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ORIGINAL ARTICLE

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Angiogenesis as a new target for cancer treatment

Abstract Neovascularization is often required for rapid growth of solid tumors and also limits vascular metastasis of tumor cells. Neovascularization-targeting agents are a recent innovation that may be a novel means of anticancer therapy. These antiangiogenic drugs have been developed by targeting cell proliferation of vascular endothelial cells, basement-membrane-degrading enzymes, angiogenic factors/receptors, extracellular matrix, angiogenesis signaling, and cell-cell/cell-matrix interactions. In this report, we describe how tumor angiogenesis occurs and how antiangiogenic agents are developed.

Key words Tumor angiogenesis · Antiangiogenic agents · Vascular endothelial cells · Angiogenic factors

Introduction

Neovascularization is involved in tumor growth [10, 16], and the transition from limited to rapid tumor growth often accompanies angiogenesis [8, 11]. The development of blood vessels within tumor tissues is also closely correlated with invasion and metastasis of cancer cells in breast cancer, melanoma, lung cancer, prostatic cancer, and other cancers [12, 21, 32, 37, 38]. Endogenous positive and negative angiogenic factors released by tumors and stroma components are thought to control tumor angiogenesis [9, 18, 20].

Vascular-targeting agents induce damage to vascular endothelial cells, resulting in a block of the blood supply to tumors and in the metastatic process [3, 10, 16]. Various antiangiogenic agents have been developed in in vitro and in vivo models. The discovery of angiostatin and thrombospondin are recent highlights in this area. Angiostatin, with more than 98% homology to an internal fragment of plasminogen, suppresses endothelial cell proliferation in vitro and angiogenesis in remote metastasis in vivo [27]. Another endogenous angiogenesis inhibitor is thrombospondin, a multifunctional, heparin-binding glycoprotein that competes with fibroblast growth factors (FGFs) for binding to matrix elements [7]. Platelet factor 4 (PF 4) [34], interferon-α (IFN-α), and antibodies against vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) [2, 17] and integrin αvβ3 [4, 5] also modulate tumor neovascularization.

We have established tumor angiogenesis models using vascular endothelial cells in vitro and the dorsal air sac of mice in vivo [1, 23, 24]. We have previously reported that enhanced expression of tissue-type plasminogen activator is required for angiogenesis in vitro [22, 31]. In the tumor-angiogenesis model systems we have also screened antiangiogenic drugs. Synthetic derivatives of α-guaiaconic acid, inhibitors of arachidonic acid/prostaglandin metabolism [15], and irsogladine, an anti-gastric ulcer drug [30], have been found to have some antiangiogenic activities, and the latter strengthens gap-junction intercellular communication [14, 35]. Irsogladine inhibits tubular morphogenesis and tissue-type plasminogen activator synthesis in vascular endothelial cells [30], and our recent study indicates that it could inhibit tumor neovascularization in mice [26]. In this report, we describe our angiogenesis models and some properties of antiangiogenic drugs.

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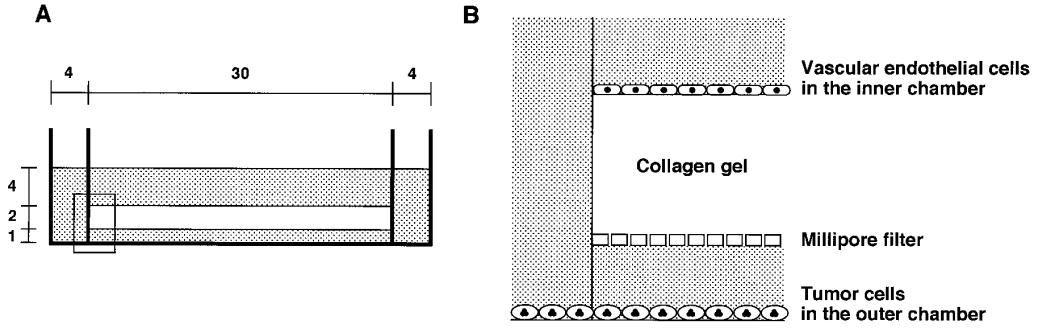

