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Meeting Report

# The biological basis for antiangiogenic therapy

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## 1. Introduction

In November 1999, an 'Inside Track' Conference of the European School of Oncology (ESO) on the 'Biological Basis for Antiangiogenic Therapy' was held in Milan, Italy. This conference provided an opportunity to discuss the biological rationale for different antiangiogenic therapies, along with the optimisation of clinical trial designs using single or multiple agents and the analysis of the outcomes and future directions. The conference was timely, with a booming interest in angiogenesis as a therapeutic target and its clinical trials, in particular with endostatin, recently occurring.

Approximately 400 participants, both basic scientists and clinicians attended the conference, a fact that demonstrates the wide interest raised by the basic research on the angiogenic process and its clinical applications. More than 100 abstracts were submitted to the conference for poster/oral presentation [1], providing a stimulating ground for discussion and a network of interactive collaboration between scientists and clinicians. This report briefly summarises the topics discussed at the conference focusing on the newest findings and controversial issues presented by the faculty members.

## 2. The process of angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing ones, and the permeability of blood vessels are regulated by a family of vascular endothelial growth factors and receptors. One concept that emerged during the conference is that at least two of these factors are also involved in lymphangiogenesis [2]. Vascular endothelial growth factor receptor (VEGFR)-3 and its ligand, VEGF-C, are involved in embryo mouse development and in some models of angiogenesis in adult life. VEGFR-$3^{-/-}$ mouse embryos died after 10.5 days of gestation with alterations in the pericardium and vascular remodelling, while VEGFR-$3^{+/-}$ mice did not show any defects. In early embryonic life, this receptor is expressed on vascular endothelial cells, but it is then downregulated and appears in lymphatic vessels. In adult life, it is exclusively present on lymphatic vessels in mice as well as in humans. However, it is transcribed *in vivo* in proliferating endothelial cells at the sites of angiogenesis (e.g. angiogenesis in breast cancer). Mutations in its kinase domain were found in patients affected by autosomal dominant hereditary lymphoedema. VEGF-C has been defined as the ligand of VEGFR-3. However, VEGF-C can also bind VEGFR-2 and trigger vascular endothelial cell activation in adults as well. By using the mutant VEGF-C 156S, which does not bind VEGFR-2, it was established that VEGFR-3 is involved in cell migration and sprouting. In oncogenesis, VEGF-C seems to play an important role in lymphatic metastasis. Indeed, Rip-1–Tag-2 transgenic mice develop pancreatic insulinomas, which do not metastasise, whereas the tumours spread by lymphatic metastases in Rip-1–Tag-2/Rip-1–VEGF-C transgenic mice.

Hypoxia plays a complex role in tumour growth acting as an on switch for inducible tumour angiogenesis factors. *VEGF-A* gene transcription is actually upregulated

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by hypoxia [3]. Low $pO_2$ (partial oxygen pressure) acts on *VEGF-A* mRNA levels by increasing the rate of synthesis and stabilisation of the transcript and, at the translational level, through a hypoxic-refractory internal ribosome entry site (IRES). During development, the $pO_2$ controls the formation of the vasculature by influencing *VEGF-A* levels: hypoxia upregulates the hypoxia-inducible factor (HIF)-1α, which binds to the *VEGF-A* promoter, starting its transcription. In fact, in HIF-$1\alpha^{-/-}$ mice, hypoxia, as well as hypoglycaemia, do not upregulate *VEGF-A* expression. In immature vessels, hyperoxia downregulates *VEGF-A* expression and promotes vascular regression. However, when vessel formation is completed and pericytes have been recruited, capillaries are no longer sensitive to the survival effect of VEGF-A and hyperoxia does not induce their regression. This is well demonstrated in the human glioblastoma model, where vessels lacking pericytes are strictly dependent on VEGF-A.

In fact, hypoxia activates the transcription of a family of hypoxia-inducible factors, specifically HIF-1α and HIF-2α [4]. These factors have a different role according to the genetic background of the cell population, as shown by the observation that under hypoxic conditions, the apoptotic and death rate of normal cells decreases if HIF-1α is knocked-out, while for tumour cells the rate of apoptosis increases. Clearly, the expression of HIFs could be considered as a biological marker of tumour behaviour, and the hypoxia signalling pathway is now proposed as a therapeutic target for gene therapy.

