Tsukada, M. Momose, J. Nakayama, T. Taki, Anti-neovascular therapy using novel peptides homing to angiogenic vessels, Oncogene 21 (2002) 2662–2669.
[11] T. Asai, K. Shimizu, M. Kondo, K. Kuromi, K. Watanabe, K. Ogino, T. Taki, S. Shuto, A. Matsuda, N. Oku, Anti-neovascular therapy by liposomal DPP-CNDAC targeted to angiogenic vessels, FEBS Lett. 520 (2002) 167–170.
[12] N. Maeda, Y. Takeuchi, M. Takada, Y. Sadzuka, Y. Namba, N. Oku, Anti-neovascular therapy by use of tumor neovasculature-targeted long-circulating liposome, J. Control. Release 100 (2004) 41–52.
[13] N. Maeda, Y. Takeuchi, M. Takada, Y. Namba, N. Oku, Synthesis of angiogenesis-targeted peptides and hydrophobized polyethylene glycol conjugate, Bioorg. Med. Chem. Lett. 14 (2004) 1015–1017.
[14] M.R. Hamblin, J.L. Miller, I. Rizvi, H.G. Loew, H.T. Hasan, Pegylation of charged polymer–photosensitiser conjugates: effects on photodynamic efficacy, Br. J. Cancer 89 (2003) 937–943.
[15] O. Bourdon, I. Laville, D. Carrez, A. Croisy, P. Fedel, A. Kasselouri, P. Prognon, P. Legrand, J. Blais, Biodistribution of meta-tetra(hydroxyphenyl)chlorin incorporated into surface-modified nanocapsules in tumor-bearing mice, Photochem. Photobiol. Sci. 1 (2002) 709–714.
[16] N. Oku, Y. Tokudome, T. Asai, H. Tsukada, Evaluation of drug targeting strategies and liposomal trafficking, Curr. Pharm. Des. 6 (2000) 1669–1691.
[17] H. Maeda, J. Fang, T. Inutsuka, Y. Kitamoto, Vascular permeability enhancement in solid tumor: various factors, mechanisms involved and its implications, Int. Immunopharmacol. 3 (2003) 319–328.
[18] J. Fang, T. Sawa, H. Maeda, Factors and mechanism of "EPR" effect and the enhanced antitumor effects of macromolecular drugs including SMANCS, Adv. Exp. Med. Biol. 519 (2003) 29–49.
[19] D.E. Dolmans, A. Kadambi, J.S. Hill, C.A. Waters, B.C. Robinson, J.P. Walker, D. Fukumura, R.K. Jain, Vascular accumulation of a novel photosensitizer, MV6401, causes selective thrombosis in tumor vessels after photodynamic therapy, Cancer Res. 62 (2002) 2151–2156.
[20] D.E. Dolmans, A. Kadambi, J.S. Hill, K.R. Flores, J.N. Gerber, J.P. Walker, I.H. Rinkes, R.K. Jain, D. Fukumura, Targeting tumor vasculature and cancer cells in orthotopic breast tumor by fractionated photosensitizer dosing photodynamic therapy, Cancer Res. 62 (2002) 4289–4294.
[21] Y. Takeuchi, K. Kurohane, K. Ichikawa, S. Yonezawa, H. Ori, T. Koishi, M. Nango, N. Oku, Polycation liposome enhances the endocytic uptake of photosensitizer into cells in the presence of serum, Bioconj. Chem. 74 (2003) 790–796.
[22] Y. Takeuchi, K. Ichikawa, S. Yonezawa, K. Kurohane, T. Koishi, M. Nango, Y. Namba, N. Oku, Intracellular target for photosensitization in cancer antiangiogenic photodynamic therapy mediated by polycation liposome, J. Control. Release 97 (2004) 231–240.
[23] F. Pastorino, C. Brignole, D. Marimpietri, M. Cilli, C. Gambini, D. Ribatti, R. Longhi, T.M. Allen, A. Corti, M. Ponzoni, Vascular damage and antiangiogenic effects of tumor vessel-targeted liposomal chemotherapy, Cancer Res. 63 (2003) 7400–7409.
[24] R.M. Schiffelers, G.A. Koning, T.L. ten Hagen, M.H. Fens, A.J. Schraa, A.P. Janssen, R.J. Kok, G. Molema, G. Storm, Anti-tumor efficacy of tumor vasculature-targeted liposomal doxorubicin, J. Control. Release 91 (2003) 115–122.
[25] M. Kondo, T. Asai, Y. Katanasaka, Y. Sadzuka, H. Tsukada, K. Ogino, T. Taki, K. Baba, N. Oku, Anti-neovascular therapy by liposomal drug targeted to membrane type-1 matrix metalloproteinase, Int. J. Cancer 108 (2004) 301–306.

Expert Opinion

Oncologic, Endocrine & Metabolic

Antineovascular therapy, a novel antiangiogenic approach

Kosuke Shimizu, Tomohiro Asai & Naoto Oku†
†*University of Shizouka, Department of Medical Biochemistry and COE Programme in the 21st Century, School of Pharmaceutical Sciences*

Angiogenesis is a crucial event in tumour growth, since the growth of tumour cells depends on the supply of essentials such as oxygen and nutrients. Therefore, suppression of angiogenesis is expected to show potent therapeutic effects on various cancers. Additionally, this 'antiangiogenic therapy' is thought not only to eradicate primary tumour cells, but also suppress tumour metastases through disruption of haematogenous metastatic pathways. Tumour dormancy therapy does not aim to disrupt newly formed angiogenic vessels but aims to inhibit further formation of neovessels through inhibiting certain processes of angiogenesis. This raises a question of whether or not these antiangiogenic agents bring complete cure of tumours as complete cut-off of oxygen and nutrients is not expected by the treatment with these agents. This paper will review a novel antiangiogenic therapy, antineovascular therapy (ANET). ANET is categorised in antiangiogenic therapy but is different from tumour dormancy therapy using conventional angiogenic inhibitors: ANET aims to disrupt neovessels rather than to inhibit neovessel formation. ANET is based on the fact that angiogenic endothelial cells are growing cells and would be effectively damaged by cytotoxic agents when the agents are effectively delivered to the neovessels. The complete eradication of angiogenic endothelial cells may cause complete cut-off of essential supplies to the tumour cells and lead to indirect but strong cytotoxicity instead of cytostasis caused by the inhibition of angiogenesis. For the purpose of ANET, an angiogenic vasculature-targeting probe has been developed, by which cytotoxic anticancer agents are actively delivered to the angiogenic endothelial cells by using drug delivery system (DDS) technology. Another way to damage newly formed vessels by cytotoxic agents is achieved by metronomic-dosing chemotherapy. This chemotherapy shifts the target of chemotherapeutic agents from tumour cells to angiogenic endothelial cells by selective dosing schedule. Similarly, the shift of target from tumour cells to angiogenic endothelial cells enhanced therapeutic efficacy of cancer photo-dynamic therapy (PDT): in this antiangiogenic PDT, photosensitizers are delivered more to neovessel endothelial cells than to tumour cells. These therapeutic strategies would be clinically applied in the future.

Keywords: antiangiogenic photodynamic therapy, antiangiogenic therapy, antineovascular therapy, drug delivery system, liposome, metronomic-dosing chemotherapy, tumour dormancy therapy

Expert Opin. Ther. Targets (2005) 9(1):63–76

1. Introduction

1.1 Tumour angiogenesis

All cells require certain essentials such as oxygen and nutrients for preservation and growth. In particular, tumour cells require such essentials more because they proliferate endlessly. Therefore, in most tumour tissues, tumour cells remain under hypoxic and starving conditions because of an excess requirement of these essentials.

Ashley Publications
www.ashley-pub.com

10.1517/14728222.9.1.63 © 2005 Ashley Publications Ltd ISSN 1472-8222

To overcome such conditions, tumour cells switch on a signal, often mediated by hypoxia inducible factor (HIF)-1, and induce angiogenesis to acquire complemented pathway for obtaining essentials [1]. Tumour malignancy correlates well with a density of new blood vessels in tumour tissues, and most tumour cells tend to metastasise to distal organs through this angiogenic vasculature. Therefore, inhibition of angiogenesis promises suppression of tumour growth and metastasis by interrupting supplemental and metastatic pathways.

In recent years, the molecular events that play the role in angiogenesis have been elucidated [2,3]. Angiogenesis is generally regulated by many cytokines produced by stromal, endothelial and tumour cells in a tumour-bearing body [4]. In physiological angiogenesis, such as wound healing and female reproductive cycle, both pro- and antiangiogenic factors cooperate in a balanced manner to control appropriate angiogenesis. However, in pathological conditions, angiogenesis is often out of control. For example, in age-related macular degeneration (AMD), uncontrolled angiogenesis causes blindness [5]. Tumour angiogenesis is also out of control. Pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), angiopoietin, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and IL-8, may function strongly compared with the actions of antiangiogenic factors such as angiostatin, endostatin, and thrombospondin [4]. Pro-angiogenic factors often stimulate endothelial cells in a receptor-dependent manner and induce angiogenesis. For example, VEGF, the major pro-angiogenic cytokine, stimulates endothelial cells through binding to VEGF receptors, such as KDR/Flk-1 and Flt-1 expressed on endothelial cells, and promotes angiogenesis [6,7]. The binding of VEGF to KDR/Flk-1 initiates a signal transduction in endothelial cells and these stimulated endothelial cells come to proliferate, migrate, and subsequently construct new vessels through the activation of mitogen activated protein kinase (MAPK), focal adhesion kinase (FAK), Akt etc [6,8,9]. However, antiangiogenic factors also control angiogenesis in various patterns of mechanism. For example, tissue inhibitor of metalloproteinases (TIMPs) generally inhibit activation of matrix metalloproteinases, which play an important role in migration, and invasion of endothelial cells into extracellular matrix (ECM) prerequisite for angiogenesis [10]. Although TIMP-2 inhibits angiogenesis in a matrix metalloproteinase (MMP)-dependent manner [11], it also inhibits proliferation of endothelial cells through direct binding to integrin $\alpha 3\beta 1$ and blocking of VEGF-induced signal transduction [12]. Additionally, TIMP-3 inhibits angiogenesis by competing with VEGF on receptor binding and stabilising death receptors, resulting in apoptosis of endothelial cells [13,14]. Therefore, TIMPs are focused as a natural angiogenic inhibitor.