Fig. 1 **A** Coculture assay system for tubule formation. In this system, each well is composed of 2 chambers: an outer chamber (38×7 mm) and an inner chamber (30×7 mm). Tumor cells were grown in medium containing 10% serum in the outer chamber, and at confluence the medium was replaced with fresh medium. Vascular endothelial cells were seeded on type I collagen gels (*nonhatched area*) in 2 ml of 10% serum-containing medium in the inner chamber with a filter at the bottom of the chamber. At confluence, the concentration of serum in the medium was decreased to 1% (*hatched area*) in both chambers and the inner chamber was transferred into the outer chamber. After incubation for 3 days, tubule formation by vascular endothelial cells in collagen gels was determined. **B** Detail of the diagram shown in **A** (indicated by the *rectangle*)

Materials and methods

Tube formation by vascular endothelial cells in type I collagen gels and quantitative analysis

Human microvascular endothelial cells were plated onto the surface of type I collagen gels in M-199 medium containing 10% fetal bovine serum (FBS). When the cells reached confluence, the medium was replaced with M-199 medium containing 1% FBS and various growth factors and the cells were incubated for 3 days. The medium was changed on the 2nd day. On the 3rd day, phase-contrast microscopic pictures of each dish were recorded using a still video camera recorder (R5000H; Fuji, Tokyo, Japan), and the total length of tube-like structures per field was measured using a Cosmozone Image Analyzer IS (Nikon, Tokyo, Japan) as described previously [24, 25, 31]. Eight random fields per dish were measured, and the total length per field was calculated.

Assay of tube formation in the coculture system

We have established an assay system with which tumor-cell-dependent tube formation by vascular endothelial cells can be determined in type I collagen gels [1, 25, 33, 35] (Fig. 1). Tumor cells were cultured in the outer chamber of six-well plates (each well 38×7 mm; Corning Glass Works, Corning, N.Y., USA) in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. At confluence the medium was changed to 2 ml of DMEM containing 1% FBS. Vascular endothelial cells were seeded separately in 2 ml of M-199 containing 10% FBS on type I collagen gels (1 ml) on culture plates with 0.4-μm filters (Millicell-CM; Millipore Laboratory Products, Bedford, Mass., USA) in the inner chamber (30×7 mm). When the endothelial cells reached confluence the FBS content of the medium was reduced from 10% to 1% and the inner chamber was transferred into the outer chamber. In this system, tubulogenesis should occur in endothelial cells in the collagen gel in the inner chamber when tumor cells cocultured in the outer chamber secrete angiogenic factors that pass through the filter of the inner chamber.

After a 3-day period of incubation, tube formation in the endothelial cells was quantified by recording on a floppy video disk. The total length of tube formation was analyzed using the COSMOZONE program (NEC PC-9801; NEC, Tokyo, Japan).

Tumor angiogenesis assay in mice

A dorsal air sac was created in 5- to 7-week-old male mice by injection of 10–15 ml of air according to the method published previously [1]. Various cell lines were suspended in phosphate-buffered saline at a concentration of 1×10^7 cells/ml; 0.2 ml of this suspension was injected into a chamber (Millipore) consisting of a ring (Millipore) with filters (0.22-μm pore size; Millipore) on both sides. Chambers containing bovine serum albumin (10 mg/ml) or tumor cells were implanted into mouse dorsal air sacs.

Five mice in each group were killed and carefully skinned on day 5. After the implanted chamber had been removed from the subcutaneous air-sac fascia, a ring without filters was placed on the same site and photographed. The area of the air-sac fascia with a dense capillary network was quantified from the photograph using an image processor (Nexus, Tokyo, Japan).