The angiopoietins (Ang) have recently joined the VEGFs as the only known growth factor families that are highly specific for the vascular endothelium. VEGFs and Ang work together in a complementary and coordinated manner in all aspects of vascular biology [5]. Four angiopoietin molecules have been cloned. Ang-1 and -4 activate the kinase activity of their receptor, Tie-2. Ang-2 and -3 are natural antagonists of this receptor. Ang-1 is a survival molecule for endothelial cells and in Ang-$1^{-/-}$ mice there is a defect in capillary network maturation, characterised by a lack of contact between endothelial cells and pericytes. In the embryo, Ang-1 is instrumental for vessel maturation by recruiting pericytes and probably smooth muscle cells. In stabilised adult vessels, Ang-1 appears to constitutively engage its receptor, and therefore probably does not play a role in vessel maintenance. In this case, Ang-2 destabilises the interaction between the endothelium and pericytes and renders endothelial cells responsive to VEGF-A triggering of angiogenesis. In the absence of VEGF-A, vessels disappear. The co-optation mechanism of angiogenesis, characterised by the tumour growth around pre-existing vessels, seems to be operative in the C6 glioblastoma model. The presence of Ang-2 and the absence of VEGF-A induces necrosis of the central area of the tumour. The tumour cells then release angiogenic molecules, which induce neoangiogenesis from the surrounding capillaries.

## 3. Targeting angiogenic factors

Inducers and inhibitors of angiogenesis are candidates for modulation during tumour development and are a potential target for therapeutic intervention. In a study by Ferrara and colleagues, the importance of VEGF in new blood vessel formation and survival was again emphasised [6]. VEGF expression is a requirement for growth and survival in neonatal mice. Two independent approaches were used to inactivate the VEGF protein in newborn mice: (1) inducible, *Cre–loxP*-mediated gene targeting; or (2) administration of mFlt(1-3)–IgG, a soluble VEGF receptor chimeric protein. Partial inhibition of VEGF achieved by inducible gene targeting resulted in increased mortality, stunted body growth and impaired organ development, most notably of the liver. Administration of mFlt(1-3)–IgG, which achieves a higher degree of VEGF inhibition, resulted in nearly complete growth arrest and lethality with signs of liver and renal failure. Endothelial cells isolated from the liver of mFlt(1-3)–IgG-treated neonates demonstrated an increased apoptotic index, indicating that VEGF is required not only for proliferation, but also for the survival of endothelial cells. Administration of mFlt(1-3)–IgG to juvenile mice instead failed to induce apoptosis in liver endothelial cells. Thus, VEGF is essential for the growth and survival in early postnatal life, but VEGF dependence is eventually lost some time after the fourth postnatal week. In the fully developed animal, VEGF is likely to be primarily involved in active angiogenesis processes such as corpus luteum development, as shown by the capacity of mFlt(1-3)–IgG to inhibit hormonally-induced ovulation in female rats. Furthermore, systemic administration of Flt-(1-3)–IgG to 24-day-old mice revealed a role for VEGF in hypertrophic cartilage remodelling, ossification and angiogenesis during endochondral bone formation. These observations raise the question of the possible biological consequences of therapeutic administration of VEGF/VEGF-receptor antagonists on physiological angiogenesis. Valuable information on these aspects will be obtained from the results of the ongoing clinical trials with humanised anti-VEGF antibody in prostate, lung and breast tumour patients.

