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Vascular endothelial growth factor gene expression in the human breast cancer cell line MX-1 is controlled by O_2 availability *in vitro* and *in vivo*

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Summary. The vascular endothelial growth factor (VEGF) plays an important role in angiogenesis. Mediated by the hypoxia-inducible transcription factor HIF-1α/β, a reduction in O_2 tension (pO_2) leads to increased VEGF gene expression in nonmalignant tissues. In tumor cells VEGF mRNA levels are often constitutively elevated. We examined pO_2-dependent VEGF mRNA expression and VEGF protein formation in the human breast cancer cell line MX-1 *in vitro* and *in vivo*. For *in vitro* study MX-1 cultures were grown on dishes with a gas-permeable bottom to expose the cells to defined O_2 concentrations (from 95% to 0%) for 4 h. Northern blot analysis showed significant VEGF mRNA in MX-1 cultures under normoxic conditions which was further increased by hypoxia. The amount of secreted VEGF was also elevated in hypoxic cultures. Western blot analysis revealed a correlation between the severity of hypoxia and HIF-1α protein amounts in the nucleus. Furthermore, DNA-binding activity of HIF-1 could be demonstrated by gel-shift assays. For *in vivo* study immunodeficient nude mice bearing MX-1 tumor transplants were exposed to inspiratory hypoxia (10% O_2). Northern blot and immunohistochemical analyses of MX-1 tumor transplants showed that VEGF mRNA and VEGF protein levels were increased in mice 17 h after the induction of inspiratory hypoxia. Thus, pO_2-dependence of VEGF gene expression can be maintained in cancer cells, even *in vivo*, which may be relevant in regard to therapeutic attempts to inhibit tumor angiogenesis by increasing tumor oxygenation.

Key words: VEGF – Hypoxia – Hyperoxia – HIF-1 – Tumor xenograft

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Introduction

The growth of solid tumors is dependent on sufficient supply of O_2 and nutrients and on the removal of metabolic endproducts (Shweiki et al. 1992). In an avascular tumor nodule the central cells exist in an environment of low O_2 tension (pO_2). When the size of the tumor exceeds about 1 mm in diameter, its growth may be transiently arrested. Further growth and metastases require the sprouting of new capillaries from adjacent blood vessels into the tumor. The transition to this vascularized phenotype is called angiogenic switch (Hanahan and Folkman 1996).

Blood vessel formation is stimulated by a variety of cytokines (Bussolino et al. 1997; Folkman 1995). A major angiogenic factor is vascular endothelial growth factor (VEGF), which is a potent mitogen for endothelial cells (Ferrara and Davis-Smyth 1997). In non-malignant tissues a lowered O_2 availability is the main stimulus for the production of VEGF. On the induction of hypoxia the transacting, dimeric protein hypoxia-inducible factor 1 (HIF-1α/β) binds to the VEGF gene promoter and increases transcription (Forsythe et al. 1996; Liu et al. 1995). Furthermore, other mechanisms contribute to elevated VEGF amounts at low pO_2 like increased VEGF mRNA stability (Levy et al. 1996) as well as the use of an internal ribosomal entry site in the 5′-untranslated region of the VEGF mRNA (Stein et al. 1998).

Here, the question may be raised whether tumor angiogenesis is truly an example of hypoxia-driven physiological processes or whether it is primarily a genetically determined reaction associated with tumor-specific elements (D'Amore and Shima 1996). In cell culture studies of 26 tumor cell lines, White et al. (1995) have shown that most tumor cells exhibit constitutive overexpression of

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VEGF mRNA but are relatively refractile towards a hypoxic challenge. At its most extreme, VEGF synthesis by tumor cells may proceed independent of changes in O_2 supply.

We used a human breast cancer cell line (MX-1) as a model for study of hypoxia-inducible VEGF production by tumor cells. First, pO_2- and HIF-1α/β-dependent VEGF mRNA and VEGF protein formation was studied *in vitro*. Second, MX-1 transplants were grown in immunodeficient nude mice to investigate whether VEGF mRNA and VEGF protein levels in solid tumors increase on the induction of inspiratory hypoxia. These studies were considered relevant with respect to therapeutic attempts to alleviate tumor hypoxia.