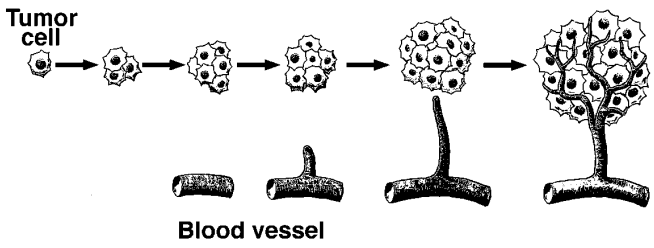

Fig. 2 Development of tumor angiogenesis. Tumor cells proliferate and tumor size is enlarged. Further tumor enlargement requires a blood supply through newly developed capillary networks. Tumor cells in concert with vascular endothelial cells are thought to produce/activate angiogenic factors and relevant proteases, resulting in induction of migration, digestion of basement membranes, proliferation, and, finally, tube formation by vascular endothelial cells. Development of new capillary networks is also thought to provide a pathway for metastasis of tumor cells

Results and discussion

Tumor angiogenesis models for screening of antiangiogenic drugs

Tumor angiogenesis (Fig. 2) is a complex phenomenon that involves at least three sequential steps: degradation of vascular basement membrane and matrixes by endothelial cells, migration and proliferation of endothelial cells, and formation of capillary loops by endothelial cells [15]. The proliferation, migration, and tubular morphogenesis of vascular endothelial cells are often assayed to determine

Table 1 Endogenous angiogenic factors (*HGF* Hepatocyte growth factor, *EGF* epidermal growth factor, *HB-EGF* heparin-binding epidermal growth factor-like growth factor, *PD-ECGF* platelet-derived endothelial-cell growth factor)

Positive factors	Negative factors
Acidic FGF basic FGF	Angiostatin
HGF	TGF-β
VEGF/VPF	IFN-α/IFN-β
IL-8	PF 4
Placenta growth factor	Prolactin fragment
TGF-α/EGF/HB-EGF	Thrombospondin
Angiogenin	Tissue metalloproteinase inhibitors
TNF-α (low dose)	TNF-α (high dose)
Prostaglandins E1, E2	Cartilage-derived inhibitor
Integrin αvβ 3	
Thymidine phosphorylase (PD-ECGF)	

angiogenic activity in vitro. In vivo angiogenesis assays include the chicken chorioallantoic membrane (CAM) assay, the cornea vasculature assay, and the dorsal air sac assay (see Materials and methods), among others.

We have established a model system for tumor angiogenesis in vitro using endothelial cells cocultured with tumor cells and have examined whether the tumor cells can induce angiogenesis [1, 24, 25] (Fig. 1). In this assay system, human cancer cells that produce high levels of transforming growth factor alpha (TGF-α) or basic FGF (bFGF) induce tube formation of vascular endothelial cells in type I collagen gels [1, 24, 25]. Using this coculture assay system, we analyzed tube formation by bovine aortic endothelial cells and human microvascular endothelial cells cocultured with tumor cells. It was expected that tubulogenesis would be induced in vascular endothelial cells on the surface of the collagen gel in the inner chamber when human tumor cells cultured in the outer chamber secreted any potent angiogenic factor.

Endogenous regulators of angiogenesis are known (Table 1) [9], and we tried to determine which factor was involved in angiogenesis by human glioma cells. In the coculture model system, we observed that human glioma cells producing higher levels of bFGF, interleukin 8 (IL-8), or VEGF/VPF induced tubular morphogenesis by vascular endothelial cells [1, 36]. Glioma cells producing high levels of bFGF also developed capillary networks in the dorsal air sac assay in mice [1], suggesting that bFGF is highly angiogenic in bovine aortic endothelial cells and in mice.