Development of low molecular weight compounds able to inhibit VEGF receptor-mediated signalling appears to be a fruitful approach for the discovery of potent antiangiogenic compounds. Inhibitors of VEGF receptor tyrosine kinase(s) are already under investigation in clinical trials. The tyrosine kinase (TK) inhibitors SU5416 and SU6668 have entered phase II/III and

phase I clinical trials, respectively [7]. Both molecules act as adenosine triphosphate (ATP) competitive inhibitors for the TK domain of growth factor receptors. SU5416 shows a certain specificity for the VEGF receptor Flk-1/KDR and SU6668 is a potent inhibitor of VEGF, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) receptors. SU5416 and SU6668 inhibit the growth of various human tumour cell lines implanted in nude mice, decreasing the number of metastases and increasing animal survival. Interestingly, SU6668 was able to cause tumour regression in lung A431 and colon Colo205 carcinoma xenografts, even when treatment was started in animals with a large tumour burden. These data, together with its oral availability and low toxicity, point to SU6668 as a potentially interesting candidate for anti-angiogenesis therapy.

The possibility to inhibit angiogenesis and experimental tumour growth by using selective TK inhibitors is supported by the results obtained with ZD4190 [8]. ZD4190 is a potent and selective inhibitor of the TK activity of VEGF receptors, Flt-1 and KDR. *In vivo*, ZD4190 inhibits VEGF-induced acute hypotension and causes epiphyseal hypertrophy in growing rat femurs by inhibiting bone angiogenesis. In addition, it inhibits tumour growth when tested on a panel of tumour xenografts. However, tumour growth resumed at a normal rate when therapy was interrupted.

The results of ongoing clinical trials with humanised anti-VEGF antibody and low molecular weight TK-VEGF receptor inhibitors are eagerly awaited.

## 4. Targeting endothelial cell functions

Genetic and biochemical pathways govern the assembly and disassembly of the vasculature during tumour growth, thus endothelial cell functions have become targets for anti-angiogenic approaches. The signalling and cross-talk among vascular cells are governed by a family of interendothelial junctions. The adhesion molecule vascular endothelial (VE)-cadherin homotypically links endothelial cells and is instrumental not only in the proper assembly of vascular structures during the remodelling and maturation of the vasculature, but it could be an exciting new template for the development of new anti-angiogenic drugs [9].

The importance of the biochemical machinery of endothelial cells in the control of the balance between degradation and matrix re-assembly of the vascular network during angiogenesis has been demonstrated by the efficacy of the matrix metalloprotease inhibitors (MMPIs) in blocking endothelial and tumour cell invasion, and ultimately angiogenesis. Human ovarian carcinomas (HOC) produce high levels of MMP-2 and MMP-9 that correlate with tumour progression. Batimastat (BB-94), used as a standard agent, affects tumour growth and spread in the HOC xenograft model. However, as is expected for this type of cytostatic-like therapy, tumour inhibition is only partial. Treatment with MMPIs in combination with cytotoxic therapy (i.e. cisplatin) increased the antitumour effects, while prolonged treatment with MMPIs maintained the response to cytotoxic therapy [10]. This finding opens the issue of using a combination of antiangiogenic compounds with conventional therapies.

A critical issue in cancer therapy is the avoidance of acquired tumour cell drug resistance by the targeting of endothelial cells. A link exists among the oncogenes affecting tumour growth via the induction or upregulation of angiogenic factors [11]. Many drugs developed to block the signal transduction cascades triggered by oncogenes may also function 'accidentally' as anti-angiogenic compounds in experimental tumours.

The use of standard chemotherapies administered at low, frequent doses was also broadly discussed at the meeting. Special interest was raised by the fact that such a strategy could control tumour growth of drug-resistant tumours by acting on the tumour vasculature.

## 5. Targeting the vascular endothelium

Vascular targeting is an innovative way to develop treatment strategies that rely on their effect on the already formed vasculature that could lead to advances in cancer treatment. By an *in vivo* selection, phages selectively homing to different tissues are recovered from a phage display peptide library following intravenous administration in mice [12]. Using this strategy, several organ- and tumour-homing peptides were isolated. These peptides bound to different receptors selectively expressed on the vascular cells of the target tissue. The tumour-homing peptides bound to receptors that are upregulated in the vasculature of angiogenic tumours. Taking advantage of this, these peptides could be used to deliver cytotoxic drugs to the tumour vessels. Successful delivery of doxorubicin demonstrated the proof-of-principle for this approach. It is clear that this technology could be extended to targeting various drugs and radioisotopes to tumours, potentially reducing the systemic damage that occurs following radio- or chemotherapy. R. Pasqualini also described the isolation of a specific gelatinase (type IV collagenase) inhibitor from phage display peptide libraries. The sequence HWGF is present in potent and selective inhibitors of MMP-2 and MMP-9 that do not inhibit other MMP family members. Synthetic peptides containing the sequence HWGF (the angiogenic 'zip code') inhibit the migration of endothelial and tumour cells *in vitro* and prevent tumour growth *in vivo*. Molecular diversity of the vascular beds was therefore proven by the targeting