Material and methods

Cell Culture Experiments. MX-1 is a human breast cancer cell line that was originally established from a 29 y old woman at the National Cancer Institute in Bethesda, USA. The present cultures were obtained from the German Cancer Research Center, Heidelberg (TZB No. 630148). Cells were maintained in RPMI 1640 culture medium (Gibco BRL, Paisley, Scotland) with 10% fetal bovine serum (Sigma, Deisenhofen, Germany) and 20 IU/ml penicillin and 20 µg/ml streptomycin. The studies were carried out with passages 26 to 45. For experiments, cells were grown on dishes with a gas-permeable hydrophilic fluoroethylene-propylene copolymer (FEP) Teflon membrane of 25 µm thickness as the cell support surface (∅ 60 mm; Petriperm®; Heraeus, Hanau, Germany). In these dishes the pericellular pO_2 is similar to the pO_2 in the surrounding gas atmosphere (Wolff et al. 1993). The cultures were exposed to different O_2-concentrations for 4 h. Just prior to experiments the culture medium was renewed and the dishes were placed in a 600 ml acrylic glas chamber which contained a perforated tray over a water level to allow free access of the ambient gas. Water-saturated gases containing different concentrations of O_2, 5% CO_2 and balanced N_2 were mixed and pumped through the chamber by serial gas mixing pumps (SA 27-2F and SA 18-2F; Wösthoff, Bochum, Germany) with flow rates of ~300 ml/min at 37 °C.

VEGF protein and lactate determination. After the incubation period the medium was immediately removed, chilled on ice, apportioned and stored at –80 °C for analysis. VEGF protein was measured by commercial enzyme-linked immunoassay according to the supplier's instructions (ELISA; Quantikine™; R & D Systems, Minneapolis, MN). In our hands, the intra- and interassay coefficients of variance of this assay are 5% and 7.5%, and the lower detection limit 9 pg/ml (Heits et al. 1997). Lactate was measured by means of an enzymatic kit (Boehringer, Mannheim, Germany) with 1 : 10 reduced volumes of all reagents.

RNA isolation and Northern Blot Analysis. For RNA isolation the cultures were washed with ice-cold phosphate-buffered saline (PBS, pH 7.40) and cells were lysed using a mono-phasic solution of phenol and guanidinium isothiocyanate (TRIzol™ Reagent, Gibco BRL). For Northern blot analysis total RNA from cultured cells or tumor tissue (15 µg RNA of each sample) was subjected to electrophoresis in denaturing 1.3% agarose gels containing 0.7 M formaldehyde. RNAs were blotted onto Nylon membranes (Nytran Plus; Schleicher and Schuell, Dassel, Germany) with a vacuum blotting apparatus (Pharmacia, Uppsala, Sweden). Filters were cross-linked with UV-light and dried at 80 °C for 2 h. A VEGF-specific PCR fragment generated with primers 5′ – GAG GAG GGC AGA ATC ATC AC – 3′ and 5′ – AGG CCC ACA GGG ATT TTC TTG TC – 3′ served as probe. Probe labelling was done with [α-^{32}P]-dCTP using a commercial DNA labeling kit (MBI Fermentas, St. Leon Roth, Germany). Membranes were prehybridized for at least 4 h and hybridized for 3 days at a temperature of 42 °C and sequentially washed reaching a final buffer concentration of 15 mM NaCl, 1.5 mM sodium citrate, 0.1% sodium dodecylsulfate at 60 °C. VEGF-specific radioactive signals were recorded by phosphoimaging (BAS 1000 reader; Fuji-Film, Düsseldorf, Germany) and normalized in relation to 28S rRNA signals (Zhong and Simons 1999).

Western Blot Analysis. For Western blotting, nuclear extracts from MX-1 cultures were produced as described (Hellwig-Bürgel et al. 1999). Samples were run on SDS/7.5% polyacrylamide gels and transferred electrophoretically (Trans-Blot SD; BioRad, München, Germany) to nitrocellulose membranes (Amersham, Braunschweig, Germany). Equal loading and transfer efficiency were verified by staining with 2% Ponceau S. Membranes were blocked overnight with PBS/5% fat-free skim milk and then incubated for 2 h at room temperature with 1 : 500 diluted monoclonal mouse antibody against human HIF-1α (Transduction Laboratories, Heidelberg, Germany). For detection, horseradish peroxidase-linked anti-mouse IgG antibody (1 : 2 000, 1 h at room temperature; Santa Cruz, Heidelberg, Germany) and enhanced chemiluminescence substrate (Amersham) were used.

