Advances in Brief

Mammary Fibroblasts May Influence Breast Tumor Angiogenesis via Hypoxia-induced Vascular Endothelial Growth Factor Up-Regulation and Protein Expression

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**Abstract**

Recent studies demonstrate the relationship of microvessel density to malignant progression in breast cancer (N. Weldner, J. P. Semple, W. R. Welch, and J. Folkman, N. Engl. J. Med., 324: 1–8, 1991), underscoring the importance of angiogenesis in this tumor. Crucial in tumor angiogenesis are the paracrine actions of tumor-secreted factors (e.g., vascular endothelial growth factor), which have been thought to derive from the tumor epithelial cells themselves. We demonstrate that in response to hypoxic conditions, human mammary fibroblasts dramatically up-regulate vascular endothelial growth factor mRNA and increase vascular endothelial growth factor protein levels in accordance with the degree of oxygen deprivation. Thus, mammary stromal cells, only recently considered in the regulation of breast carcinomas, may play a hitherto unrealized role in breast cancer angiogenesis.

**Introduction**

Due to irregular and inadequate tumor vasculature, oxygen levels can vary considerably over microregions within tumors. Within breast carcinomas, as in all solid tumors, cell proliferation must be accompanied by angiogenesis to provide adequate oxygenation (1), and recent studies demonstrate the relationship of tumor microvessel density to malignant progression in breast cancer (2). Tumor angiogenesis depends on the interaction between different tumor components, e.g., paracrine stimulation of endothelial proliferation by tumor epithelial cells. Above it, stromal and vascular elements, yet a role for stromal fibroblasts in the angiogenesis of breast tumors has not been considered. Could stromal fibroblasts contribute to the growth and progression of breast carcinomas by the secretion of angiogenesis factors? Folkman and Shing (3) describe the up-regulation of angiogenesis factors in tumor epithelial cells as inherent to the “switching” of these tumor cells to an angiogenic phenotype in the process of malignant transformation. If tumor fibroblasts are important in production of angiogenesis factors it would likely be by an alternative mechanism since they are not themselves transformed. Up-regulation of VEGF, a major angiogenesis factor (4), has been shown to be hypoxia inducible in human glioblastoma cells (5), mouse fibroblast L cells (6), and rat cardiac cells (7). This environmentally triggered up-regulation of an angiogenesis factor bears similarity to that seen in macrophages during wound healing (7). We reasoned that if a similar mechanism of hypoxic induction of VEGF exists in human mammary tumor fibroblasts, it could provide an avenue whereby fibroblasts might influence breast tumor angiogenesis. Although angiogenesis is currently under intense investigation in breast cancer, the expression of VEGF in this tumor has yet to be studied. We show here evidence of hypoxic induction of VEGF up-regulation in human mammary fibroblasts, suggesting the possibility of paracrine influences by fibroblasts on endothelium within hypoxic regions of these tumors.

**Materials and Methods**

Cells were grown in monolayer cultures for detection of VEGF mRNA via protection assays, following hypoxic exposure [VEGF is also called vascular permeability factor (8, 9)]. Cells were grown in multicellular sandwich cultures to study both the levels and the subcellular location of VEGF protein on a cell-by-cell basis over oxygen/nutrient gradients via immunohistochemistry and digitized imaging.

**RNA Detection.** Monolayers of early culture (third) passage mammary fibroblast cells, isolated from both breast tumor (B437) and normal breast tissue derived from reduction mammoplasty (B106) were plated in DMEM (GIBCO) with 20% fetal bovine serum (GIBCO) at 4 × 10⁶ cells/60-mm glass Petri dish. Cells were obtained from Dr. Michael Gould (Department of Human Oncology, University of Wisconsin). Fibroblast origin was verified by immunostaining with a monoclonal antibody against vimentin (Dako). Hypoxia (>100 ppm O₂) was achieved using a Bactron IV anaerobic chamber (Sheldon) with a self-contained incubator unit that uses a 5% H₂–5% CO₂–90% N₂-injected atmosphere and a palladium catalyst. Some cultures that had undergone a 24-h hypoxic period were reoxygenated with an additional 24-h oxygen (21%) incubation. For mRNA collection cells were lysed on site in 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% N-lauroylsarcosine and 0.1 M 2-mercaptoethanol, and total RNA was isolated using acid phenol. From human VEGF-165 cDNA (Calbio California Technology), a 146-base pair clone extending from nucleotides 440 to 585 was subcloned in a Bluescript II-KS vector (Stratagene) and utilized as the probe template. The solution hybridization RNase protection assay was performed using a high specific activity antisense riboprobe transcribed in vitro (10). For reference, cyclophilin mRNA was analyzed in each sample simultaneously with the VEGF mRNA. This technique afforded the simultaneous probing of the cyclophilin control and the VEGF bands, allowing for a reproducible probing difference due to variations in gel workup. Human granulosa-lutein cells were used as positive controls (11). Total RNA was dissolved in 25-μl hybridization solution (4 M guanidinium thiocyanate-0.1 M EDTA, pH 7) and mixed with 250 pg of each probe. The protected materials were resolved through nondenaturing 5% PAGE. Quantitation of the bands was performed using a phosphomager (Molecular Dynamics).

