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Overview

Angiogenesis Research up to 1996. A Commentary on the State of Art and Suggestions for Future Studies

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THE MAIN aim of this special issue of the *European Journal of Cancer* is to give the reader a comprehensive and updated report on the most relevant fields of research on angiogenesis. Because the Journal is generally read by colleagues involved both in experimental and clinical oncology, and because one of the main topics the editors wish to see covered is translational research, we have tried to put together a balance of contributions from basic and clinical research.

Historically, modern angiogenesis research made its appearance in the mid-1960s, but only during the 1980s was there an escalation of important scientific contributions from several independent laboratories. This was due to the discovery and molecular sequencing of endothelial growth factors, endogenous angiogenesis inhibitors and other molecules of the extracellular matrix (ECM) involved in neovascularisation. During the early part of the 1990s there was a great impetus to the field with the discovery of both natural angiogenesis inhibitors and pharmacological drugs capable, by different mechanisms of action, of suppressing specific steps of the complex cascade of events necessary for the neovascularisation of tumours. The development of several angiosuppressive agents as well as independent *in vivo* experimental studies have provided direct evidence that tumours are, in general, angiogenesis-dependent diseases. This means that angiogenesis is necessary for primary tumour growth, progression and metastasis and that its inhibition leads to tumour remission. The anticancer efficacy of the various forms of modulation of angiogenesis is variable, depending on the biological characteristics of the tumour, the potency of each angiosuppressive agent, its pharmacodynamic and pharmacokinetic properties, and on the complexity of the experimental model used. Rarely are complete tumour remissions observed suggesting that although inhibition of angiogenesis is a new promising therapeutic strategy, suppression of angiogenesis alone seems insufficient for complete control of tumour progression. During the last 3–4 years, angiogenesis research has had a clinical impact as a consequence of two main fields of endeavour. The first is the determination of the angiogenic activity of tumours as a diagnostic and prognostic tool, and the second is the early clinical development (phase I–II trials) of angiogenesis inhibitors. Another recent major advance in experimental oncology was the switch from studies orientated to answer a single phenomenon related to angiogenesis (for example the discovery of the mechanisms of action of new angiogenic peptides or natural angiogenesis inhibitors or of ECM molecules and their influence on the behaviour of the tumour), to the understanding of some co-ordinated and complex pathways of control. These pathways of tumour growth, in which angiogenesis is involved, but is not the sole regulating biological phenomenon, include the following examples:

(i) The genetic or epigenetic alterations involved in angiogenesis. A major pathway links p53/p21 with thrombospondin (TSP), $\alpha_v\beta_3$ integrin, hypoxia and vascular endothelial growth factors (VEGFs). Other important mechanisms of activation of VEGF is through certain oncogenes including *V-HA-RAS*, *V-RAF*, *K-RAS*, *FOS*, *SRC* and *HER-2(neu)*. The two main mechanisms of promoting angiogenesis are via a loss of a tumour suppressor regulator or via upregulation of one or more angiogenic peptides;

(ii) The control of angiogenesis exerted by ECM. An excess of VEGF activates integrin $\alpha_v\beta_3$ and a cascade of enzymatic pathways involving urokinase-tissue factor leading to modification of the microenvironment that permits the formation of new blood vessels. Also, basic fibroblast growth factor (bFGF) works through ECM by activating the uPA/PAI-I system. Another known proteolytic pathway involves integrin $\alpha_v\beta_3$, metalloproteinases and collagens. Therapy with either $\alpha_v\beta_3$ antagonists or metalloproteinases inhibitors is capable of blocking these steps of neovascularisation with consequent tumour regression in some animal models;

(iii) Dormant metastasis. A picture is emerging in which angiogenesis and apoptosis concur in the control of growth of metastasis (from a dormant to an active status). Both the presence of endogenous circulating inhibitors, i.e. angiostatin, or systemic treatment of animals bearing metastatic tumours with anti-angiogenic drugs are capable of inducing metastasis dormancy. Angiosuppression sustains an enhanced apoptotic index of tumour cells, but does not affect the proliferation rate of surviving tumour cells;

(iv) The mutual interaction between tumour and stromal cells. Through paracrine mechanisms and the production of