Gel-shift assay. Determination of HIF-1 DNA-binding activity was done by use of the gel-shift assay as described (Hellwig-Bürgel et al. 1999). Nuclear extracts were incubated with a radioactively labelled, double-stranded oligonucleotide (TfHBSww) containing two HIF-1 response elements (sequence derived from the transferrin gene promoter). Specificity testing was achieved by competition and supershift experiments. For competition experiments a 500 fold excess of unlabelled double-stranded oligonucleotide, either the TfHBSww oligonucleotide or an unrelated oligonucleotide containing a NF-κB binding site, was added to the binding reaction. For supershift analyses 1 µl of undiluted anti-HIF-1α antibody was added at the end of the reaction and incubated for an additional hour at room temperature.

Mouse Model. MX-1 cells transplanted subcutaneously into thymus aplastic nude mice (nu/nu) form solid tumors with the characteristic features of undifferentiated breast carcinomas with very few necrotic areas (Mendoza et al. 1995). In the present study, tumor tissue grown on the neck of a donor mouse was explanted, cut into small cubes of less than 1 mm^3, and transplanted to the right (for RNA extraction) or both (for additional histochemical studies) hind paws of 14 other nu/nu mice. The animals were allowed to eat and drink *ad libitum*. After three weeks, tumors had grown to ovoids of about 180 mm^3. For study of the effect of hypoxia on VEGF mRNA and VEGF protein levels in the tumor tissue, mice were placed into a chamber of acrylic glass containing an atmosphere of 10% O_2 for various periods of time (up to 17 h). At the end of the experiments, mice were anaesthesized by an i. p. injection (16 µl/g body weight) of a solution composed of 2 µg/µl Nembutal and 1 µg/µl Rompun in saline. For RNA analysis, the extirpated tumor was immediately frozen in liquid nitrogen. For immunohistochemical studies the animals were perfused with heparinized Ringer solution.

Immunohistochemistry. For the immunohistochemical studies the animals were perfused through the left cardiac ventricle with

Ringer solution containing heparin (10 IU/ml) to wash out the blood. Tissues were fixed with 4% formalin in PBS (pH 7.40). After 10 min of fixation the tumor was excised and cut into 3 pieces for a further 24 h fixation period. Then the pieces were immersed into a 30% sucrose solution for cryoprotection. Tissue sections of 12 µm thickness were cut at –20 °C on a Leica cryostat microtome. After repeated rinsing steps in 100 mM PBS nonspecific binding of the specimens was blocked by incubation in 10% normal goat serum (Dako, Hamburg, Germany) for 30 min. The sections were incubated with an anti-VEGF antibody (Oncogene Science, Diagnostics Inc., Cambridge, MA) in a 1 : 20 dilution in a humid chamber for 12 h. Antibody binding was visualized using a secondary antibody-horseradish peroxidase conjugate with diaminobenzidine tetrahydrochloride (DAB) as substrate for the chromogenic reaction. The chromogenic reaction was stopped under microscopic control by washing out the substrate. The primary antibody was not strictly species-specific and reacted with both human and mouse VEGF.

Results

MX-1 cell cultures. To demonstrate the ability of MX-1 cells to respond to hypoxia with increased VEGF gene transcription, Northern hybridizations were carried out. Even under normoxic conditions (20% O_2) VEGF mRNA was clearly detectable and, moreover, VEGF mRNA levels increased when the cultures were maintained at low oxygen concentration (3% O_2) for 4 h (Fig. 1). In contrast, VEGF mRNA was not reduced below the normoxic level when the cells were exposed to hyperoxia (95% O_2) for 4 h (Fig. 1).

To address the question if the elevated VEGF mRNA in hypoxic MX-1 cells is translated, cell culture supernatants were tested for the presence of secreted VEGF protein. The amount of immunoreactive VEGF increased in parallel with the induction of VEGF mRNA when the O_2 concentration was reduced below 3%. The accompanying stimulation of lactate formation was indicative of cellular hypoxia (Fig. 2).