**Tumor-mimetic Sandwich Cultures.** Mixed-cell sandwich cultures were set up by plating on microscope slides breast-fibroblasts (isolated either from tumor or normal mammary tissue after reduction mammoplasty) along with MCF7 cells, each at a density of 1.7 × 10⁶ cells/slide. Single-cell sandwiches were cultured using either tumor or normal mammary fibroblasts plated at 1.7 × 10⁶ cells/slide. After 24-h incubation, a top slide (coated with Protil) was placed on the cultures, sandwiching the cells and restricting cell access of medium to the 75-μm gap between the slides (Fig. 2). The medium reservoir functions as the vasculature feeding the cell population. In much the same manner as in a tumor, cell consumption and diffusion constraints (due to the narrow gap between slides) decrease the oxygen/nutrient availability as a function of distance into the sandwich. In addition to tumor mimetics, the...
induced gradient acts to provide experimental controls by displaying on the same slide adjacent regions with varying microenvironmental conditions.

**Protein Detection.** Immunohistochemical detection of VEGF was made for slides after various times in sandwich culture. Top slides were removed and the cells were washed with PBS, fixed in cold acetone/methanol (1:1) for 10 min, and air dried. Immunodetection were commenced by rehydrating in PBS, followed by blocking of endogenous peroxidase activity in 1% hydrogen peroxide in double distilled H2O for 10 min, and blocking of the nonspecific sites with 10% normal swine serum before applying the primary antibody in a 3 μg/ml concentration in 0.05 M Tris-HCl. The antibody was polyclonal rabbit anti-human VEGF (Santa Cruz Biotechnology), and for staining we used a sensitive three-step streptavidin-biotin peroxidase procedure (LSAB-2 Kit, Dako). As negative controls, slides were incubated with normal rabbit serum (1:500) in PBS in place of the primary antibody. The chromogen diaminobenzidine (Sigma Chemical Co.) was used, and slides were counterstained with Mayer hematoxylin and mounted. Densitometric quantitation of antibody per cell was done using a Quamit 570 computerized imaging facility, utilizing a Reichert-Jung Polvar microscope with automated stage integrated to an imaging computer via a JVC 3-CCD camera.

**Results and Discussion**

Monolayers of early culture human fibroblasts, derived from both tumor and normal breast tissue, were subjected to hypoxic conditions for 6, 12, and 24 h. RNase protection assays were performed to display the expressed VEGF mRNA (Fig. 1). Dramatic VEGF up-regulation was observed in human mammary fibroblasts in response to hypoxia. Tumor fibroblasts showed a 30-fold increase in the expression of VEGF mRNA after 6 h of hypoxia. For the fibroblasts from normal breast tissue, an almost 10-fold increase was seen after 6 h of hypoxia. Demonstrating the reversibility of this up-regulation, VEGF mRNA levels essentially returned to background following a 24-h reoxygenation period subsequent to 24 h of hypoxia. This reversibility highlights the difference between the concept of a switched-on “persistent” up-regulation of angiogenesis factors in tumor epithelium and the microenvironmentally triggered transient process demonstrated here for tumor fibroblasts (Fig. 1).