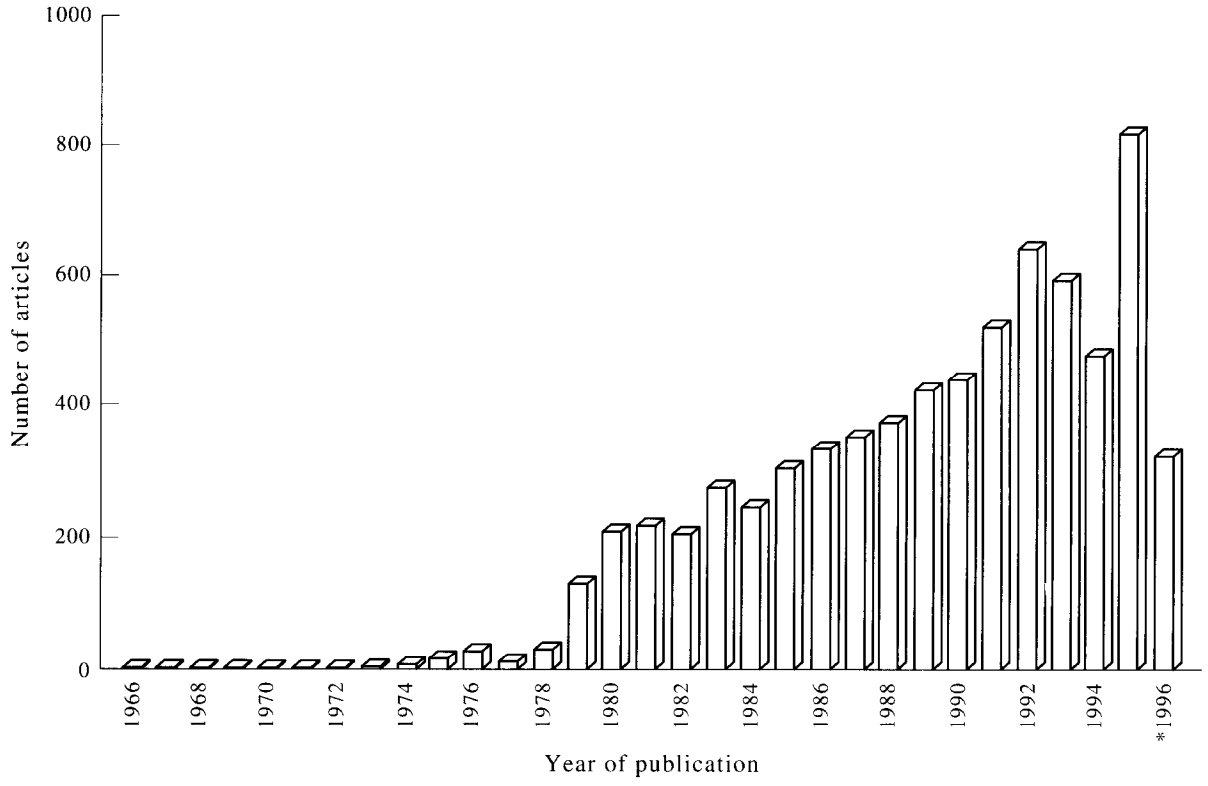

Figure 1. Number of articles published annually on "angiogenesis" or "neovascularisation" as listed in MEDLINE (National Library of Medicine, Bethesda, Maryland, U.S.A.) from 1966 to the first 6 months of 1996 (*).

growth factors and cytokines, activated endothelial cells, macrophages and fibroblasts stimulate tumour growth. During progression, the cell-to-cell relationships may be modified and may lead to different effects on both angiogenesis and tumour growth. Currently, angiogenesis may be considered as one of the most diffuse and topical areas of research in oncology, as documented by the large and increasing number of publications in the field (Figure 1).

Within this Special Issue of the *European Journal of Cancer*, the results of original studies or overviews in basic research are presented, which contribute to our knowledge of the main sequence of events involved in tumour angiogenesis: (i) acquisition of the angiogenic phenotype; (ii) role of the structure and cells of the stroma, mainly endothelial cells; (iii) the mechanisms regulating formation of new vessels within a tumour; (iv) the role played by angiogenesis in primary tumour growth and metastasis in experimental models; and ultimately, (v) its pharmacological inhibition.

The molecular basis for the acquisition of the angiogenic phenotype is an important issue. The complexity of angiogenesis implies the presence of multiple controls including certain genetic and physiological mechanisms. Some pathways are presumed to act in specific cell types and tissues, whilst others seem to have a wider action. Hypoxia *per se* is able to stimulate, for example, VEGF-induced angiogenesis under different conditions.