Recently, it has been clarified that progenitor cells derived from bone marrow often participate in angiogenesis [15]. In general, these progenitor cells are related with vasculogenesis in embryonic development. However, these cells also flock to an angiogenic site during tumour angiogenesis and differentiate into endothelial cells [16,17]. The mechanism of recruitment of endothelial progenitor cells into angiogenesis is not fully understood but mobilisation of Kit-ligand (KitL) by MMP-9 in the stromal area seems to involve recruitment of progenitor cells from bone marrow [18,19]. Thus, MMP-9 contributes to promote angiogenesis [20]. In this way, tumour angiogenesis is established not only with stimulated local endothelial cells, but also recruited endothelial progenitor cells [21]. The understanding of tumour angiogenic process provides us new targets for angiogenic inhibitors.

In this review, the authors will briefly describe some of the regulatory molecules of angiogenesis and their inhibitors, and then discuss the feature of antineovascular therapy (ANET) and its effectiveness as a novel strategy in cancer chemotherapy.

2. Molecules playing central roles

2.1 Vascular endothelial growth factor and its receptors

As described above, angiogenesis is regulated by various kinds of cytokines. Most of these factors act in a receptor-dependent manner to promote angiogenesis. VEGF is one of the most important cytokines among them. VEGF is secreted by various kinds of cells such as tumour cells. Although VEGF expression is regulated by various kinds of factors, hypoxia seems to play an important role, since the HIF-1 binding site exists in the 5'-promoter region of the *vegf* gene and thus HIF-1 regulates transcription of the *vegf* gene [22,23]. VEGF binds to its receptors (VEGF-R) expressed on the plasma membrane of the endothelial cells and regulates angiogenesis.

It is known that KDR/Flk-1 and Flt-1, which are members of VEGF-R family, are mainly involved in angiogenesis because abnormal vessels in the fetal period are observed and caused fetal death in each gene knockout model mouse [24]. Thus, blockage of binding VEGF to VEGF-R enables angiogenesis to be inhibited. In fact, blockage of VEGF-binding by anti-KDR/Flk-1 antibody induced apoptosis of endothelial cells and subsequently suppressed of tumour growth [25,26]. Functional peptide, derived from the phage-displayed peptide library with *in vitro* biopanning, also blocks interaction between VEGF and its receptor and inhibits angiogenesis following regression of solid tumour [27]. Additionally, when anticancer agents were administered with anti-KDR/Flk-1 antibody in tumour-bearing mice, tumour growth was effectively suppressed in comparison with the treatment of the anticancer agent alone or of the antibody alone because of synergistic effect of the tumour cell killing and the inhibition of angiogenesis [28]. However, soluble Type VEGF receptor, sFlt-1, is focused as an angiogenic inhibitor. In general, Flt-1 that lacks VEGF signal transduction, has higher affinity compared with KDR/Flk-1 and regulates VEGF-binding to KDR/Flk-1, and sFlt-1 similarly controls angiogenesis through modulating the binding of VEGF to KDR/Flk-1 [29]. Thus, overexpression of sFlt-1 at angiogenic site by gene

transfer promises inhibition of angiogenesis by inhibiting binding of VEGF to KDR/Flk-1 [30,31].

2.2 ECM-degradative enzyme, matrix metalloproteinase

Activated endothelial cells become to proliferate, migrate and invade into the ECM in order to form new sprouts from pre-existing vessels and subsequent angiogenic vasculature [32]. In order to invade into the ECM, endothelial cells or surrounding stromal cells express or secret various kinds of degradative enzymes and digest ECM. MMPs are respective enzymes among them. MMPs, which are zinc dependent proteases and consist of ~20 members, play roles in angiogenesis and metastasis [33]. Each MMP has substrate-specificity. For example, MMP-2 and MMP-9, which were originally known as gelatinase, specifically digest some types of collagen to promote migration of angiogenic endothelial cells through the ECM. Thus, the blockage of the MMP activity potentially inhibits angiogenesis. For this purpose, various kinds of MMP inhibitors have been developed [34]. A recent study indicates that laser-induced choroidal neovascularisation is strongly attenuated in mice deficient in the expression of both MMP-2 and -9 compared with single deficient mice [35]. Therefore, an inhibitor for both MMP-2 and -9 would be useful for the suppression of angiogenesis.

2.3 Integrins and other adhesion molecules

Endothelial cell migration is an important step in angiogenesis. Cell migration depends on both cell motility and adhesion to ECM. Integrins expressed on endothelial cells seems to be important molecules in cell adhesion [36]. They bind to ECM components such as vitronectin and fibronectin and also bind to cell membrane ligands [37]. Integrins are heterodimeric receptors composed of both α- and β-chains, and their intracellular domain makes complex with certain skeleton-related proteins such as actin and vinculin. Integrins also bind to FAK and their activation signals are transmitted through this molecule. Integrin $\alpha_v\beta_3$ is the most focusing integrin in angiogenesis because this molecule is upregulated on angiogenic endothelial cells [38]. It is important as not only an adhesion molecule, but also as a MMP mediator [39]. Thus, integrin $\alpha_v\beta_3$ mediates endothelial proliferation, adhesion and invasion [40]. Vitaxin (LM609) has been developed as a neutralising antibody against integrin $\alpha_v\beta_3$. It inhibits bFGF-induced angiogenesis and induces endothelial cell apoptosis [41]. Small molecule inhibitors, such as a small peptide containing RGD (Arg-Gly-Asp) sequence or RGD peptide mimetics, also inhibit angiogenesis through blockage of integrin adhesion to extracellular matrix and subsequent induction of cell apoptosis [42,43].

Vascular endothelial-cadherin (VE-cadherin) has recently been focused as adhesion molecule, which is localised within specialised structures at cell – cell contact, referred to as an adherence junction [44,45]. VE-cadherin is specifically expressed on endothelial cells and plays an important role in endothelial cell growth, migration, adhesion, and, in particular, tube formation [46,47]. Therefore, this molecule is expected as a novel target molecule in antiangiogenic therapy. In fact, selective targeted antibody against VE-cadherin inhibits angiogenesis and subsequent tumour growth without affecting vascular permeability and other side effect [48].

3. Typical inhibitors of angiogenesis

3.1 Endogenous inhibitors (angiostatin, endostatin, and thrombospondin-1)

Angiogenesis is generally regulated with the balance between pro-angiogenic factors and antiangiogenic factors. However, in abundant angiogenesis such as tumour angiogenesis, pro-angiogenic factors mainly exist at angiogenic sites compared with antiangiogenic factors, and induce excessive angiogenesis. Thus, if antiangiogenic factors become predominant at the angiogenic site, angiogenesis might be smoothly suppressed. O'Reilly *et al.* originally found an endogenous antiangiogenic factor, which is known as angiostatin [49]. Angiostatin consists of four kringle domains of plasminogen and effectively suppresses angiogenesis by preventing endothelial cell proliferation and migration [50]. There are some opinions about targeting molecules of angiostatin, but accurate antiangiogenic mechanisms of angiostatin have not yet been elucidated, except that angiostatin binds to ATP synthase, angiomotin, and annexin II [51,52]. Endostatin is another endogenous angiogenic inhibitor, isolated from the murine endothelioma cell line [53]. Endostatin is composed of C-terminal fragments of collagen XVIII and also inhibits endothelial cell proliferation, migration and invasion. The molecular mechanism of its action is not yet clear, although it has recently been suggested that endostatin inhibits not only catalytic activity of MMPs, but also VEGF-induced phosphorylation of KDR/Flk-1 receptor and subsequent ERK, p38 MAPK, and FAK [54]. These endogenous angiogenic inhibitors induce tumour dormancy, which means the unvaried condition in tumour volume, by inhibiting angiogenesis.

Thrombospondin-1 (TSP-1) is a large glycoprotein secreted by various cells and composed of ECM. TSP-1 has multifunctions, such as platelet aggregation and vascular homeostasis. Among them, it is known that TSP-1 acts as an endogenous angiogenic inhibitor [55]. TSP-1 inhibits endothelial cell proliferation and migration by inhibiting MMP-9 activation and subsequent VEGF mobilisation from ECM [56]. Furthermore, TSP-1 induces endothelial cell apoptosis through the CD36 receptor [57]. Additionally, type I repeat synthetic peptide of TSP-1, which mimicked antiproliferative activity in TSP-1 inhibits angiogenesis and induces endothelial apoptosis [58].

