic/antitumour activity (Ref. 216), and finally, endostatin-expressing Ad vectors encapsulated in cationic liposome to allow delivery to cancer cells lacking Ad receptors (Refs 217, 218), have all found success in preclinical trials, although not yet in humans. The criteria of what is a better biomarker of a successful clinical trial might also need to be revisited. Antiangiogenesis prevents blood vessel formation that shrinks tumours, rather than by being cytotoxic; this is a slow process that might need time for characterisation and recognition of the clinical end point. Antiangiogenesis remains an attractive therapeutic application, but it would appear that its sources need to be scrupulously and completely understood to translate them into beneficial clinical applications.

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Further reading, resources and contacts

Website

For detailed information on clinical trials using anti-VEGF therapies, endostatin or the TSP1-derived ABT-510 in treatment of cancer, see:

http://clinicaltrials.gov

Review articles

Bonnet, C.S. and Walsh, D.A. (2005) Osteoarthritis, angiogenesis and inflammation. Rheumatology 44, 7-16

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Further reading, resources and contacts *(continued)*

This review discusses how angiogenesis promotes osteoarthritis and the importance of the antiangiogenic approach in designing therapies for osteoarthritis.

Zheng, M.-J. (2009) Endostatin derivative angiogenesis inhibitors. Chinese Medical Journal 122, 1947-1951

The review discusses peptide derivatives from endostatin and their success in preclinical trials.

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This review describes the various antiangiogenic peptides derived from several different classes of biological molecules and their trials and tribulations.

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The article is in Chinese and therefore we were unable to review it. It is the only published Phase III clinical trial for endostatin in humans, and readers may be interested in the abstract that is available in English in PubMed (PMID: 21108883), which suggests a beneficial response in NSCLC patients treated with endostatin when combined with chemotherapy.

Faye, C. et al. (2009) The first draft of the endostatin interaction network. Journal of Biological Chemistry 284, 22041-22047

This article describes an endostatin network that involves physical interaction with other proteins including TSP1 and SPARC identified by surface plasmon resonance studies, indicating the extremely complicated mode of endostatin behaviour.

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Goel, S. et al. (2011) Normalization of the vasculature for treatment of cancer and other diseases. Physiological Reviews 91, 1071-1121

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Features associated with this article

Figures

Figure 1. Schematic representation of the cartilage matrix showing many of the molecules present in cartilage and some of their interactions.

Figure 2. The antitumour activity of the type IIB collagen protein derived PIIBNP propeptide.

Figure 3. The multimodal antiangiogenic attributes of various molecules on endothelial cells.

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Features associated with this article *(continued)*

Table

Table 1. Summary of cartilage-derived antiangiogenic molecules, their modes of inhibition in endothelial cells and outcomes in clinical trials.

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