The original studies by Hanahan and colleagues (pages 2386–2393) using transgenic mouse models, provide a suitable and elegant method for studying the basis for the angiogenic switch from hyperplastic lesions to pre-invasive and invasive cancers (i.e. tumorigenesis). Results obtained using three models of multistage tumorigenesis (RIP-Tag, BPV1.69 and K14-HPV16 transgenic mice) suggest that: (i) angiogenesis is activated via different pathways during the preneoplastic stages that precede the appearance of solid tumours; (ii) the mechanisms regulating the angiogenic switch may be tissue-specific and are discrete and rate-limiting steps of tumorigenesis; and (iii) transgenic mice represent an important model for testing the efficacy of anti-angiogenic drugs, particularly chemopreventive agents which prevent the angiogenesis step of tumour-growth. In fact, the authors observed that early treatment of RIP-Tag transgenic mice with a combination of anti-angiogenesis inhibitors was capable of retarding angiogenesis, increasing tumour cell apoptosis and suppressing tumour growth.

The studies by Stellmach and colleagues (pages 2394–2400) provide evidence for the concept that loss of natural angiogenesis inhibitory signals is a relevant mechanism in the angiogenesis switch. At least five human tumour suppressor genes have been shown to influence angiogenesis in different cell types. Dr Bouck's group have demonstrated that the wild-type *TP53* tumour suppressor gene regulates angiogenesis through the production of anti-angiogenic levels of thrombospondin-1 (TSP-1) by fibroblasts, platelets and human breast carcinoma cells. However, the authors found that one human fibrosarcoma cell line was highly angiogenic even in the presence of TSP-1. In this tumour, TSP-1 function was overcome by very high levels of bFGF and VEGF produced by these cells as compared to tumorigenic fibroblasts that lost wild-type *TP53*.

A common message from both the Hanahan and Stellmach studies is that different tumour types may be regulated by different positive and negative angiogenic factors and that genetic alterations, such as loss of *TP53*, can influence angiogenesis in various ways in different tumours.

Angiogenesis is under tight regulatory control in normal tissues where the angiosuppressory pathways predominate. A paradigm is emerging which postulates that some endogenous angiogenesis inhibitors are stored as cryptic parts of larger molecules that themselves lack angiogenic activity. The capability of releasing the biologically active fragments (angiostatin, the 29 kDa fragment of fibronectin, the 16 kDa fragment of prolactin) may be a major pathway of maintaining quiescent endothelia in normal tissues. The identification of angiostatin has provided evidence that systemic administration of this 38 kDa fragment of plasminogen induces macroscopic primary tumour regression in nude mice bearing different types of human carcinomas. Analysis of histological sections of treated tumours show both angio-involution and enhanced apoptotic rates in the surviving tumour cells. Overall, recent studies from Folkman's group suggest the concept that tumour dormancy can be induced by modulation of angiogenesis in both primary tumours and in micrometastases, when cell proliferation is balanced by apoptosis, the latter being sustained by angio-inhibition. However, the cellular sources of natural angiogenesis inhibitors, the conditions under which their production is stimulated, whether some of these inhibitory factors act like hormones, and their physiological targets still need to be elucidated.

The reports by Bussollino and associates (pages 2401–2412) and Ferrara (pages 2413–2422) overview the characteristics and biological activities of the most important endothelial growth factors currently identified and sequenced. Bussolino and colleagues summarise the role of some angiogenic peptides in promoting angiogenesis. Endothelial growth factors include endogenous and exogenous polypeptides, such as bFGF, hepatocyte growth factor (HGF), placental growth factor (PGF) and HIV-1-*Tat*, which modify the genetic programme of endothelial cells and induce them to migrate, enter cell-cycle and then differentiate again to form new vessels. Among these, bFGF also seems to be relevant in angiogenic autocrine pathways, suggesting that endothelial cells themselves can sustain an angiogenic response. HGF is another example of a pleiotropic growth factor, which plays a pivotal role in organogenesis. It is able to promote the formation of an organised epithelial structure as well as the formation of new vessels. PGF is a growth factor which shares the receptor with VEGF and has a prominent role in angiogenesis of normal adult organs.

An interesting finding is that a tumour type preferably produces specific endothelial growth factors and that the levels of production of each one of these may change during malignant progression even in the same tumour, i.e. VEGF and PGF in human thyroid tumours. From a functional point of view, compelling data suggest that some angiogenic peptides (VEGF and PGF) are specific for endothelial cells, whereas others, i.e. HGF, are pleiotropic and also stimulate other stromal cells, i.e. monocytes and macrophages, to release endothelial growth factors. Finally, HIV-1-*Tat*, the transactivator of HIV-1 genes, can be released by infected cells and modifies functions of vascular cells, thus in part explaining the vascular disorders present in AIDS. Also, some autacoids, including platelet activating factor (PAF) and nitric oxide, two vasodilating molecules, are also angiogenic. The angiogenic activity of PAF is characterised by the ability to induce migration of endothelial cells and to recruit macrophages, which release other angiogenic factors. Nitric oxide seems to be implicated in selected angiogenic pathways, triggered by inflammatory mediators and VEGF, but not by bFGF.