3.2 Molecular-targeting agents

As described above, angiogenesis is regulated by many cytokines and, in particular, VEGF is known as one of the most potent pro-angiogenic factors among them. These cytokines mainly transmit their signals through their

receptors. Once they bind to their receptor, these receptors are activated by receptor-mediated tyrosine phosphorylation. Recently, some approaches to inhibit receptor-mediated phosphorylation have been considered in order to inhibit angiogenesis. These inhibitors are generally known as molecular-targeting agents. There are two types of the agents; direct and indirect angiogenic inhibitors. Direct angiogenic inhibitors generally prevent receptor tyrosine phosphorylation in the endothelial cells such as KDR/Flk-1 receptor phosphorylation and inhibit subsequent signal transduction [59]. Because phosphorylation of the KDR/Flk-1 receptor induces pro-angiogenic signalling, such as MAPK and FAK, the direct angiogenic inhibitor prevents endothelial cells from proliferation, migration, invasion, and subsequent angiogenesis. For example, the molecular targeting inhibitor SU5416, specifically prevents VEGF receptor phosphorylation and subsequent signal transduction [60]. As a result, SU5416 effectively suppresses angiogenesis without side effects because VEGF receptor phosphorylation specifically occurs in angiogenic endothelial cells [61].

On the other hand, indirect angiogenic inhibitors prevent expression or secretion of pro-angiogenic factors in tumour cells and subsequently inhibit angiogenesis. In some cases, tumour cells depend on EGF receptor-mediated signalling such as MAPK to grow [62]. This signalling causes generation of pro-angiogenic factors such as VEGF and PDGF. Therefore, prevention of EGF receptor phosphorylation leads to the subsequent inhibition of the release of pro-angiogenic factors from the tumour cells. Gefitnib (ZD1839), was initially developed as an inhibitor of EGF receptor phosphorylation for preventing tumour growth, but it has now been clarified that Gefitinib shows antiangiogenic effect in order to both preventing production of angiogenic factors and direct inhibition of EGF-mediated angiogenesis in angiogenic endothelial cells [63]. Therefore, Gefitinib suppressed tumour growth effectively [64]. Trustuzumab also shows indirect antiangiogenic effect. Trustuzumab is a monoclonal antibody against human epidermal growth factor receptor-2 (HER-2) that is often expressed on breast cancer cells. Trustuzumab effectively suppresses tumour growth through original antitumour effect but it also inhibits angiogenesis because of adaptive antiangiogenic effect [65]. These molecular targeting agents are expected as a new type of anticancer agent with decreased side effects.

A fumagillin derivative, TNP-470 (AGM1470), which was isolated from *Aspergillus fumagilus*, strongly inhibits *in vitro* angiogenic endothelial cell growth and *in vivo* angiogenesis with decreased side effects [66,67]. The antiangiogenic mechanism of TNP-470 is not fully understood but some results indicate that TNP-470 binds to methionine aminopeptidase-2 (MetAP-2) which is highly expressed during cell proliferation and decreases cyclin D1 expression [68,69]. Marchio *et al.* recently reported that a peptide specifically bound to aminopeptidase A suppressed migration and proliferation of endothelial cells, inhibited angiogenesis, and suppressed tumour growth [70].

It has been known that expression of cyclooxygenases, in particular cyclooxygenase-2 (COX-2), is upregulated and induces tumour cell proliferation but a new function of COX-2 in angiogenesis becomes focused [71,72]. COX-2 is a catalytic enzyme, which transforms arachidonic acid into some inflammation-related molecules such as prostaglandins (PGs), and thromboxane (TX). The expression of COX-2 correlates well with expression of pro-angiogenic factors such as VEGF in tumour cells [73]. PGs also induce expression of HIF-1 and VEGF [74]. These observations indicate the close relationship between angiogenesis and COX-2. In fact, the non-selective COX inhibitor, indometacin, and selective COX-2 inhibitor, SC-236, inhibited angiogenesis in a dose-dependent manner [75]. Thus, it is expected that COX-2 inhibitors are useful in cancer chemotherapy because of their antitumour and antiangiogenic effects.

Thalidomide is an agent that has once been developed as potential sleeping pills. However, this agent was restricted and subsequently avoided because of dangerous teratogenesis and phocomelia (stunted limb growth). It has been clarified that these severe side effects are caused by strong inhibition of vessel formation during the fetal period. Thalidomide was re-evaluated as a potential agent for reducing the pain of Hansen's disease and as a potential angiogenesis inhibitor in a limited dose [76]. Thalidomide and its analogue effectively inhibited tube formation *in vitro* and angiogenesis, and suppressed tumour growth [77]. Although further research is needed in order to clarify the cellular targets and to define how they work, thalidomide may be a potential angiogenic inhibitor if historical and social issues are faded.

4 Antineovascular therapy

4.1 Concept of antineovascular therapy

Antineovascular therapy (ANET) is targeting angiogenesis; however, the concept is different from conventional antiangiogenic therapy where agents inhibit neovessel formation through inhibiting a certain step of angiogenesis. ANET targets newly formed vessels and causes lethal damage of the vessels which leads to indirect damage of tumour cells and inhibits further vessel formation. Tumour angiogenic vasculature is thought to be an ideal target site for a drug delivery system. Drugs or drug carriers injected into the bloodstream firstly meet neovessels prior to entering the disease tissue. Therefore, targeting endothelial cells may be easier than targeting to specific cells that exist outside of the bloodstream.

Angiogenic vasculature shows enhanced permeability compared with pre-existing vessels because of its rough construction (**Figure 1**). Macromolecules, such as liposomes, passively accumulate in tumour tissues as a reflection of the feature [78]. In particular, small sized liposomes having long circulating characteristics are known to accumulate passively in tumour tissue due to enhanced permeability and retention effect (EPR effect) [79,80] (**Figure 1**): Avoidance of the trapping of liposomes in reticuloendothelial system (RES), such as liver and spleen,

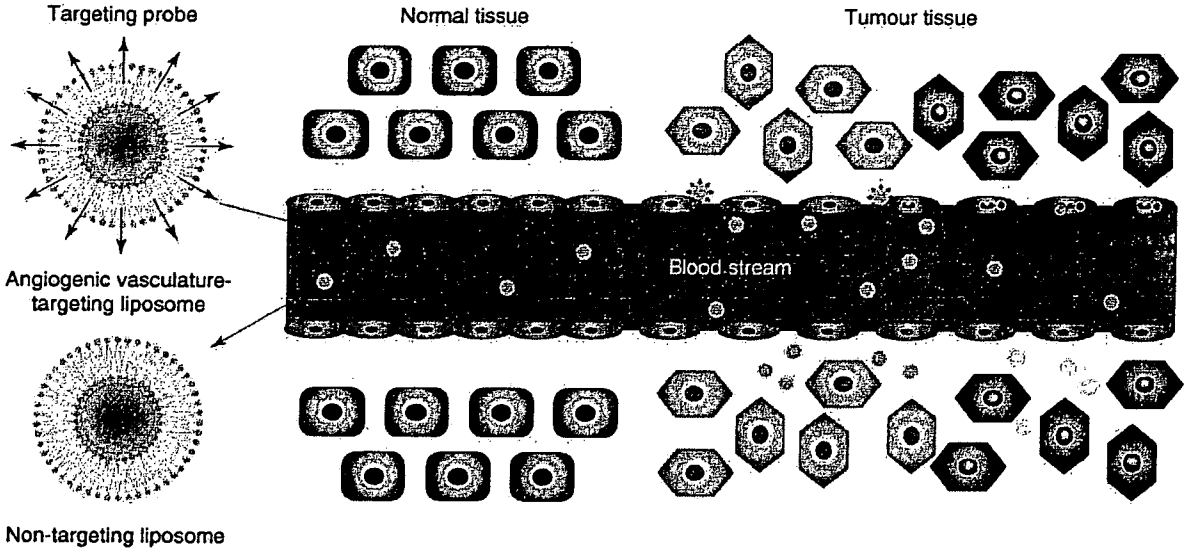

Figure 1. Scheme of ANET by using angiogenic vasculature-targeting liposomes. Angiogenic vasculature-targeting liposome is shown in the upper left. Liposomes are modified with a targeting probe specific for angiogenic endothelial cells. For example, ADM-encapsulated liposomes composed of distearoylphosphatidylcholine, cholesterol and stearoyl-APRPG, 10/5/2 as a molar ratio, are prepared as follows: lipids dissolved in chloroform are dried under reduced pressure. The thin lipid film is hydrated in 0.3 M citric acid solution (pH 4.0), frozen and thawed for three cycles, and extruded three times through a 100-nm-pored filter. After the solution is neutralized, ADM, or other antitumour drugs, is loaded into liposomes by an incubation at 60°C for 15 min. For preparing long circulating liposomes, distearoylphosphatidylethanolamine-PEG-APRPG or other probe-conjugated PEG lipids are used for the modification of liposomes. These liposomes pass through normal tissues but interact with angiogenic endothelial cells and damage them after endocytosed. This vessel damage causes regression of tumour due to cut-off of the nutrients and oxygen (upper right). On the other hand, non-targeted long circulating liposomes, which are usually prepared by PEG-modification, accumulate in tumour tissues by EPR effect, and damage tumour cells by sustained release of antitumour drugs (lower right).
ADM: Adriamycin; ANET: Antineovascular therapy; APRPG: Ala-Pro-Arg-Pro-Gly; EPR: Enhanced permeability and retention; PEG: Polyethylene glycol.

results in endowing liposomes with long circulating characteristic, since the RES trapping is the major clearance route of particulate drug carriers including liposomes. Therefore, liposomalisation of anticancer cytotoxic agents enhances their activity with decreased side effects [81,82]. Doxil® (Ortho Biotech Products, LP) and DaunoXome® (Gilead) are typical liposomal anticancer agents that have been used in clinical chemotherapy. However, neovascular density of tumours is considerably different among cancerous organs. In case of low-vascularised tumours, macromolecules such as liposomes could not accumulate effectively because of decreasing EPR effect. In fact, the commercialised anticancer liposomes are adopted for high-vascularised tumours such as breast and ovarian cancer but not low-vascularised tumours such as stomach, colorectal and pancreas cancer. Targeted liposomes to angiogenic endothelial cells may also be useful for such low-vascularised tumours, since they could meet objective cells in the bloodstream. Moreover, since those commercialised liposomal anticancer agents target tumour cells, the therapeutic efficacy would be varied with the kinds of tumour cells.