To test whether decreasing oxygen concentrations are able to induce nuclear accumulation of the α subunit of the transcription factor HIF-1 (hypoxia inducible factor 1), the supposed main regulatory factor in hypoxia induced VEGF transcription, Western blot analyses were performed. In the range from 5%–1% O_2 the HIF-1α protein concentrations increased steadily with declining oxygen concentrations and were maximal at 1% O_2) (Fig. 3a). Further lowering of pO_2 towards anoxia led to less HIF-1α protein in the nuclei compared to 1% O_2. Additionally, activity of nuclear accumulated HIF-1α/β heterodimer was demonstrated by means of gel-shift assays. Incubation at 3% O_2 for 4 h increased HIF-1 DNA-binding activity to the TfHBSww oligo-

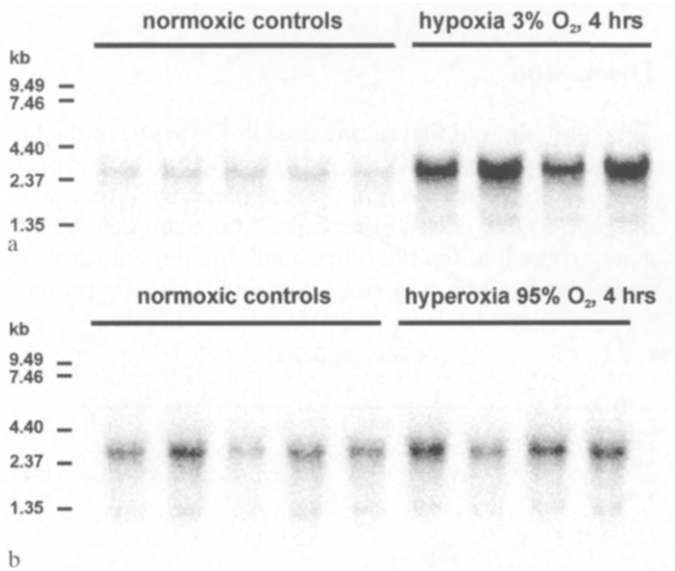

Fig. 1. a: Northern blot analysis demonstrating induction of VEGF mRNA on exposure of MX-1 cultures to low O_2 concentration (3 % O_2) for 4 h, and b: lack of suppression of VEGF mRNA on exposure to hyperoxia (95 % O_2). Results are shown for 5 and 4 separate subcultures, respectively.

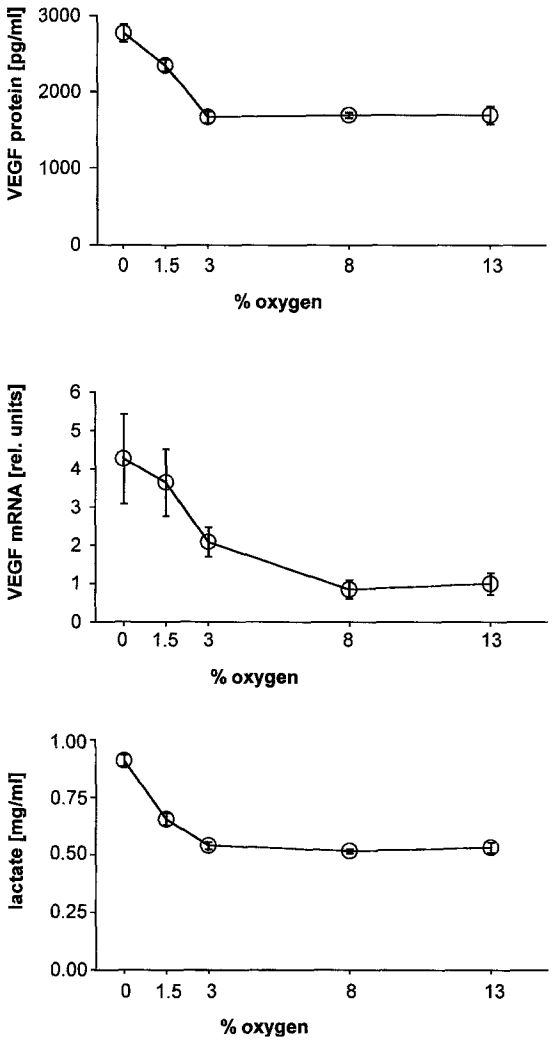

Fig. 2. Secreted VEGF protein and lactate concentrations in the culture supernatant and relative VEGF mRNA levels of MX-1 cultures. Cultures were maintained at various O_2 concentrations for 4 h. Relative VEGF mRNA units were created by normalization to 28 S rRNA and defining the mean of the 13 % group to be 1. Shown are means ± SD (n = 3).