Although these data demonstrate that hypoxia stimulates mammary fibroblasts to strongly up-regulate VEGF, it remained to be determined whether, under tumor-mimetic oxygen gradients, there is a corresponding increase in detectable protein levels. (In the studies on the hypoxic up-regulation of VEGF in human glioblastoma (5) and rat cardiac (6) cells, protein increases were not investigated). In tumors, as in normal tissue, the distance a cell is from vasculature determines its ambient oxygen concentration. But in tumors, due to inadequate vascularization and vascular compression (12), much steeper oxygen gradients exist. As a result of unregulated cell proliferation, cells can be pushed further and further from existing vasculature, down the oxygen gradient. Within a solid tumor mass, cells at distances sufficiently removed from vascularization (~200 μm) experience extreme oxygen shortages, and eventually undergo necrosis. Thus, the microenvironment a tumor cell experiences is dynamic; it alters as cell proliferation expands the tumor mass. If a tumor stromal cell produces VEGF in response to the amount of oxygen it senses, consistent with the need for angiogenesis, its production would need to be variable and constantly adjusted in accordance with changing microenvironmental condition. Using tumor-mimetic in vitro cultures coupled with immunohistochemical techniques, we investigated whether VEGF protein is adjusted in accordance with the time and degree of microenvironmental oxygen deprivation.

To achieve dynamic, microenvironmental gradients in culture that mimic those in tumors, the sandwich culture system was utilized (13). In these cultures, as in the widely used multicellular spheroid system (14), cell-created diffusion and consumption gradients are induced by restricting the access of cells to the source of oxygen and nutrients. In

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**Fig. 1. Up-regulation of VEGF mRNA under hypoxia in tumor and normal mammary fibroblasts. (Top) Autoradiogram of RNase protection assay for VEGF. Lane 1, double stranded pBR322 DNA digested with MspI; Lane 2, probe alone digested with RNase. Lanes 3–10 refer to tumor-derived fibroblasts (B437): Lane 3, 6 h of O2; Lane 4, 12 h of O2; Lane 5, 24 h of O2; Lane 6, 6 h of hypoxia; Lane 7, 12 h of hypoxia; Lane 8, 24 h of hypoxia; Lane 9, 24 h of hypoxia, followed by a 24-h reoxygenation period; Lane 10, 24 h under hypoxia in media containing 20 μg/ml transforming growth factor β1,2,3-blocking antibody (Genzyme). Lanes 11–14 refer to normal fibroblasts (B106) derived from reduction mammaplasties: Lane 11, 6 h of O2; Lane 12, 24 h of O2; Lane 13, 6 h of hypoxia; Lane 14, 24 h of hypoxia. For reference, cyclophilin mRNA was probed in each sample simultaneously with the VEGF mRNA. Human granulosa-lutein cells were used as positive controls (not shown). (Bottom) Relative values of VEGF mRNA/cyclophilin mRNA for the autoradiogram. For both tumor and normal fibroblasts relative values are reported as ratios to their respective 6-h O2 points. These calculations demonstrate that VEGF mRNA levels return essentially to background following reoxygenation (compare Lanes 8 and 9) and that addition of the transforming growth factor β1,2,3-blocking antibody did not perturb the up-regulation of the VEGF mRNA (compare Lanes 8 and 10), indicating that transforming growth factor β is probably not a major intermediary in the hypoxic up-regulation of VEGF. Although there is no significant decrease in cyclophilin levels after 6 h of hypoxia, by 24 h of hypoxia cyclophilin levels drop. This drop in cyclophilin with prolonged hypoxia has the effect of suggesting a more pronounced increase of VEGF mRNA at 24 h of hypoxia, as compared to that at 6 h of hypoxia, than would have existed without the drop in cyclophilin. For the tumor fibroblasts in particular, most of the VEGF up-regulation probably occurs within the first hours of hypoxia. This would imply that these VEGF up-regulations could well occur under the durations of hypoxia expected in tumors.

sandwiches, as in tumors, the distance between a cell and the perfusion source (medium reservoir or vasculature, respectively) determines the local oxygen/nutrient environment of that cell. Sandwiches were set up either as cocultures of mammary fibroblasts mixed with MCF7 breast carcinoma cells or as cultures of mammary fibroblasts alone. Even in coculture sandwiches, the mammary fibroblasts grew relatively well (a high percentage of mitotic figures were observed) under extended periods of O2/nutrient deprivation, indicating that fibroblasts may indeed maintain viability in poorly perfused areas of breast tumors (Fig. 2).