The phenotype of angiogenic endothelial cells is different from that of pre-existing vessels due to the activation by many selective factors including pro-angiogenic factors. Thus, angiogenic endothelial cells express specific molecules on their surface. This characteristic may provide active targeting guides for cancer treatment. Based on these backgrounds, the authors established novel cancer chemotherapy which directly eradicate angiogenic vasculature by allowing cytotoxic agents to deliver to angiogenic endothelial cells (Figure 1 upper scheme). Activated endothelial cells receiving angiogenic signalling grow rapidly. Therefore, cytotoxic agents against growing cells cause damage of neovascular endothelial cells, resulting in indirect lethal damage of tumour cells. Tumour cells often acquire drug resistance due to their genetic instability; however, neovascular endothelial cells would not be expected to acquire drug resistance. For the purpose of ANET, the authors isolated the peptide specific for tumour angiogenic vessels, and liposomes modified with the peptide was used as a cytotoxic drug carrier for delivering the drug to the vessels. The results demonstrated that ANET using newly developed liposomes markedly suppressed tumour growth through damaging angiogenic endothelial cells.

4.2 Isolation of an angiogenic vasculature-targeting probe

At first, the authors isolated an angiogenic vasculature-targeting probe by use of a phage-displayed random peptide library [83,84]. For obtaining peptides specifically homing to angiogenic

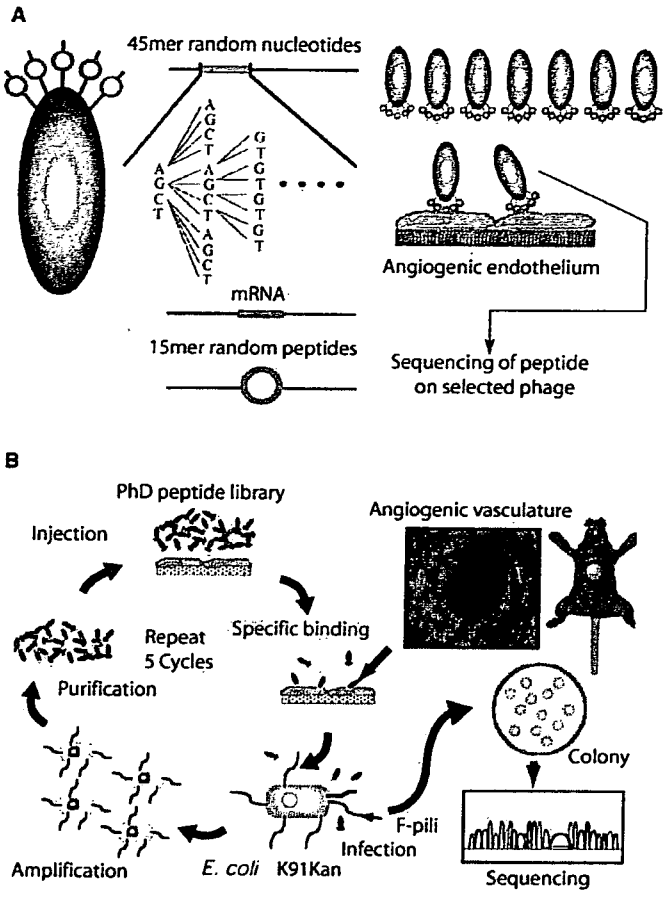

Figure 2. Isolation of a peptide homing to neovessels by using a phage-displayed random peptide library. A) Principle of a phage-displayed peptide library. A phage-displayed random peptide library expressing pentadecamer amino acid residues at the N terminus of pIII phage coat protein of M13 phage was used for *in vivo* biopanning. **B)** Experimental procedure of *in vivo* biopanning. *In vivo* biopanning was performed by a modified method as described by Pasqualini et al (Nat. Biotechnol. 15, 542-546, 1997). The phage-displayed peptide library (1 x 1013 cfu) was injected into angiogenesis model mice via a tail vein. The mice were deeply anesthetized with pentobarbital sodium and snap frozen in liquid nitrogen 4 min after the injection. The skin attached to the Millipore chamber ring, where the angiogenic vessels had been formed, was dissected, minced, and homogenized with ice-cold DMEM containing 1 mM phenyl methyl sulfonyl fluoride. This homogenate was washed 3 times with ice-cold DMEM containing 1% bovine serum albumin, and the accumulated phages were recovered by infecting E. coli K91KAN with them. A part of the phages in the homogenate was used for the titration of the accumulated phages, and the remaining phages were amplified in E. coli K91KAN and purified. A second round of biopanning was then performed similarly as for the first round. These biopanning steps were repeated for 5 cycles. After selected phages were cloned, sequences of the peptides presented on the selected phage clones were determined
cfu: Colony-forming units; DMEM: Dulbecco's modified eagle medium.

vasculature, the authors performed *in vivo* biopanning by use of dorsal air sac model mice instead of tumour-bearing mice (Figure 2). *In vivo* biopanning is useful method for obtaining peptides specific for vasculature of various organs including tumour tissues [85,86]. Therefore, the obtaining phage clones express the peptides specific for angiogenic endothelial cells but not for tumour cells. Moreover, biopanning eliminates phage clones having affinity for pre-existing endothelial cells. After cloning the selected phages, each phage clone was injected intravenously into tumour-bearing mice to obtain phage clones showing high affinity for neovessels. In case of injection into B16BL6 melanoma-bearing mice, PRPGAPLAGSWPGTS, DRWRPALPVVLFPLH, and ASSSYPLIHWRPWAR peptide-presented phage clones accumulated in the tumour more than 20-fold compared with the accumulation of the original phage library. These clones also accumulated in implanted Meth A sarcoma. Furthermore, the accumulation of the phage clones was competitively suppressed by the synthetic peptides having the corresponding sequences. By the competitive inhibition assay, the authors determined the epitope sequence of peptide for showing the highest affinity to neovessels as Ala-Pro-Arg-Pro-Gly (APRPG).

To deliver the cytotoxic agent to angiogenic endothelial cells, the authors selected liposomes as a drug carrier and

modified liposomal surface with APRPG peptide after the peptide had been hydrophobised with palmitoyl group. When the biodistribution of APRPG-modified liposomes (APRPG-Lip) was examined, APRPG-Lip significantly accumulated in tumours [83].

Then, the applicability of APRPG-containing peptides to human was examined since the peptide was selected in the murine angiogenic model. Binding capacity of APRPG-Lip and control liposome to HUVECs was determined with confocal laser scanning microscope. NBD-labelled liposome bound to VEGF-activated HUVECs only when liposome was modified with APRPG. This binding was cancelled in the presence of excess APRPG peptide. Interestingly, the specific binding of APRPG-Lip was not observed without stimulation of HUVECs with VEGF. Furthermore, histochemical analysis demonstrated that biotinylated PRPGAPLAGSWPGTS specifically bound to angiogenic endothelial cells in human islet cell tumour of the pancreas and glioblastoma. In this case also, pretreatment with an excess of synthetic PRPGAPLAGSWPGTS inhibited the binding of biotinylated PRPGAPLAGSWPGTS on the glioblastoma specimens [83]. These data indicate that APRPG-containing peptides have affinity to some molecule(s) on human angiogenic endothelial cells.

4.3 Antineovascular therapy by using angiogenic vasculature-targeting liposomes

Organ-selective targeting of agents promises enhancement of their activity and reduction of the side effects. Since APRPG-modified liposomes showed high accumulation in tumours, APRPG-Lip was used for ANET: the authors encapsulated adriamycin (ADM) into the APRPG-Lip (APRPG-LipADM) and injected into the bloodstream of tumour-bearing mice.

In therapeutic experiments, modification of liposomes with APRPG enhanced the antitumour activity of ADM and reduced toxicity due to targeting effect (Figure 3) [83]. These effects of APRPG seemed to be independent of tumour type, because enhanced tumour accumulation of APRPG-Lip was observed in Meth A sarcoma- and Colon26 NL-17 carcinoma-bearing mice. The enhanced antitumour activity of APRPG-LipADM may be explained partly by the increase of the local concentration of ADM in the tumour. However, it is also considered that ADM damages neovascular endothelial cells, since APRPG-LipADM is expected to bind these cells efficiently from the results of both confocal observation and histochemical staining.

To discover which factor is predominant between direct toxicity against tumour cells and indirect tumour growth suppression through toxic action against angiogenic endothelial cells, the authors examined ANET by using a hydrophobic anticancer drug, 5'-*O*-dipalmitoylphosphatidyl derivative of 2'-C-*cyano*-2'-deoxy-1-β-D-*arabino*-pento-furanosylcytosine (DPP-CNDAC) [87]. The therapeutic activity of DPP-CNDAC was also enhanced by the APRPG-liposomal formulation. As lipophilic drugs should be delivered to the cells in liposomal form, the therapeutic efficacy reflects the damage of the cells to which liposome accesses rather than change in local concentration of the agent in tumour tissue. The therapeutic efficacy of APRPG-liposomal DPP-CNDAC is superior to non-modified liposomal DPP-CNDAC, suggesting that the destruction of angiogenic endothelial cells is superior to the direct destruction of tumour cells in the tumour treatment [87].