of various tissues by means of different peptide motifs. Organ- and tissue-specific heterogeneity of the vasculature could serve as a selective molecular 'address' for therapy.

There are promising compounds that selectively target tumour vasculature currently under development for cancer treatment. The tubulin-binding agent combretastatin A-4 phosphate is selectively toxic for proliferating endothelial cells *in vitro* and acts on tumours *in vivo* by inducing vascular shutdown [13]. The selective cytotoxic effect on proliferating endothelial cells of combretastatin A-4 seems to be mediated by apoptosis rather than by necrosis. The strong antitumour effects obtained by combretastatin A-4 were associated with the irreversible reduction of tumour blood flow and impairment of the nutritional supply into the tumour tissue. This agent has recently entered phase I clinical trials as a vascular targeting agent.

The selective occlusion of the tumour vasculature by the formation of blood clots (thrombosis) within the tumour vessels is another successful way to target tumour vasculature [14]. This result was achieved by targeting clotting agents to the cell surface domain of human tissue factor, by means of a bispecific antibody. Another example of targeting the endothelial cells of the tumour vasculature, rather than the tumour cells themselves, is the use of an antibody against endoglin, an endothelial cell proliferation marker upregulated on tumour endothelial cells. The anti-endoglin antibody might have therapeutic value by selectively killing the dividing endothelial cells that are prevalent in tumours. Other potential candidate molecules for targeting are the adhesion molecules vascular cell adhesion molecule (VCAM)-1, and E-selectin, the $\alpha v\beta 3$ integrin, and the VEGF-receptor complex.

## 6. Clinical applications

The prognostic relevance of the histopathological microvessel count and the clinical value of the surrogate markers of angiogenesis were the object of controversial discussions. A comprehensive lecture on the clinical significance of the determination of angiogenesis in breast cancer [15], reported that, in the 36 retrospective studies analysed in the literature, the microvessel count in the tumour 'hot spot' have convincingly demonstrated the existence of a strong correlation of this marker to prognosis. Nevertheless, the critical issue of a lack of international standardisation for the methodology is still a strong limitation to its use on a routine basis for all types of cancer. In this scenario, the measure of angiogenesis parameters and their correlation with progression and response to treatment appears to be a very promising approach to be adopted for breast cancer at the clinical level.

The hypothesis has been explored that resistance to antiangiogenic treatment could be explained by the existence of non-angiogenic tumours that take their blood supply from pre-existing vessels of the surrounding tissue [16]. A different phenotype of the vessels present in angiogenic and truly non-angiogenic primary non-small cell lung cancer (NSCLC) was demonstrated, as well as differences in microvessel density (MVD) among primary breast cancers and lymph node or lung metastases from those tumours. By analysing the intratumour vessel expression of $\alpha v\beta 3$ (expressed on new vessels), of LH39 (expressed on mature vessels) and of cell determinant (CD)31, one can conclude that the phenotype of the vessels present in putative non-angiogenic NSCLC is that of the pre-existent mature vessels and not the immature phenotype expected from a new angiogenesis. Furthermore, statistically significant differences in the MVD between primary breast cancers and synchronous lung and or lymph node metastases have been observed. The MVD in lung metastases was always higher when compared either with their primary tumour (angiogenic or non-angiogenic) or to the lymph node tumours. This intriguing observation also supports the hypothesis that angiogenesis might be influenced by the site of metastases.