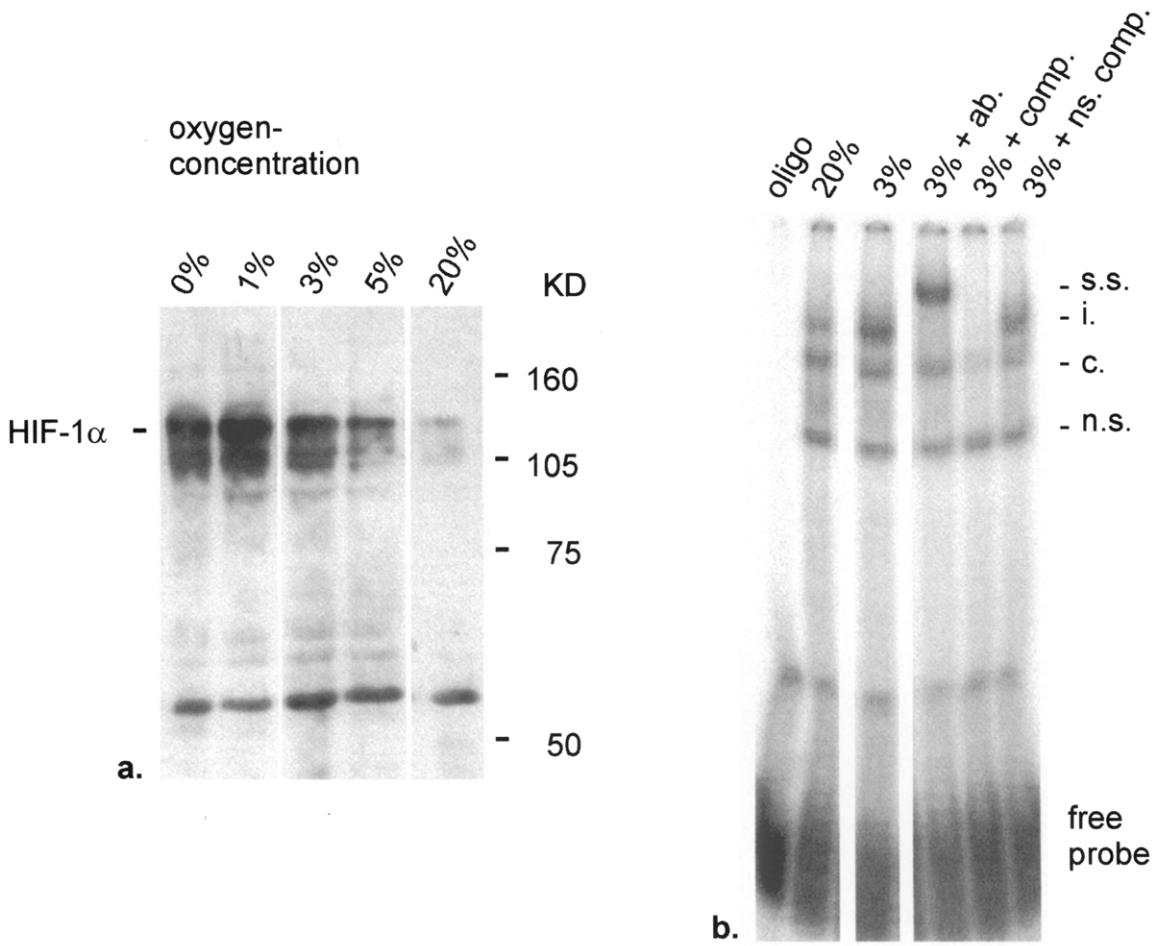

Fig. 3. a: Detection of HIF-1α protein. Western blot with nuclear extracts from MX-1 cells challenged with various oxygen concentrations for 4 h. b: gel-shift assay with nuclear extracts from MX-1 cells exposed to 20% or 3 % O_2 for 4 h. Additions to binding reactions were: + ab. = 1 µl anti-HIF-1α antibody; + comp. = 500 fold excess of cold TfHBSww oligonucleotide; + ns. comp = 500 fold excess of cold NF-κB oligonucleotide. oligo = labelled TfHBSww oligonucleotide without nuclear extract; i. = inducible DNA-binding; c. = constitutive DNA-binding activity; n. s. = non specific DNA binding; s. s. = supershift.

nucleotide in comparison to the normoxic control (Fig. 3 b). Nevertheless, even under normoxic conditions significant HIF-1 DNA-binding was observed.