Ferrara, in his comprehensive overview, describes the VEGF gene, the mechanisms for regulation of VEGF gene expression, the functions of the VEGF isoforms, and updates the complex biological activities of the VEGF family. Heterozygous mutations inactivating the VEGF gene and homozygous mutations inactivating the *Flt*-1 or *Flk*-1/KDR genes block vasculogenesis, leading to early intra-uterine death. The VEGF/VEGF-receptor system is an important mediator not only in vasculogenesis, but also in angiogenesis-related diseases. In the latter, it is suggested that anti-VEGF antibodies may be of therapeutic value for the cure of cancer.

In two separate reports, Brooks (pages 2423–2429) and Polverini (pages 2430–2437) explain the role played by ECM, adhesion molecules and stromal cells in modulating angiogenesis. Both the invasiveness and the formation of new vessels in tumour cells depend on the co-operation between adhesive and proteolytic molecules operating in the ECM. Both are involved in directional cell migration and permit cell invasion and proliferation. A complex functional co-operation exists between adhesive molecules (integrins, selectin and immunoglobulin supergene families) and proteolytic enzymes (matrix metalloproteinases, the plasminogen system and serine proteinase families).

β_1 and $\alpha_v\beta_3/\alpha_v\beta_5$ integrins are involved in angiogenesis and their ligand binding provides a critical survival signal necessary for the growth and proliferation of blood vessels during the angiogenesis cascade. In fact, recent experimental research suggests that ligand binding of $\alpha_v\beta_3$ integrin promotes a specific adhesion-dependent cell survival signal leading to the inhibition of *TP53* activity, downregulation of p21 and suppression of the *bax* cell death pathway in human endothelial cells. Indeed, $\alpha_v\beta_3$ integrin not only promotes cell migration on a wide range of matrix proteins, but also acts as a receptor for metalloproteinases on the surface of invasive cells, facilitating their invasiveness. Thus, the single cell-surface receptor $\alpha_v\beta_3$ is involved in both cell migration and matrix degradation in cancer tissues.

Polverini gives further support for the role of ECM in angiogenesis and emphasises the functions of tumour-recruited host cells, mainly macrophages, for remodelling of ECM and angiogenesis. Activated macrophages produce a variety of growth factors and cytokines, including angiogenic peptides and angiogenesis inhibitors such as TSP-1. During tumour progression, changes in ECM composition can be induced by soluble factors produced not only by tumour cells, but also by activated stromal cells. The angiogenic molecules may have different biological effects on neovascularisation depending on the tumour-induced modification of ECM and on the functional status and the quantity of the macrophage infiltrate.

Rak and associates (pages 2438–2450) detail the reciprocal interactions operating between tumour and endothelial cells, and how the reciprocal paracrine stimulation contributes to tumour progression. Activated endothelial cells in the stroma of solid tumours are a rich source of bioactive molecules acting via an autocrine, juxtacrine, paracrine and perhaps also endocrine manner. It has been proven that more than 20 bioactive products, including endothelial

growth factors, interleukins, macrophage and granulocyte-macrophage-colony stimulating factors, endothelin-1, nitric oxide and prostaglandins, can be produced by endothelial cells and affect tumour growth. Endothelial cells not only respond to tumour secreted growth factors, but also actively stimulate tumour cell proliferation through the production of these soluble factors. The authors hypothesise that the relationship of tumour and endothelial cells may change in parallel with tumour progression in a three-dimensional tumour growth geometric model.

Currently, we have only a partial knowledge of the phenotypic changes in tumour-associated endothelial cells. Activated endothelium overexpresses a number of molecules and receptors including *KDR*, *Flt*-1, *tie*-1, tyrosine kinases, endoglin and $\alpha_v\beta_3$ integrin, that are presumed to play a relevant role in regulating its proliferation and function. The authors also suggest that understanding the mechanisms operating in the complex network of tumour and endothelial cell interactions may be the key to a better knowledge of tumour progression and may provide the identification of new targets for anticancer therapy.