Since APRPG peptide is originally isolated by biopanning of phage-displayed library as mentioned above, APRPG-Lip tends to avoid the RES trapping. For further enhancement of circulation time of the liposomes, we conjugated APRPG to the edge of polyethylene glycol (PEG) of PEG-coated liposomes. APRPG-PEG-LipADM accumulated more in tumour than PEG-uncoated liposomes and displayed enhanced anti-tumour activity [88].

Pastorino *et al.* reported antiangiogenic chemotherapy by use of NGR peptide-modified long-circulating liposomes encapsulating doxorubicin (ADM) [89]. NGR peptide targets aminopeptidase N on angiogenic endothelial cells. The concept is quite similar to the authors' concept. They showed not only drastic therapeutic efficacy against tumour-bearing mice was demonstrated but also pronounced destruction of the tumour vasculature by use of NGR peptide-modified liposomal anticancer drug.

Schiffelers *et al.* used RGD peptide as a targeting tool for integrin $\alpha_v\beta_3$ which were expressed on angiogenic endothelial cells. They developed angiogenic vasculature-targeting liposomes by conjugating the RGD peptide with the edge of PEG-coated long circulating liposomes (LCL). They demonstrated that RGD-LCL specifically bound to endothelial cells *in vitro* and RGD-LCL encapsulating ADM effectively suppressed tumour growth in C26 colon carcinoma model [90]. These observations indicate that angiogenic vasculature-targeting ANET may be a new approach to potent cancer chemotherapy [91,92].

4.4 Metronomic-dosing chemotherapy

Most anticancer agents directly damage tumour cells in a cell cycle-dependent manner and these cytotoxic agents are used in traditional cancer chemotherapy. In clinical applications, cytotoxic agents are used for killing tumour cells and/or for preventing recurrence in metastatic sites after surgical removal of the primary tumour. In these cases, cyto-toxic agents are generally administered at maximum tolerated dose (MTD), which is called 'conventional schedule'. However, cancer patients usually bear a great burden of severe side effects, such as myelosuppression, dehairing, acute body weight decrease and etc. Conventional schedule requires non-treatment period for reducing these side effects. However, unfortunately, this period also allows tumour cells to grow. Of course, angiogenesis also occurs in this term and promote tumour growth and metastasis. For these reasons, cancer patients often receive unsatisfactory treatment in cancer chemotherapy.

As cancer chemotherapy damages rapidly growing cells, not only tumour cells but also normal growing cells such as bone marrow and intestinal cells are damaged. These normal cell damages appear as side effects. Chemotherapy may also damage angiogenic endothelial cells, since these cells are growing cells, although this effect had not been well noticed since this effect is included in main effect of the antitumour agent.

Browder *et al.* determined an appropriate administration scheduling for cancer treatment [93]. This schedule was characterised by low dose injection at short intervals, and named 'antiangiogenic schedule', because they observed apoptosis of angiogenic endothelial cells prior to tumour cell killing. The antiangiogenic schedule showed marked suppression of angiogenesis, since a shorter treatment-free period might not allow reconstruction of new blood vessels. It was also demonstrated that this enhanced antiangiogenic effect brought marked suppression of tumour growth. It was confirmed in various kinds of cancer models. Additionally, this schedule could be applied to drug-resistant tumour and p53-deficient tumour cell lines, since the mechanism was based on disruption of angiogenic vasculature [94]. Furthermore, administration of liposomal formulation of cytotoxic agent in antiangiogenic schedule showed more enhanced antitumour effect compared with free formulation of it: liposomal formulation might allow sustained drug release. Combinational administration of both cytotoxic agent and angiogenic inhibitor in antiangiogenic schedule also showed more enhanced antitumour effect compared with administration of cytotoxic agent alone [93].

Kerbel *et al.* reported that repeated low-dose administration (metronomic dosing) showed potent therapeutic effect and prolongation of survival time of mice in various cancer models [94]. They also demonstrated the effectiveness of metronomic dosing in drug-resistant cancer model. This therapeutic scheduling is now waiting to be taken into clinical cancer chemotherapy [95].

4.5 Antiangiogenic photodynamic therapy

Photodynamic therapy (PDT) promises potent efficacy against neoplastic and abnormal tissues such as tumour tissue. PDT uses a combination of photosensitiser, such as porphyrin, chlorin, or phthalocyanine derivatives, and tissue-penetrating visible laser light. In brief, laser light promotes the photosensitiser into an excited state, and when it comes back to ground state, activated oxygen, such as singlet oxygen, is generated by interaction with oxygen. Activated oxygen then directly kills tumour cells [96]. As laser irradiation can be localised around the tumour, severe side effects that are usually observed in chemotherapy can be avoided. Benzoporphyrin derivative monoacid ring A (BPD-MA) is a second generation of photo-sensitiser, and their liposomal formulation is commercialised as Visudyne™ (Quadra Logic Technologies/Novartis) for the treatment of AMD in which uncontrolled angiogenesis occurs. Specific laser irradiation at angiogenic site causes eradication of angiogenic endothelial cells, resulting in disruption of angiogenic vasculature [97]. Based on this idea, the authors established a novel photodynamic cancer therapy targeting to angiogenic vasculature, namely antiangiogenic PDT. This therapy also targets on the growing angiogenic endothelial cells of newly formed vessels.

The authors previously determined antiangiogenic scheduling of photodynamic therapy for cancer therapy, which performed laser irradiation 15 min after administration of photosensitiser (15-min PDT). In general, laser irradiation is performed 3 – 5 h after administration of BPD-MA because the photosensitiser highly accumulates in tumour tissue at those times. However, at earlier times such as 15 min after administration, the photosensitiser mainly exists in bloodstream and may be incorporated in angiogenic endothelial cells rather than in tumour cells. In fact, antiangiogenic PDT scheduling (15 min PDT) effectively damaged angiogenic vasculature compared with 3-h PDT by use of dorsal air sac model. Furthermore, in a therapeutic experiment, 15-min PDT using liposomal BPD-MA effectively suppressed tumour growth and showed prolonged survival time of solid tumour-bearing mice [98,99].

To enhance electrostatic interaction of liposomal BPD-MA with angiogenic endothelial cells, the authors used positively charged liposomes as a carrier for BPD-M, because the surface of endothelial cells are negatively charged [100]. For this purpose we used polycation liposomes (PCLs): liposomal surface was coated with polyethylenimine. BPD-MA-encapsulated PCLs showed strong binding to endothelial cells and enhanced cytotoxic effect against endothelial cells after laser irradiation *in vitro* [101,102]. Corresponding to this *in vitro* data, BPD-MA-encapsulated PCLs showed potent therapeutic effect such as tumour regression and prolonged survival time in solid tumour-bearing mice after irradiation of laser 15 min after administration [103]. Furthermore, it has been clarified that enhanced tumour regression by BPD-MA PCL-mediated PDT depends on disruption of angiogenic vasculature. These observations indicate that antiangiogenic PDT is expected to be efficient cancer therapy compared with traditional PDT.

5. Conclusion

Tumour angiogenesis is a critical event for solid tumour growth, and research has been carried out to develop inhibitors for blocking certain step in angiogenesis. Antiangiogenic therapy is thus proposed. There are many good reviews on antiangiogenic therapy which aims to inhibit certain angiogenic processes such as VEGF-mediated signalling and MMP-related ECM degradation, and so on [104]. Recently, it has become noticed that cancer chemotherapy damages angiogenic endothelial cells as well as tumour cells, because angiogenic endothelial cells are growing cells, such as tumour cells. And new strategy for antiangiogenic therapy is proposed, which aims to damage pre-formed neovessels. In this review, the authors described three different approaches;