MX-1 tumors in vivo. Northern blot analysis of RNA extracted from tumor tissue of mice maintained at normal inspiratory O_2 concentrations revealed significant amounts of VEGF mRNA. Exposure of the animals to inspiratory hypoxia (10% O_2, balanced N_2) for 17 h led to a 3fold increase in tumoral VEGF mRNA levels (Fig. 4). Shorter hypoxic periods (1 h, 6 h, 9 h) did not alter the basic VEGF mRNA levels.

Immunohistochemical staining of thin sections of the extirpated tumors from mice after 17 h of hypoxic exposure revealed VEGF protein in the cytoplasm of the MX-1 cells, but not in the surrounding non-tumorous murine cells. The intraluminal surface of the endothelium of capillaries, however, was also positive for VEGF protein thus indicating the presence of either receptor- bound or free humoral VEGF (Fig. 5). Shorter hypoxic periods did not suffice to increase VEGF-protein in thin sections. This parallels the results regarding the VEGF mRNA.

Discussion

This study showed that normoxic cells (20% O_2) of the human breast cancer cell line MX-1 constitutively express VEGF mRNA *in vitro*. On the one hand, hyperoxic incubation (95% O_2) of cultured cells did not suppress VEGF gene expression. On the other hand, the physiological response to hypoxia was still maintained. HIF-1α protein,

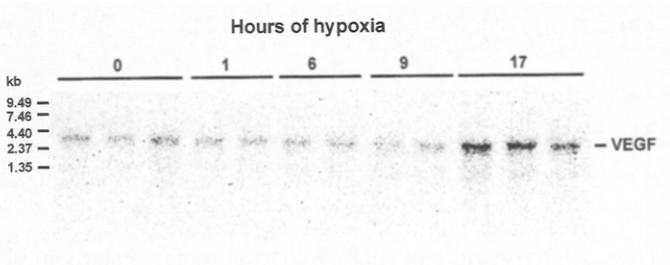

Fig. 4. Northern hybridization with a VEGF specific probe on total RNAs from tumor tissues from each 2, and 3 mice respectively maintained under normal air or 10 % O_2 for various time intervals up to 17 h.