The paper by Ellis and Fidler (pages 2451–2460) examines the role of angiogenesis in metastasis. The central concept they propose is that angiogenesis is necessary but, is not sufficient for the development of metastasis. This is because metastasis is a highly selective process made up of complex sequential interdependent steps that favour and permit the survival, in distant organs, of a subpopulation of metastatic cells pre-existing within the primary tumour. The attachment and survival of metastases in the target tissue also depend upon the net balance of systemic, local, endogenous, angiogenic and angiosuppressive factors and the micro-environment. The acquisition of metastatic potential is determined by the convergence of multiple molecular mechanisms. For example, a tumour cell, even if it has acquired the angiogenic phenotype, does not produce metastases if this characteristic is not coupled with motile-invasive and proliferative capacities. Therefore, it appears that anti-angiogenic therapy is a promising antimetastatic therapy particularly if given in combination with other biological response modifiers (BRMs). These combinations may interfere, for example, with the activity of adhesion molecules or other structures of ECM involved in cell migration and invasiveness. Drugs capable of inducing apoptosis in tumour cells or of modulating the immune response of the host may also act in concert with anti-angiogenic therapy.

The papers by Teicher and associates (pages 2461–2466) and Lin and colleagues (pages 2467–2473) describe two different experimental approaches to blocking tumour angiogenesis, including anti-angiogenic therapy, aimed at inhibiting endothelial cell growth (Teicher and associates) and vascular targeting, aimed at directly destroying the established vasculature, using antibodies capable of distinguishing normal versus tumour-activated endothelium (Lin and associates). Teicher's studies not only demonstrate the (partial) antitumour efficacy of some specific anti-angiogenic drugs alone, but also prove that the strategy of concurrently targeting both endothelial and tumour cells by using a combined therapy of anti-angiogenic and cytotoxic drugs or radiation therapy leads to an enhanced rate of primary tumour and metastases remissions, without an increase in systemic toxic effects. These results are in agreement and support the 'angiogenesis progression' hypothesis of Rak and Kerbel and are worthy of clinical testing.

The results of the study by Lin and associates, from Dvorak's laboratory, show that the tumour-cell secreted and the microvessel-bound VEGF/VEGF-receptors system seems to be a useful target for diagnostic imaging and anticancer therapy using neutralising antibodies.The authors injected ^{125}I-Ab-VEGF-N into syngeneic mice bearing transplantable solid tumours or malignant ascites, and found that this antibody accumulated preferentially within tumour microvessels. The authors suggest the theoretical advantages of the use of an antibody therapy directed against newly generated blood vessels. Furthermore, they speculate that the Ab-VEGF-N is a promising vehicle for targeting tumour microvessels and destroying tumour vascularisation by delivering toxins within the tumour mass.

This Special Issue also includes papers dealing with the clinical applications of research on angiogenesis in oncology. Considering that we are now at the 'dawn' of the translational application of research on angiogenesis, two clinical applications seem to be promising.

First, quantitation of intratumoral microvessel density (IMD) in histological specimens, using specific endothelial markers and immunohistochemical techniques, has been found to be of prognostic value in a variety of solid tumours. The general finding is that patients with highly vascularised primary tumours have an increased likelihood of developing metastasis and dying. Even though the determination of vascularity is a relatively crude static method for estimating tumoural angiogenic activity, it is quite a successful predictor of prognosis because presumably IMD reflects the balance of positive and negative angiogenic factors. Moreover, high vascularity increases the vascular area, facilitating the escape of tumour cells into the circulation and amplifying the paracrine effects exerted by endothelial cells on tumour cells.

A consensus paper (Vermeulen and associates (pages 2451–2460)) emphasises the importance of standardisation and prospective validation of the methodology and criteria of evaluation of tumour vascularity. Vermeulen co-ordinated the collaborators from independent institutions to reach an international consensus. The proposed standard method for assessment of IMD suggests: quality controls for the selection of the tissue sample; immunostaining the area(s) for microvessel quantitation; and a technique of evaluation in order to facilitate both the comparison of the results among different institutions and the organisation of confirmatory studies on the prognostic value of IMD in prospective controlled multicentre trials.

The best endothelial marker for assessing IMD has not yet been identified. However, at least eight panendothelial markers are currently available, most of which work well in paraffin-embedded pathological material. Six other antibodies seem to be more specific for activated/proliferating endothelium and blood vessels, giving no or poor staining of lymphatic and normal quiescent blood vessels. These latter markers mainly react with fresh or frozen tissues and, as compared with the panendothelial markers, may provide more accurate prognostic information. Their use, therefore, seems to be particularly appropriate for future prospective studies.