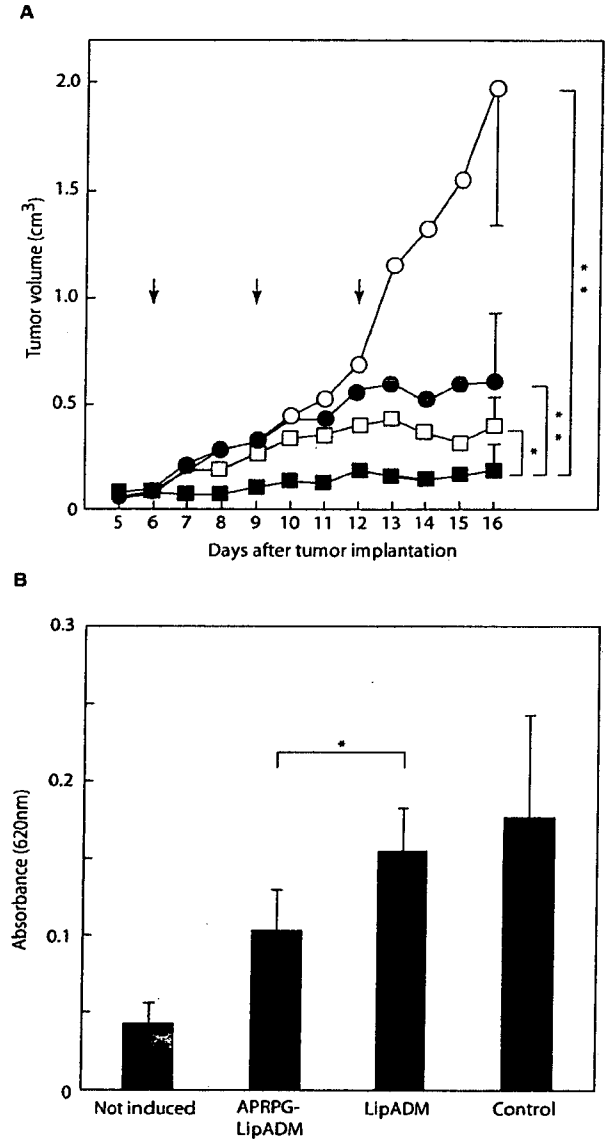

Figure 3. Antitumour (antineovascular) therapeutic experiment with adriamycin-encapsulated liposomes. ADM-encapsulated liposome was prepared by a modification of the remote-loading method, and the encapsulation efficiency was > 90% throughout the experiment. **A**) Suppression of tumour growth by APRPG-LipADM in Meth A sarcoma-bearing mice. ADM encapsulated in control liposome (white square = LipADM) or in liposome-modified with stearoyl APRPG (black square = APRPG-LipADM) (10 mg/kg as ADM), ADM alone (black circle = 10 mg/kg), or 0.3M glucose solution (white circle = control) were injected intravenously into tumour bearing BALB/c mice (n = 6) at days 6, 9 and 12 after implantation of Meth A sarcoma cells. The size of the tumour and body weight of each mouse were monitored daily. Tumour volume was calculated using the formula 0.4 (a x b2), where "a" was the largest and "b" was the smallest diameter of the tumour. Variance in a group was evaluated by the F-test, and differences in mean tumour volume were evaluated by Student's t-test. Data are presented as mean tumour volume and SD. SD bars are shown only for the last points for the sake of graphic clarity. Arrows show the day of treatment. Significant differences are indicated (*, $p < 0.05$; **, $p < 0.01$). **B**) Suppression of in vivo angiogenesis by APRPG-LipADM. For assay of antineovascular activity, a chamber ring loaded with Colon 26 NL-17 cells (1 x 107 cells/ring) was dorsally inoculated into five-week-old BALB/c male mice. At 2 days after inoculation, LipADM, APRPG-LipADM (10 mg/kg as ADM), or 0.3M glucose solution were injected intravenously into DAS model mice. At 4 days after inoculation, 1% Evans Blue solution was injected intravenously into the mice. After 1 min, they were sacrified and the pigment in the skin attached to the ring was extracted for the measurement of absorbance at 620 nm. The data represent an absorbance of the pigment at angiogenic site and significant difference from the LipADM is indicated (*, $p < 0.05$).

ADM: Adriamycin; APRPG: Ala-Pro-Arg-Pro-Gly; DAS: Dorsal air sac; LipADM: Liposome adriamycin; PRP: Platelet-rich plasma; SD: Standard deviation.

firstly one aims to deliver cytotoxic agents to angiogenic endothelial cells, secondly to function cytotoxic agents more to angiogenic endothelial cells than to tumour cells by altering administration scheduling of the agents, and thirdly to damage angiogenic endothelial cells by use of appropriate scheduling and appropriate carrier of photosensitiser in PDT. To clarify the difference of this strategy from conventional antiangiogenic therapy, the authors named this as 'anti-neovascular therapy (ANET)'.

Inhibition of angiogenic process is a promising approach, however, it sometimes brings only tumour dormancy. Moreover, most of the angiogenesis of these inhibitors need an abundant dose and frequent administration to suppress tumour growth. Selective but not drastic activities of these inhibitors may make clinical studies difficult. On the contrary, ANET aims to disrupt angiogenic vasculature by delivering cytotoxic agent to angiogenic vessels. Therefore, ANET is expected not only to suppress tumour growth, but also to eradicate tumour cells through complete cut-off of oxygen and nutrients. In general, cancer chemotherapy is accompanied with strong side effects and acquired drug resistance. Therefore, drug delivery systems which selectively deliver the drugs to the target tumour are awaited. Recently, vascular targeting has become a focus of interest, since certain drugs or drug carriers first meet neovasculature before extravasation in the tumour. In particular, targeting of a tumour angiogenic vasculature is promising for cancer treatment since these vessels have properties different from those of the pre-existing systemic vasculature. Furthermore, angiogenic endothelial cells are growing cells, and are effectively damaged by antitumour drugs if the drugs are appropriately delivered to or functioned on the neovessel cells. In this review, the authors have shown that the direct eradication of angiogenic endothelial cells is actually more potent to eradicate tumours than the direct damaging of tumour cells by angiogenic vasculature-targeting 'ANET'. ANET including metronomic-dosing chemotherapy and antiangiogenic PDT would be a hopeful treatment modality for cancer patients.

6. Expert opinion

Since cancer became one of the higher fatality diseases in developed countries, various kinds of anticancer agents have been developed for cancer therapy. Although they show effective direct cytotoxicity against tumour cells, most of them accompany severe side effects, such as myelosuppression, because they also damage some growing normal cells. This problem is mainly caused by low selectivity of the drugs, since most anti-cancer agents show their dramatic cytotoxicity in a cell cycle-dependent manner. Due to this problem, clinical MTD of anticancer agents often fails to show enough therapeutic efficiency. Additionally, acquirement of drug resistance in tumour cells sometimes causes difficulty in cancer therapy. For these reasons, development of novel cytotoxic agents becomes more difficult. Thus, introduction of drug delivery system (DDS) technology and a novel approach for cancer therapy are now expected.

Since Folkman and co-workers stated the importance of angiogenesis in tumour growth in the earlier 1970s [105] and discovered angiostatin in 1996 [49], angiogenesis in cancer research has been considered. Up till now, the angiogenic processes and involvement of angiogenic factors and signal transducing molecules have been elucidated. According with this interest, cancer therapy targeting angiogenesis has been focused. In the present review, the authors have firstly introduced various kinds of targeting molecules for antiangiogenic therapy and their antitumour effect. As a result, it has been clarified that angiogenesis is processed with complex stages where angiogenic endothelial cells play an important role. In brief, angiogenesis initiates with interaction of angiogenic factors with their receptors, following with signal transduction, endothelial cell proliferation, migration, invasion, and tube formation. Antiangiogenic therapy aims to inhibit one or several steps of angiogenesis and subsequently to suppress tumour growth. However, it is questionable whether: an injectable dose could completely suppress tumour angiogenesis; suppression of angiogenesis leads to complete eradication of tumour cells; whether antitumour effect lasts long-term; and whether it is applicable for any stage or various kinds of tumours. In fact, some cases are reported: natural angiogenic inhibitor, endostatin showed effective inhibition of angiogenesis in early stage of tumour but not shown in late stages. Additionally, although various kinds of antiangiogenic agents have been developed, some doses do not show satisfactory antitumour effect in clinical study. One of the reasons for this is based on the alternative functions of a variety of pro-angiogenic factors in various stages of angiogenesis. To overcome this problem, a novel approach in antiangiogenic therapy has been expected.

Since conventional cytotoxic anticancer drugs target angiogenic endothelial cells as well as other growing cells, the authors developed one of a novel antiangiogenic therapy, ANET. ANET includes angiogenic vasculature-targeting chemotherapy, metronomic-dosing therapy, and antiangiogenic photodynamic therapy. Angiogenic vasculature-targeting chemotherapy was achieved with active-targeting tools and DDS technology: anticancer drug-encapsulating liposomes modified with a peptide specifically bound to angiogenic endothelial cells were used. This liposomal anti-cancer drug suppressed tumour growth in a tumour-bearing mice model. Furthermore, usage of DDS technology decreased side effects by lowering administration dose of cytotoxic agents and by altering biodistribution of the agents because of the targeting effect. Metronomic-dosing chemotherapy aims to shift the action site of cytotoxic agents from tumour cells to angiogenic endothelial cells with continuous low-dose administration scheduling and shows potent anti-tumour effect against various cancers including drug-resistant cancer. Antiangiogenic PDT, which also shifts

the target from tumour cells to angiogenic endothelial cells, shows efficient antitumour activity with reduced side effects. Thus, these strategies promise complete cancer cure with overcoming the conventional problems such as severe side effects and drug selection. We hope that ANET will be applied in clinical cancer therapy in the future.