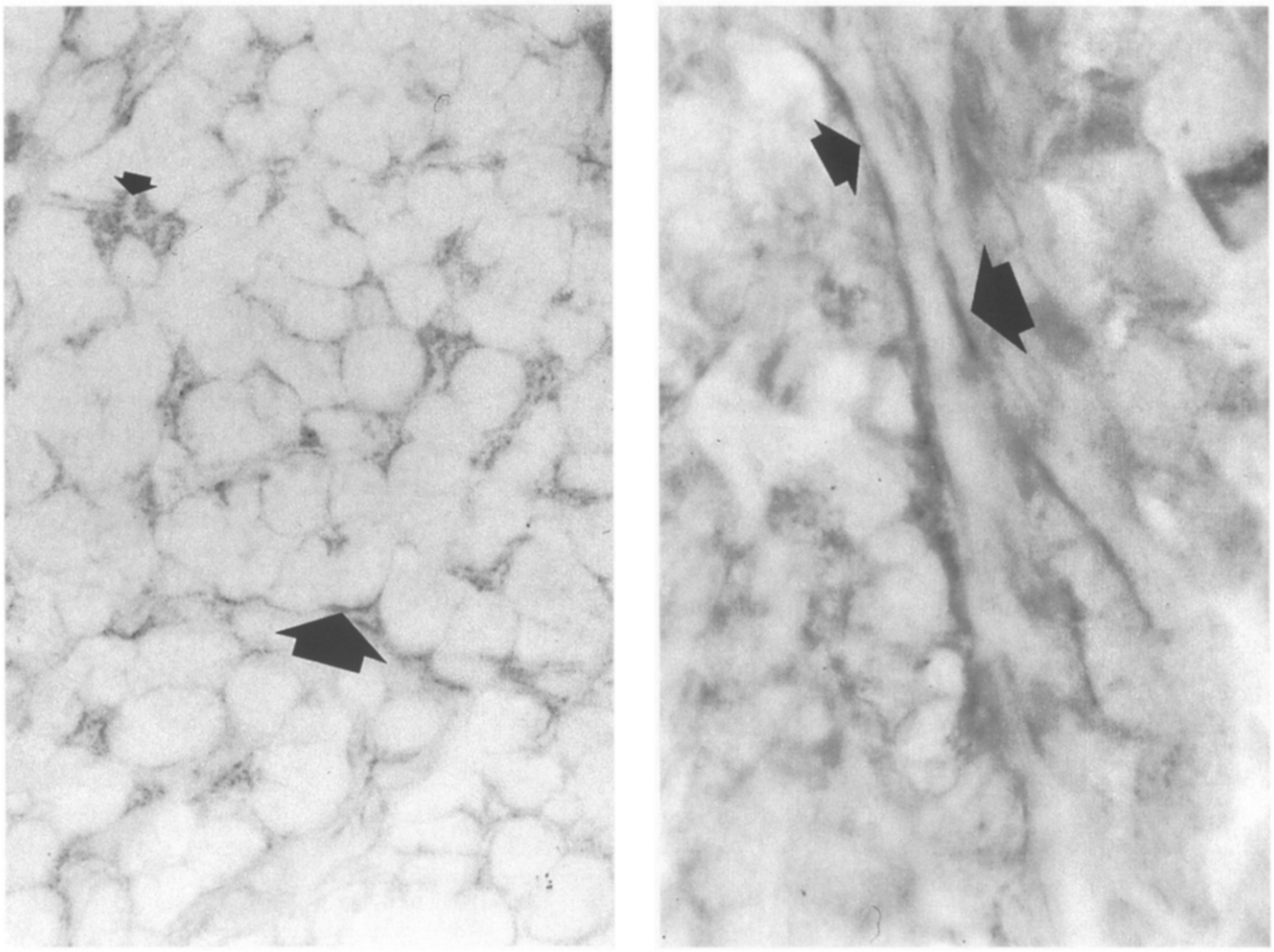

Fig. 5. Immunostained sections of intratumoral areas from a tumor of a mouse exposed to 10 % O_2 for 17 h. Left: VEGF (large arrow; dark precipitates) was localized around the immunonegative karyoplasm of MX-1 cells. The area between tumor cells did not show any precipitates (small arrow). Right: Besides some immunoreactive MX-1 cells a positive immunoreaction (arrows) in the wall of a small vessel was obvious. (1 000×)

VEGF mRNA and VEGF protein levels all increased on exposure of the cultures to 3% O_2. To further elucidate the pO_2 dependence of VEGF production additional experiments were carried out with various O_2 concentrations (13%–0%). VEGF mRNA and VEGF protein levels increased exponentially with a notable boost under severe hypoxia. The increased VEGF production is the result of a more efficient transcription and translation of the gene (Forsythe et al. 1996; Levy et al. 1996; Liu et al. 1995; Stein et al. 1998). The activation of HIF-1 by hypoxia is considered the main transcriptional mechanism leading to increased VEGF gene expression. Although nuclear HIF-1α protein was hardly detectable under normoxic conditions, the highly sensitive gel shift assay revealed a very efficient DNA-binding of functional HIF-1 complexes. This may contribute to the basal transcription of the VEGF-gene in MX-1 cells. Thorough examination of the VEGF response of MX-1 cultures to hypoxia showed half maximum levels between 1.5% and 3% O_2 and concomitant maximum HIF-1α protein levels around 1.5% O_2. The decrease in nuclear HIF-1α protein when the pO_2 approached 0 indicates that under anoxic conditions transcription factors other than HIF-1 probably play important roles in transcribing the VEGF-gene. Our results are in good agreement with those of Jiang et al. (1996) who reported half-maximum HIF-1α protein levels in human hepatoma cultures at 1.5% to 2% O_2 concentrations and a reduced HIF-1α protein concentration and HIF-1 DNA-binding activity under exposure to anoxic conditions.