The papers by Gasparini, Pezzella and associates, Chung and associates and Weidner overview clinical studies on the prognostic significance of IMD in invasive breast, lung, gas-

tro-intestinal and genito-urinary cancers, respectively. Overall, taking into account that meta-analyses on retrospective studies are subjected to biases related to the diverse clinicopathological characteristics and heterogenous treatments of the series and to the different techniques and criteria of evaluation adopted to assess angiogenesis, determination of IMD emerges as one of the most interesting biological indicators to assess prognosis. In fact, data from 21 published studies revealed that most authors found a statistically significant association of IMD with prognosis in mixed, node-negative or node-positive series of breast cancer patients (Gasparini, pages 2485–2493). Of particular relevance, 69% and 80% of the studies with a multivariate analysis found that IMD is of prognostic value for relapse-free survival and overall survival, respectively.

Pezzella and associates (pages 2494–2500) show that most of the seven authors who have published results of IMD in primary non-small cell lung cancer have found a statistically significant association of vascularity with metastasis. More controversial appears to be the association of IMD with prognosis within the same stage of disease. A novel finding reported by Pezzella and colleagues, is the analysis of the pattern of vascularisation of lung metastases originating from solid primary tumours. They observed the presence of either angiogenic (more frequent) or alveolar (non-angiogenic) patterns. Among the tumours with an angiogenic pattern, the IMD of the metastatic lesion was higher than that of the primary cancer. Finally, in some well-vascularised metastases, the bcl-2 protein was expressed. Because bcl-2 protein inhibits certain pathways of apoptosis, the preliminary results of Pezzella and colleagues indirectly support experimental data from Folkman's laboratory showing that metastasis dormancy is sustained by angiosuppression and a high apoptotic index. Chung and colleagues (pages 2501–2505) provide the results of both experimental and clinical studies indicating the central role played by VEGF in promoting colon cancer angiogenesis, progression and prognosis. The authors selected the highly metastatic cell line LM5 from the parental human colon cancer cell line OCUC-LM. LM5 cells, when transplanted into nude mice, cause frequent and numerous liver metastases. The high metastatic potential of LM5 cells is related to the production of an endothelial growth factor having the characteristics of VEGF. The clinical studies of Chung and associates show that IMD is significantly associated with the expression of VEGF and that both are predictive of recurrence in patients with gastro-intestinal tumours, mainly in the subgroup of cases with haematogenous metastasis.

The paper by Weidner (pages 2506–2512) overviews the clinical significance of angiogenesis in patients with male or female urogenital cancers. Overall, IMD seems to be a potentially useful prognostic tool in patients with prostate, bladder and ovarian cancers. Although less data are available and are more controversial, it appears that IMD may be of prognostic value in testicular, cervix or endometrial neoplasms.

Toi and associates (pages 2513–2519) report their experience of the determination of some angiogenic peptides either in primary tumours or in the serum of cancer patients. They mainly show the results on VEGF, platelet-derived endothelial cell growth factor, HGF and metalloproteinases. The determination of endothelial growth factors seems to be a reasonable alternative to IMD of assessing angiogenesis. In series of breast cancer patients, the authors found a statistically significant association of the expression of some angiogenic peptides with high vascularisation and prognosis. Other authors found similar relationships in other solid tumours. Furthermore, a higher frequency of aberrant levels of VEGF and HGF was found in the serum of cancer patients as compared to normal subjects. An association of high VEGF or HGF serum levels with tumour progression was demonstrated in patients with recurrent breast carcinoma. Preliminary findings suggest that determination of circulating concentrations of angiogenic peptides may give clinical information potentially useful as both tumour and prognostic markers.

Angiosuppression is a new therapeutic strategy for the cure of cancer patients. Castronovo and Belotti (pages 2520–2527) and Talbot and Brown (pages 2528–2533) report the preliminary results of phase I–II clinical trials performed with a drug affecting endothelial cell proliferation (TNP-470) or using various metalloproteinases inhibitors, respectively. Both these reports also include an overview of the mechanisms of action, target structures, and the pharmacodynamic and pharmacokinetic characteristics of the investigated drugs. Although most of the clinical studies are

Table 1. Anti-angiogenic therapy. Possible targets and mechanisms of action

Mechanisms	Target	Therapy
• Block of proteolytic pathways occurring in the extracellular matrix	Metalloproteinases and their substrates	Metalloproteinase inhibitors (BB-94 and BB-2516)
• Block of adhesion of molecules involved in angiogenesis	β_1, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins	Neutralising antibodies (LM-609)
• Block of endothelial cell migration	*In vitro* tests for cell migration	Linomide
• Block of proliferating endothelial cells	Activated endothelium (TEC-11; E-9; $\alpha_v\beta_3$ integrin)	Specific growth inhibitors (TNP-470, angiostatin, IL-12)
• Block of angiogenic peptides	VEGF, bFGF and others	Growth factor inhibitors (suramin and analogues); neutralising antibodies
• Gene therapy	TSP-1, angiostatin and platelet-factor 4	Transfection of neoplastic cells with genes encoding endogenous angiogenic inhibitors

VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; TSP-1, thrombospondin-1.