The angiogenic pathway is controlled by the local balance between molecules that stimulate (FGF2, VEGF, type IV collagenase, etc.) or inhibit this process [17]. The modulation of these molecules represents the first rational therapeutic approach to angiogenesis. Transforming growth factor (TGF)$\alpha$ present in tumours can induce hyperplasia that produces angiogenic molecules used by the tumour to expand. The interferons $\alpha$ and $\beta$ have a role in inhibiting angiogenesis through the downregulation of the transcription and protein production of FGF2, collagenase IV and the metalloprotease MMP-9. For example, the angiogenic growth of haemangiomas and melanomas in the presence of FGF2 and VEGF was associated with a local deficiency of interferon (IFN) that, on the contrary, was present nearby in the normal tissue. Furthermore, 78% of human carcinomas have a deletion of the short arm of chromosome 9 where the *IFN* gene is located. The use of IFNs as antiangiogenic agents is intriguing because there is no direct dose–effect relationship, i.e. a higher dose did not produce better results and the administration schedule was very important. This was clearly demonstrated in an experimental model using IFN-$\alpha$ against human bladder cancer cells (resistant to the antiproliferative effect of IFNs) implanted in the bladder of nude mice: daily low doses of IFN-$\alpha$ were active in inhibiting FGF2 and MMP-9 expression and impaired tumour growth.

Several novel proteins with antiangiogenic properties have been discovered. Specifically, a new cryptic endogenous inhibitor of angiogenesis, a 53 kDa fragment of

antithrombin III generated by proteolytic digestion by an unknown protease [18] was reported. It inhibits the proliferation of endothelial cells *in vitro* and the growth of a neuroblastoma cell line *in vivo*. Its therapeutic efficacy was more that 95% and similar to that of endostatin and angiostatin. It is now known that angiostatin and endostatin can be generated by proteolytic cleavage of plasminogen by MMP-2, and of collagen XVIII by elastase, respectively. These endogenous inhibitors are normally released by primary tumours and they appear to inhibit the growth of metastases. Actually, in animal models (e.g. Lewis Lung carcinoma) and in humans, removal of the primary lesion is often followed by rapid formation of metastases. It is possible to hypothesise that other endogenous angiogenic inhibitors could be generated by larger molecules (for instance other zymogens participating in the coagulation cascade). With endostatin having just entered clinical trials, its efficacy in controlling the growth of several human and murine tumour cell lines highlighted problems concerning the stability of the molecule. The central role of $Zn^{2+}$ and of pH for the activity of endostatin has been revisited. Finally, J. Folkman showed encouraging results that have been obtained with young patients affected by haemangiomas and treated with IFN$\alpha$. The treatment with this cytokine induced the regression of the lesion without any appearance of drug resistance, a common feature of conventional therapies.

Several anti-angiogenic compounds have entered clinical trials [19]. There are two classes of angiogenesis inhibitors resulting from the preclinical model system. One type can be defined as 'angiotoxic' because the agents are cytotoxic, targeting angiogenic endothelial cells to a greater extent than normal or tumour cells. These agents include TNP-470, thalidomide, combretastatins, colchicines, squalamine and some chemotherapeutic agents. They might have their clinical activity detected according to fairly standard approaches, where tumour shrinkage is the basis for scoring a tumour response. A second class of agents are those having targets which are uniquely expressed by proliferating or migrating endothelial cells. They include anti-angiogenic factor antibodies (i.e. anti-VEGF), metalloprotease inhibitors, compounds that regulate genes leading to elaboration of pro-angiogenic factors, or growth-regulatory protein fragments such as endostatin or angiostatin. This type of agent may not have easily detectable effects on tumour behaviour in conventional early clinical trials. An important issue is the necessity to establish a proper methodology to detect the evidence of drug effects and biological endpoints directed at endothelial cells.

Certainly we need to understand much more about the biology of tumour angiogenesis. By identifying the key molecular targets of the angiogenesis process, selective antagonists can be developed. A growing number of angiostatic agents showing promising antitumour effects in preclinical models are now entering clinical studies. Despite the increasing number of molecules with angiostatic properties, the design of optimal regimens in combination with conventional therapy, as well as the need for surrogate markers for the selection and the optimisation of antitumour efficacy, appear to be the critical problems to be solved in the near future.

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