Bibliography

1. CARMELIET P, DOR Y, HERBERT JM *et al.*: Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* (1998) 394:485-490.
2. FOLKMAN J: Fundamental concepts of the angiogenic process. *Curr. Mol. Med.* (2003) 3:643-651.
3. CARMELIET P: Angiogenesis in health and disease. *Nat. Med.* (2003) 9:653-660.
4. RELF M, LEJEUNE S, SCOTT PA *et al.*: Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. *Cancer Res.* (1997) 57:963-969.
5. KONERDING MA: Ocular angiogenesis: translating preclinical indications to successful clinical development. *Expert Opin. Ther. Targets* (2004) 8:255-258.
6. ABEDI H, ZACHARY I: Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J. Biol. Chem.* (1997) 272:15442-15451.
7. FERRARA N: VEGF and the quest for tumour angiogenesis factors. *Nat. Rev. Cancer* (2002) 2:795-803.
8. TAKAHASHI T, UENO H, SHIBUYA M: VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. *Oncogene* (1999) 18:2221-2230.
9. GERBER HP, MCMURTREY A, KOWALSKI J *et al.*: Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J. Biol. Chem.* (1998) 273:30336-30343.
10. BURBRIDGE MF, COGE F, GALIZZI JP *et al.*: The role of the matrix metalloproteinases during *in vitro* vessel formation. *Angiogenesis* (2002) 5:215-226.
11. HAJITOU A, SOUNNI NE, DEVY L *et al.*: Down-regulation of vascular endothelial growth factor by tissue inhibitor of metalloproteinase-2: effect on *in vivo* mammary tumor growth and angiogenesis. *Cancer Res.* (2001) 61:3450-3457.
12. SEO DW, LI H, GUEDEZ L *et al.*: TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism. *Cell* (2003) 114:171-180.
13. QI JH, EBRAHEM Q, MOORE N *et al.*: A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. *Nat. Med.* (2003) 9:407-415.
14. AHONEN M, POUKKULA M, BAKER AH *et al.*: Tissue inhibitor of metalloproteinases-3 induces apoptosis in melanoma cells by stabilization of death receptors. *Oncogene* (2003) 22:2121-2134.
15. ASAHARA T, MUROHARA T, SULLIVAN A *et al.*: Isolation of putative progenitor endothelial cells for angiogenesis. *Science* (1997) 275:964-967.
16. ASAHARA T, MASUDA H, TAKAHASHI T *et al.*: Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ. Res.* (1999) 85:221-228.
17. TAMURA M, UNNO K, YONEZAWA S *et al.*: *In vivo* trafficking of endothelial progenitor cells and their possible involvement in tumor neovascularization. *Life Sci.* (2004) 75:575–584.
18. HEISSIG B, HATTORI K, DIAS S *et al.*: Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* (2002) 109:625-637.
19. HEISSIG B, WERB Z, RAFII S, HATTORI K: Role of c-kit/Kit ligand signaling in regulating vasculogenesis. *Thromb. Haemost.* (2003) 90:570-576.
20. CHANTRAIN CF, SHIMAD H, JODELE S *et al.*: Stromal matrix metalloproteinase-9 regulates the vascular architecture in neuroblastoma by promoting pericyte recruitment. *Cancer Res.* (2004) 64:1675-1686.
21. RAFII S, LYDEN D, BENEZRA R, HATTORI K, HEISSIG B: Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat. Rev. Cancer* (2002) 2:826-835.
22. KIMURA H, WEISZ A, OGURA T *et al.*: Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. *J. Biol. Chem.* (2001) 276:2292-2298.
23. SEMENZA GL: Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer* (2003) 3:721-732.
24. FERRARA N, GERBER HP, LECOUTER J: The biology of VEGF and its receptors. *Nat. Med.* (2003) 9:669-676.
25. PREWETT M, HUBER J, LI Y *et al.*: Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. *Cancer Res.* (1999) 59:5209-5218.
26. BREKKEN RA, OVERHOLSER JP, STASTNY VA *et al.*: Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. *Cancer Res.* (2000) 60:5117-5124.
27. BINETRUY-TOURNAIRE R, DEMANGEL C, MALAVAUD B *et al.*: Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis. *EMBO J.* (2000) 19:1525-1533.
28. ZHANG L, YU D, HICKLIN DJ *et al.*: Combined anti-fetal liver kinase 1 monoclonal antibody and continuous low-dose doxorubicin inhibits angiogenesis and growth of human soft tissue sarcoma xenografts by induction of endothelial cell apoptosis. *Cancer Res.* (2002) 62:2034-2042.
29. YAMAGUCHI S, IWATA K, SHIBUYA M: Soluble Flt-1 (soluble VEGFR-1), a potent natural antiangiogenic molecule in mammals, is phylogenetically conserved in avians. *Biochem. Biophys. Res. Commun.* (2002) 291:554-559.

30. MAHASRESHTI PJ, NAVARRO JG, KATARAM M *et al.*: Adenovirus-mediated soluble FLT-1 gene therapy for ovarian carcinoma. *Clin. Cancer Res.* (2001) 7:2057-2066.
31. HASUMI Y, MIZUKAMI H, URABE M *et al.*: Soluble FLT-1 expression suppresses carcinomatous ascites in nude mice bearing ovarian cancer. *Cancer Res.* (2002) 62:2019-2023.
32. KALLURI R: Basement membranes: structure, assembly and role in tumour angiogenesis. *Nat. Rev. Cancer* (2003) 3:422-433.
33. JOHN A, TUSZYNSKI G: The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. *Pathol. Oncol. Res.* (2001) 7:14-23.
34. ZUCKER S, CAO J, CHEN WT: Critical appraisal of the use of matrix metalloproteinase inhibitors in cancer treatment. *Oncogene* (2000) 19:6642-6650.
35. LAMBERT V, WIELOCKX B, MUNAUT C *et al.*: MMP-2 and MMP-9 synergize in promoting choroidal neovascularization. *FASEB J.* (2003) 17:2290-2292.
36. RUPP PA, LITTLE CD: Integrins in vascular development. *Circ. Res.* (2001) 89:566-572.
37. STUPACK DG, CHERESH DA: Get a ligand, get a life: integrins, signaling and cell survival. *J. Cell Sci.* (2002) 115:3729-3738.
38. RUEGG C, DORMOND O, MARIOTTI A: Endothelial cell integrins and COX-2: mediators and therapeutic targets of tumor angiogenesis. *Biochim. Biophys. Acta.* (2004) 1654:51-67.
39. BROOKS PC, STROMBLAD S, SANDERS LC *et al.*: Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* (1996) 85:683-693.
40. HYNES RO: A reevaluation of integrins as regulators of angiogenesis. *Nat. Med.* (2002) 8:918-921.
41. GUTHEIL JC, CAMPBELL TN, PIERCE PR *et al.*: Targeted antiangiogenic therapy for cancer using Vitaxin: a humanized monoclonal antibody to the integrin alphavbeta3. *Clin. Cancer Res.* (2000) 6:3056-3061.
42. KAWAGUCHI M, HOSOTANI R, OHISHI S *et al.*: A novel synthetic Arg-Gly-Asp-containing peptide cyclo(-RGDf==V-) is the potent inhibitor of angiogenesis. *Biochem. Biophys. Res. Commun.* (2001) 288:711-717.
43. KUROHANE K, NAMBA Y, OKU N: Liposomes modified with a synthetic Arg-Gly-Asp mimetic inhibit lung metastasis of B16BL6 melanoma cells. *Life Sci.* (2000) 68:273-281.
44. KEVIL CG, PAYNE DK, MIRE E, ALEXANDER JS: Vascular permeability factor/vascular endothelial cell growth factor-mediated permeability occurs through disorganization of endothelial junctional proteins. *J. Biol. Chem.* (1998) 273:15099-15103.
45. BLASCHUK OW, ROWLANDS TM: Cadherins as modulators of angiogenesis and the structural integrity of blood vessels. *Cancer Metastasis Rev.* (2000) 19:1-5.
46. CARMELIET P, LAMPUGNANI MG, MOONS L *et al.*: Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* (1999) 98:147-157.
47. CARMELIET P, COLLEN D: Molecular basis of angiogenesis. Role of VEGF and VE-cadherin. *Ann. NY Acad. Sci.* (2000) 902:249-262; discussion 262-244.
48. LIAO F, DOODY JF, OVERHOLSER J *et al.*: Selective targeting of angiogenic tumor vasculature by vascular endothelial-cadherin antibody inhibits tumor growth without affecting vascular permeability. *Cancer Res.* (2002) 62:2567-2575.
49. O'REILLY MS, HOLMGREN L, CHEN C, FOLKMAN J: Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat. Med.* (1996) 2:689-692.
• **This is the first paper that describes angiostatin as a factor that causes and sustains tumour dormancy.**
50. CLAESSON-WELSH L, WELSH M, ITO N *et al.*: Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. *Proc. Natl. Acad. Sci. USA* (1998) 95:5579-5583.
51. MOSER TL, STACK MS, ASPLIN I *et al.*: Angiostatin binds ATP synthase on the surface of human endothelial cells. *Proc. Natl. Acad. Sci. USA* (1999) 96:2811-2816.
52. TUSZYNSKI GP, SHARMA MR, ROTHMAN VL, SHARMA MC: Angiostatin binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells. *Microvasc. Res.* (2002) 64:448-462.
53. O'REILLY MS, BOEHM T, SHING Y *et al.*: Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* (1997) 88:277-285.
54. KIM YM, HWANG S, PYUN BJ *et al.*: Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. *J. Biol. Chem.* (2002) 277:27872-27879.
55. IRUELA-ARISPE ML, LUQUE A, LEE N: Thrombospondin modules and angiogenesis. *Int. J. Biochem. Cell Biol.* (2004) 36:1070-1078.
56. RODRIGUEZ-MANZANEQUE JC, LANE TF, ORTEGA MA *et al.*: Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. *Proc. Natl. Acad. Sci. USA* (2001) 98:12485-12490.
57. JIMENEZ B, VOLPERT OV, CRAWFORD SE *et al.*: Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat. Med.* (2000) 6:41-48.
58. MIAO WM, SENG WL, DUQUETTE M *et al.*: Thrombospondin-1 Type 1 repeat recombinant proteins inhibit tumor growth through transforming growth factor-beta-dependent and - independent mechanisms. *Cancer Res.* (2001) 61:7830-7839.
59. SHAHEEN RM, DAVIS DW, LIU W *et al.*: Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. *Cancer Res.* (1999) 59:5412-5416.
60. MENDEL DB, LAIRD AD, SMOLICH BD *et al.*: Development of SU5416, a selective small molecule inhibitor of VEGF receptor tyrosine kinase activity, as an anti-angiogenesis agent. *Anticancer Drug Des.* (2000) 15:29-41.
61. FONG TA, SHAWVER LK, SUN L *et al.*: SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res.* (1999) 59:99-106.