Table 2. Anti-angiogenic drugs

• Inhibition of endothelial cell proliferation	AGM-1470† (angioinhibin or TNP-470)
	Angiostatin, Linomide†
	Recombinant platelet-factor 4†
	Tamoxifen*
	Pentosan polysulphate†
	D-penicillamine
	Genestein
	Thalidomide
	Interferons*
	Antibody TEC-11 against endoglin
• Blockage of endothelial cell migration and formation of new capillaries	Linomide†
• Neutralisation of angiogenic peptides	Antibodies to bFGF
	Antibodies to VEGF†
	Sulphonic derivatives of distamycin A
	Suramin and analogues†
	Tecogalan sodium (DS-4152)†
• Inhibition of the synthesis and turnover of vessel basement membrane	Minocycline*
	Sulphated carboxylmethyl chitin
	Angiostatic heparin–steroid complexes
	Beta-cyclodextrin-tetra-decasulphate steroids†
	Proline analogues
	Polysaccharide-K
	Razoxane†
	Protamine†
• Inhibition of extracellular matrix proteins	Antibody LM609 against the $\alpha_v\beta_3$ integrin
	Metalloproteinase inhibitors†
• Stimulation of natural angiogenesis inhibitors	Retinoids†

*Commercially available drugs; †Drugs under early clinical trials. bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor.

preliminary and still ongoing, TNP-470 and the metalloproteinase inhibitor BB-94 induced objective responses in some patients and more frequently stable disease. In general, these treatments were well tolerated, with no relevant systemic side-effects. These results should be considered encouraging taking into account that most of the patients enrolled in these phase I–II studies had advanced metastatic disease resistant to conventional anticancer treatments, and that they were enrolled in anti-angiogenesis trials using the conventional clinical criteria used for chemotherapeutic drugs. A careful selection of the cases potentially responsive to anti-angiogenic therapy based on the assessment of specific targets of responsiveness (i.e. the fraction of proliferating endothelial cells for TNP-470 and expression of the preferred substrates for metalloproteinases inhibitors) is expected to be the key for maximising the activity of angiosuppressive agents and for minimising unnecessary toxic side-effects and costs.

The paper by Dr Folkman completes the issue (pages 2534–2539). Dr Folkman believes that "basic research designed to uncover mechanisms of the angiogenic process, has led in recent years, to new thinking about the development, progression and therapy of human cancer. These new ideas...provide explanations for some clinical phenomena that were previously puzzling." A general consideration is that the scientific community has, until recently, underestimated the role of endothelial cells (and perhaps of other stromal components) in the control of tumour growth. Experimental studies conducted in Dr Folkman's laboratory have provided important new information on the biology of cancer than may lead to novel anticancer therapeutic approaches: (i) primary tumours could suppress their metastasis by the secretion of angiogenesic inhibitors, such as angiostatin; (ii) tumour dormancy, i.e. clinically undetectable microscopic disease, in which there is no expansion of tumour size, could be sustained by balanced proliferation and apoptosis of tumour cells in the presence of angiosuppression; (iii) certain clinical patterns of presentation of metastases may have an angiogenic basis, and a co-respective experimental animal model on which to test novel thera-

Table 3. Anti-angiogenic therapy. General principles and clinical indications

- Endothelial cells represent a homogeneous target of normal (diploid) cells that are less likely to develop genetic alterations and resistance to therapy as compared with tumour cells, the latter being genetically unstable.
- Inhibitors of the switch to the angiogenic phenotype are potential candidates for chemoprevention to inhibit the passage from pre-invasive to invasive cancer.
- Anti-angiogenic agents should be mainly directed at small foci of proliferating/migrating endothelial cells. Therefore, the best clinical setting is as adjuvant treatment to prevent the growth of micrometastasis.
- In general, specific inhibitors of angiogenesis have low toxicity, are selective and need to be given for long periods of time to prolong tumour dormancy.
- Therapy directed against both the parenchymal (tumour cells) and the stromal cells (endothelial cells) represents a new therapeutic paradigm worthy of clinical testing for improving the cure of solid tumours. In fact, a mutual paracrine stimulation exists between these two cell-compartments.