In addition, MX-1 tumors were xenotransplanted to nude mice as an *in vivo* model to examine VEGF mRNA expression. Northern blot analysis of RNA extracted from extirpated tumors revealed detectable amounts of VEGF mRNA when animals were maintained under normoxic conditions. Lowering the O_2 concentration in the inspiratory gas to 10% for 17 h resulted in 3 fold increased steady state VEGF mRNA amounts in tumor tissues. Immunohistochemical studies showed that VEGF protein was also increased. Additionally, immunoreactive VEGF in the serum of these experimental animals was in-

creased, too (n = 2, data not shown). These observations indicate that VEGF gene expression in the tumor was regulated by O_2 *in vivo*. Note that the O_2 content of arterial blood in mice inspiring 10% O_2 is reduced to about 50% of the normal. This estimation is based on measurements of the arterial pO_2 in rats exposed to inspiratory hypoxia and recordings of the O_2 binding curve yielding an O_2 half saturation pressure of 41 mmHg under standard conditions (Pepelko and Dixon 1975; Petschow et al. 1978). The delayed response (in regard to the VEGF mRNA formation) *in vivo* compared to the *in vitro* experiments shows that the whole organism has a greater capacity to resist hypoxic challenges than isolated cells. Our finding that shorter hypoxic periods are not sufficient to increase steady state VEGF mRNA amounts is in accordance with Sandner et al. (1996) who reported that 10% O_2 in the inspiratory atmosphere was not able to increase VEGF mRNA levels in rats within 6 h.

Scott et al. (1998) have shown that there is considerable variation in VEGF mRNA expression across human breast cancer cell lines maintained under normoxic conditions. Furthermore, these authors demonstrated that VEGF mRNA levels increased on hypoxic stimulation, independent of the basal level, in all of the 7 cell lines under study (Scott et al. 1998). By *in situ* hybridization (Brown et al. 1995) and immunohistochemistry (Yoshiji et al. 1996), clinical studies have shown that VEGF production is confined to malignant breast cancer cells, while non-malignant breast tissue is almost negative. Breast tumor cell-derived VEGF is thought to play an important role in the vascular and stromal changes necessary for the growth and metastatic spread of breast cancer (Brown et al. 1995). In fact, there is a significant correlation between the density of microvessels in invasive breast carcinomas and the occurrence of metastases (Weidner et al. 1991).

In the present study, the tumor consisted of both human MX-1 cells and murine connective tissue and blood vessels. To determine the origin of VEGF-protein producing cells, histological analyses of thin-sections of the extirpated tumors were carried out. Immunohistochemical staining of the sections showed VEGF protein in the cytoplasm of the MX-1 cells, but not in the surrounding non-malignant mouse cells. The intraluminal surface of the endothelium of capillaries was also positive for VEGF protein, suggesting the presence of receptor-bound VEGF.

Recently, Parliament et al. (2000) and Danielsen et al. (2000) showed that VEGF expression is relatively independent of hypoxic challenge, but genetically determined in human glioma or melanoma xenografts and spheroids. Additionally, White et al. (1995) found VEGF highly inducible in cell lines with low basal VEGF production, whereas especially cells with high constitutive expression of VEGF are relatively refractile to hypoxia. Thus, the degree of hypoxic induction of VEGF expression is tumor type-specifc. In the light of our results, it may be particularly important to increase oxygenation in that subset of tumors which show relatively low constitutive expression with high hypoxic inducibility.

Mendoza et al. (1995) reported monocytes and mast cells in the perivascular tissue of the MX-1 xenografts. Hellwig-Bürgel et al. (1999) showed that the proinflammatory cytokines IL-1β and TNF-α increase HIF-1 activity under normoxic conditions. Hence, under normoxic conditions cytokine induced HIF-1 activation would offer an attractive explanation for constitutive VEGF expression.

To the best of our knowledge the present study is the first to show that VEGF production increases in tumors *in vivo* when the O_2 content of the arterial blood is lowered. The other way round, VEGF mRNA levels – and thus angiogenesis and its effect on tumor growth and metastasis – may be lowered when the O_2 supply to tumors is increased. Experiments are presently being carried out in our laboratory to study the relationships between arterial and intratumoral pO_2, VEGF mRNA levels, angiogenesis and tumor growth in MX-1 tumor transplanted mice undergoing erythropoietin and hyperbaric O_2 therapies.

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