Table 2 Antiangiogenic agents

Steroids
Carboxyamidotriazole
Peptides from laminin/fibronectin
Fumagillin derivatives (AGM-1470)
Pentosan polysulfate
Anthrobacter polysaccharides (DS-4152)
Herbimycin A, genestein
Irsogladine
Derivatives of retinoic acid or vitamin D3
Antibodies against integrins

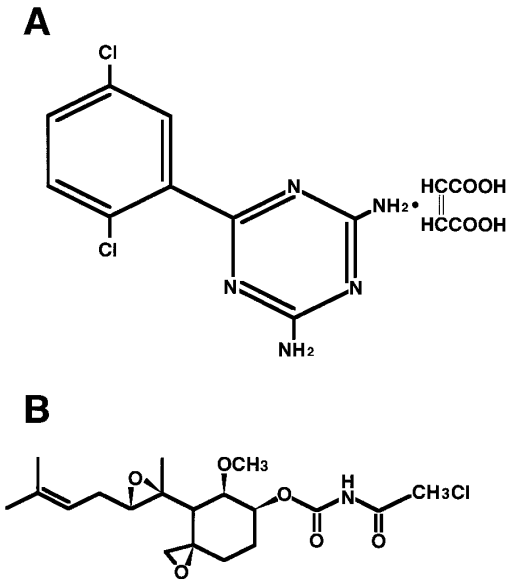

Fig. 3A,B Structures of **A** irsogladine and **B** AGM-1470 (TNP-470)

Analysis of patients with glioma has indicated that VEGF/VPF is closely related to the tumor vasculature, whereas bFGF, TGF-α, and TGF-β are not [29]. Whether bFGF or IL-8 is involved in tumor angiogenesis in human brain tumors remains to be determined. However, factors other than these are also expected to be involved in other types of human tumors.

Exogenous inhibitors of angiogenesis

Many antiangiogenic drugs have been reported (Table 2), and some are being tested in clinical trials [16]. Fumagillin and its derivative AGM-1470 (TNP-470; Fig. 3) are potent antiangiogenic drugs. AGM-1470 specifically inhibits the growth of vascular endothelial cells rather than tumor cells and also inhibits angiogenesis in the CAM and dorsal air-sac assays [13, 19]. AGM-l470 administration induces antitumor activity in mouse Lewis lung cancer and B16 melanoma in vivo [13, 19].

In contrast, irsogladine (Fig. 3), which strengthens the gap junction, is used clinically as an anti-gastric ulcer drug; it inhibits synthesis of tissue-type plasminogen activator in vascular endothelial cells and tubular morphogenesis by vascular endothelial cells [30]. Our previous studies have demonstrated that expression of the plasminogen activator gene is a prerequisite for angiogenesis in in vitro models [22, 23, 25]. Antitumor activity has also been observed in animal experiments when irsogladine is given [26].

Is tumor angiogenesis a real new target for cancer treatment?

Development of tumor angiogenesis-targeting agents is often referred to as a new concept in anticancer therapy [16], and antiangiogenic agents could have the following

clinical implications [6]: (1) they may overcome drug resistance in solid tumors; (2) identification of the angiogenic factors in serum or microvessels in tumors could allow the efficacy of the new agents to be quantified; (3) antiangiogenic agents may have low toxicity due to their selective effect on tumor vasculature; (4) their combination with anticancer agents may potentiate their antitumor effects; and (5) antiangiogenic drugs may show therapeutic effects not only in tumors but also in other neovascularization diseases such as rheumatoid arthritis, diabetic retinopathy, and psoriasis.

Endogenous and exogenous factors and drugs are known to modulate neovascularization through pleiotropic mechanisms (Tables 1, 2). A proposal by Folkman suggests that antiangiogenic drugs or factors could be favorable anticancer therapeutic agents [3, 6, 16]. However, the specific characteristics of tumor vasculature are not yet understood in detail, although recent studies have demonstrated the existence of distinctive endothelial cell-surface molecules that may discriminate tumor-associated vasculature from normal blood vessels. These include endoglin [6], endosialin [28], and integrin [4, 5]. Further efforts are needed to determine whether antiangiogenic agents are useful therapeutic agents.

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