Table 4. Study design and challenges for the clinical evaluation of angiogenesis inhibitors

Phase I studies:
- Selection of the patients: using biological criteria by identifying specific molecular targets to assess responsiveness to anti-angiogenic agents.
- End-points: pharmacokinetics characteristics, toxicity and identification of the biologically active doses.
- Schedules of administration: low and frequent doses for relatively long periods.

Phase II studies:
- Selection of the patients: biological and clinical criteria: by tumour molecular targets; patients with low deposits of metastatic disease resistant to conventional anticancer treatments.
- End-points: durable stable disease should be considered as a biological response.

Phase III studies (controlled clinical trials with a control arm receiving the 'best' conventional anticancer treatments):
- Chemoprevention: anti-angiogenic agents to prevent the angiogenic switch and local invasiveness in high-risk patients or in those with *in situ* carcinomas.
- Adjuvant: anti-angiogenic agents to prevent metastasis or to prolong dormancy of micrometastases in high-risk patients receiving surgery for solid tumours
- Combined therapy: anti-angiogenic agents given together with conventional anticancer treatments (the two-compartment therapeutic paradigm) in patients with advanced disease.

peutic approaches has been developed; and (iv) the endothelial compartment within a tumour needs to be considered as an anticancer therapeutic target. In particular, inhibition of angiogenesis seems to be a promising strategy for prolonging tumour dormancy.

Dr Folkman concludes his overview by commenting on certain misconceptions in angiogenesis that may affect the design of clinical trials for anti-angiogenic therapy. This is an important point because experimental studies on inhibitors of angiogenesis in animals indicate that there are multiple possible targets for inhibiting the various steps of tumour neovascularisation by using agents with diverse modes of action (Table 1). Indeed, several anti-angiogenic drugs have been found capable of suppressing tumour growth in animal models through the inhibition of specific pathways necessary for neovascularisation and some of these are entering clinical trials (Table 2). Table 3 shows some general principles and possible clinical indications for anti-angiogenic therapy.

A final consideration is that anti-angiogenic agents are BRMs, non-specific cytotoxic drugs, because their specific target is the angiogenic activity of a tumour, without any direct effect on tumour cells (in fact, generally, anti-angiogenic agents do not affect the *in vitro* growth of tumour cells). This notion clearly suggests that proper study designs should be adopted for the clinical development of angiogenic inhibitors. In future phase I studies, the selection of the patients for this form of therapy should be driven by the assessment of specific markers related to the mode of action of each angiosuppressive modality. Biological criteria should be added to the clinical criteria of selection of the patients. The main end-points of these studies should be the assessment of toxicity as well as the identification of the range of biologically active dosage. Indeed, the schedules of administration need to be thought out, with consideration that angiosuppression is likely to be necessary for long periods to be effective against advanced solid tumours.

In phase II studies, it is unlikely that patients with large metastatic disease will be responsive to BRMs. Angiosuppressive agents should be considered biologically active in patients with advanced stages of cancer even if the outcome is only durable stable disease.

Finally, once active and low toxic anti-angiogenic agents are available for phase III studies, experimental studies suggest that it is likely they will be more active in chemopreventive and adjuvant settings, alone or in combination with conventional anticancer treatments or other BRMs, rather than in patients with a large tumour mass (Table 4).

One should not expect that angiosuppression will be a 'magic cure' for cancer. Probably it will be an effective therapy for specific tumours, certain stages of disease, but not in all cancer patients. Indeed, systemic angiosuppression may interfere with the female reproductive function, wound and bone repair, and targeting of VEGF receptors may lead to diabetes due to the role of these receptors in the endothelium of pancreatic islets, for example.

To conclude, the strongest message from the contents of this Issue is that experimental studies on angiogenesis have contributed to a better understanding of the biological mechanisms governing tumour growth and metastasis. Moreover, these studies provide the identification of new targets for novel forms of anticancer therapy based on angiosuppression. Importantly, angiogenesis research has already translated to the clinic where it has potential value in diagnosis, prognosis and therapy of human cancers.

Well-designed clinical studies with appropriate criteria of selection of the patients, as well as specific clinical and biological end-points, are needed to evaluate properly the impact that the assessment of angiogenic activity of the tumours will have for prognostic purposes. In addition, valid trials to show how therapy with anti-angiogenic drugs will be able to prevent the passage from pre-invasive to invasive tumour stages or of enhancing the cure of patients with invasive cancers in the years to come are also needed.

In conclusion, since the schedule of production for this issue was very tight, we are grateful to the authors whose efforts have resulted in what we consider to be an excellent Special Issue, which we hope will be a valuable source of information and a basis to stimulate further research on angiogenesis.

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