62. BERGERS G, BENJAMIN LE: Tumorigenesis and the angiogenic switch. *Nat. Rev. Cancer* (2003) 3:401-410.

63. HIRATA A, UEHARA H, IZUMI K *et al.*: Direct inhibition of EGF receptor activation in vascular endothelial cells by gefitinib ('Iressa', ZD1839). *Cancer Sci.* (2004) 95:614-618.

64. HIRATA A, OGAWA S, KOMETANI T *et al.*: ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. *Cancer Res.* (2002) 62:2554-2560.

65. IZUMI Y, XU L, DI TOMASO E, FUKUMURA D, JAIN RK: Tumour biology: herceptin acts as an antiangiogenic cocktail. *Nature* (2002) 416:279-280.

66. MORI S, UEDA T, KURATSU S *et al.*: Suppression of pulmonary metastasis by angiogenesis inhibitor TNP-470 in murine osteosarcoma. *Int. J. Cancer* (1995) 61:148-152.

67. ISOBE N, UOZUMI T, KURISU K, KAWAMOTO K: Antitumor effect of TNP-470 on glial tumors transplanted in rats. *Anticancer Res.* (1996) 16:71-76.

68. LIU S, WIDOM J, KEMP CW, CREWS CM, CLARDY J: Structure of human methionine aminopeptidase-2 complexed with fumagillin. *Science* (1998) 282:1324-1327.

69. WANG J, LOU P, HENKIN J: Selective inhibition of endothelial cell proliferation by fumagillin is not due to differential expression of methionine aminopeptidases. *J. Cell Biochem.* (2000) 77:465-473.

70. MARCHIO S, LAHDENRANTA J, SCHLINGEMANN RO *et al.*: Aminopeptidase A is a functional target in angiogenic blood vessels. *Cancer Cell* (2004) 5:151-162.

71. DANIEL TO, LIU H, MORROW JD, CREWS BC, MARNETT LJ: Thromboxane A2 is a mediator of cyclooxygenase-2-dependent endothelial migration and angiogenesis. *Cancer Res.* (1999) 59:4574-4577.

72. TSUJII M, KAWANO S, TSUJI S *et al.*: Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* (1998) 93:705-716.

73. KIRKPATRICK K, OGUNKOLADE W, ELKAK A *et al.*: The mRNA expression of cyclo-oxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) in human breast cancer. *Curr. Med. Res. Opin.* (2002) 18:237-241.

74. LIU XH, KIRSCHENBAUM A, LU M *et al.*: Prostaglandin E2 induces hypoxia-inducible factor-1alpha stabilization and nuclear localization in a human prostate cancer cell line. *J. Biol. Chem.* (2002) 277:50081-50086.

75. MASFERRER JL, KOKI A, SEIBERT K: COX-2 inhibitors. A new class of antiangiogenic agents. *Ann. NY Acad. Sci.* (1999) 889:84-86.

76. FINE HA, FIGG WD, JAECKLE K *et al.*: Phase II trial of the antiangiogenic agent thalidomide in patients with recurrent high-grade gliomas. *J. Clin. Oncol.* (2000) 18:708-715.

77. NG SS, GUTSCHOW M, WEISS M *et al.*: Antiangiogenic activity of N-substituted and tetrafluorinated thalidomide analogues. *Cancer Res.* (2003) 63:3189-3194.

78. ALLEN TM, CULLIS PR: Drug delivery systems: entering the mainstream. *Science* (2004) 303:1818-1822.

79. OKU N: Anticancer therapy using glucuronate modified long-circulating liposomes. *Adv. Drug Deliv. Rev.* (1999) 40:63-73.

80. OKU N, TOKUDOME Y, ASAI T, TSUKADA H: Evaluation of drug targeting strategies and liposomal trafficking. *Curr. Pharm. Des.* (2000) 6:1669-1691.

81. SHIMIZU K, TAKADA M, ASAI T *et al.*: Cancer chemotherapy by liposomal 6-[12-(dimethylamino)ethyl]aminol-3-hydroxy-7H-indeno[2,1-clquinolin-7-one dihydrochloride (TAS-103), a novel anti-cancer agent. *Biol. Pharm. Bull.* (2002) 25:1385-1387.

82. SHIMIZU K, TAKADA M, ASAI T *et al.*: Potential usage of liposomal 4beta-aminoalkyl-4'-O-demethyl-4-desoxypodophyllotoxin (TOP-53) for cancer chemotherapy. *Biol. Pharm. Bull.* (2002) 25:783-786.

83. OKU N, ASAI T, WATANABE K *et al.*: Anti-neovascular therapy using novel peptides homing to angiogenic vessels. *Oncogene* (2002) 21:2662-2669.

•• **The first paper to propose anti-neovascular therapy, an indirect tumour regression through damaging angiogenic endothelial cells by anticancer agents.**

84. ASAI T, NAGATSUKA M, KUROMI K *et al.*: Suppression of tumor growth by novel peptides homing to tumor-derived new blood vessels. *FEBS Lett.* (2002) 510:206-210.

85. ZURITA AJ, ARAP W, PASQUALINI R: Mapping tumor vascular diversity by screening phage display libraries. *J. Control. Release* (2003) 91:183-186.

86. TREPEL M, ARAP W, PASQUALINI R: *In vivo* phage display and vascular heterogeneity: implications for targeted medicine. *Curr. Opin. Chem. Biol.* (2002) 6:399-404.

• **A review for the biopanning of a phage-displayed peptide library to obtain endothelial cell-specific peptides.**

87. ASAI T, SHIMIZU K, KONDO M *et al.*: Anti-neovascular therapy by liposomal DPP-CNDAC targeted to angiogenic vessels. *FEBS Lett.* (2002) 520:167-170.

88. MAEDA N, TAKEUCHI Y, TAKADA M *et al.*:Anti-neovascular therapy by use of tumor neovasculature-targeted long-circulating liposome. *J. Control. Release* (2004) in press.

89. PASTORINO F, BRIGNOLE C, MARIMPIETRI D *et al.*: Vascular damage and anti-angiogenic effects of tumor vessel-targeted liposomal chemotherapy. *Cancer Res.* (2003) 63:7400-7409.

90. SCHIFFELERS RM, KONING GA, TEN HAGEN TL *et al.*: Anti-tumor efficacy of tumor vasculature-targeted liposomal doxorubicin. *J. Control. Release* (2003) 91:115-122.

91. OKU N, ASAI T, WATANABE K *et al.*: Anti-neovascular therapy by use of liposomes targeted to angiogenic vessels. *J. Liposome Res.* (2003) 13:25-27.

92. KONDO M, ASAI T, KATANASAKA Y *et al.*: Anti-neovascular therapy by liposomal drug targeted to membrane type-1 matrix metalloproteinase. *Int. J. Cancer* (2004) 108:301-306.

93. BROWDER T, BUTTERFIELD CE, KRALING BM *et al.*: Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res.* (2000) 60:1878-1886.

94. KERBEL RS, KLEMENT G, PRITCHARD KI, KAMEN B: Continuous low-dose anti-angiogenic/metronomic chemotherapy: from the research laboratory into the oncology clinic. *Ann. Oncol.* (2002) 13:12-15.

95. MAN S, BOCCI G, FRANCIA G *et al.*: Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. *Cancer Res.* (2002) 62:2731-2735.

96. DOUGHERTY TJ, GOMER CJ, HENDERSON BW *et al.*: Photodynamic therapy. *J. Natl. Cancer Inst.* (1998) 90:889-905.

97. CIULLA TA, DANIS RP, CRISWELL M, PRATT LM: Changing therapeutic paradigms for exudative age-related macular degeneration: antiangiogenic agents and photodynamic therapy. *Expert Opin. Investig. Drugs* (1999) 8:2173-2182.

98. KUROHANE K, TOMINAGA A, SATO K *et al.*: Photodynamic therapy targeted to tumor-induced angiogenic vessels. *Cancer Lett.* (2001) 167:49-56.

99. ICHIKAWA K, TAKEUCHI Y, YONEZAWA S *et al.*: Antiangiogenic photodynamic therapy (PDT) using Visudyne causes effective suppression of tumor growth. *Cancer Lett.* (2004) 205:39-48.

100. RAN S, DOWNES A, THORPE PE: Increased exposure of anionic phospholipids on the surface of tumor blood vessels. *Cancer Res.* (2002) 62:6132-6140.

101. TAKEUCHI Y, KUROHANE K, ICHIKAWA K *et al.*: Polycation liposome enhances the endocytic uptake of photosensitizer into cells in the presence of serum. *Bioconjug. Chem.* (2003) 14:790-796.

102. TAKEUCHI Y, ICHIKAWA K, YONEZAWA S *et al.*: Intracellular target for photosensitization in cancer antiangiogenic photodynamic therapy mediated by polycation liposome. *J. Control. Release* (2004) 97:231-240.

103. TAKEUCHI Y, KUROHANE K, ICHIKAWA K *et al.*: Induction of intensive tumor suppression by antiangiogenic photodynamic therapy using polycation-modified liposomal photosensitizer. *Cancer* (2003) 97:2027-2034.

104. DUDEK AZ, PAWLAK WZ, KIRSTEIN MN: Molecular targets in the inhibition of angiogenesis. *Expert Opin. Ther. Targets* (2003) 7:527-541.

105. FOLKMAN J: Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* (1971) 285:1182-1186.

Affiliation

Kosuke Shimizu MSci, Tomohiro Asai PhD & Naoto Oku PhD†

†Author for correspondence

†University of Shizouka, Department of Medical Biochemistry and COE Programme in the 21st Century, School of Pharmaceutical Sciences, Japan

Tel: +81 54 264 5701; Fax: +81 54 264 5705;

E-mail: oku@u-shizouka-ken.ac.jp