Sandwich cultures were labeled with an affinity-purified antibody against human VEGF. Immunodetection revealed considerable expression of VEGF in fibroblasts of the interior, oxygen-deprived regions of sandwiches. In all sandwich cultures the degree of VEGF antibody binding per fibroblast significantly increased as a function of oxygen deprivation (Fig. 3). This was true for fibroblast sandwiches cultured for 24, 48, or 72 h with or without MCF7, with or without serum in the medium, and regardless of fibroblast source (normal breast or mammary carcinoma). Fibroblasts from tumor tissue exhibited more VEGF antibody binding than did fibroblasts from normal breast tissue under the same condition. MCF7 cells were also seen to
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Fig. 2. Sandwich culture setup depicting cell-created microenvironmental gradients over the cell population. Cells grow in monolayer on the bottom slide and O₂/nutrients diffuse through the narrow gap between slides. The medium reservoir acts as an O₂/nutrient source. Oxygen concentration decreases as a function of distance into the sandwich (13). The spatial distance of a cell into the sandwich determines its ambient microenvironment.

Fig. 3. VEGF-antibody binding in tumor fibroblast sandwiches. Cells from the outside, middle, and interior regions of sandwiches are shown after 24 and 72 h in culture. ×100. Note the minimal staining in cells in the outer, well-perfused regions and the intense immunostaining in the interior, oxygen-deprived regions. Cells were counterstained with hematoxylin; thus, cell nuclei are visible even in outer regions where antibody binding is minimal. The gradient in immunostaining intensity is counter to the oxygen gradient in each case. In the 72-h sandwich very strong perinuclear VEGF immunostaining was observed for cells in the middle and interior regions. In a single sandwich, at a given time (e.g., 24 h), the VEGF protein gradient is counter to the oxygen gradient (reading up the column). For a given position in the sandwich (e.g., inside) there is a positive variation in the amount of VEGF with time (e.g., from 24–72 h sandwich, reading across the row). Subcellular localization of protein alters with time from cytoplasmic to strikingly perinuclear.

constitutively express VEGF.⁴ In coculture sandwiches, tumor fibroblasts exhibited less VEGF antibody binding than did MCF7 cells under high oxygen tensions, although tumor fibroblasts exhibited greater VEGF antibody binding than did MCF7 cells under low oxygen conditions.

⁴ Unpublished data.

Immunodetection of VEGF in sandwiches at various times allowed further exploration of the time-dependent nature of the VEGF protein response. For a sandwich of a given age the VEGF protein gradient is counter to the oxygen gradient, and for a given position within sandwiches there is a positive variation with sandwich age. Both VEGF protein expression and subcellular location are altered in a time-dependent manner consistent with preparation for protein export from the cell (Fig. 3). For fibroblasts in the middle and interior regions of 24-h sandwiches, antibody binding was generalized throughout the cytoplasm. By 72 h, cells in these regions exhibited protein which was perinuclearly localized, presumably reflecting its accumulation in the Golgi before final vesiculization and export.

Densitometric imaging was used to quantify the amount of antibody staining per cell across the sandwich population. Plotted (Fig. 4), these data verify increased antibody binding as a function of the distance of a cell into the sandwich (i.e., as a function of oxygen deprivation). Although VEGF antibody binding is not strictly stoichiometric for protein, densitometric quantitation clearly demonstrates a monotonic increase in VEGF which parallels the monotonic decrease in oxygen tension. In contrast, when fibroblast sandwiches were stained with a monoclonal antibody to vimentin and imaged densitometrically, no alteration in vimentin antibody binding per cell was detected over the sandwich O₂ gradient.

In summary, evidence is accumulating that mammary stromal cells exert influence not only on the regulation of normal mammary gland development but also on the regulation of breast cancer (15–17). Stromal fibroblasts produce a number of potent factors which may impact tumor growth via paracrine effects on tumor epithelium (18). Stromal-epithelial interactions have been implicated in the malignant progression of breast tumors. Several proteolytic enzymes that degrade extracellular matrix and are therefore postulated to promote tumor invasiveness are secreted cooperatively by tumor epithelial cells and adjacent stromal fibroblasts (19). We have shown here that mammary stromal fibroblasts may also produce factors which exert influence on the growth and malignant progression of breast tumors via paracrine effects on tumor-associated endothelium.
Our findings demonstrate that human mammary fibroblasts dramatically up-regulate and express VEGF protein in response to hypoxic tumor-mimetic microenvironmental conditions. In light of these findings, it seems that examination of the secretion of tumor angiogenesis factors should be extended beyond tumor epithelial cells to include the role of stromal cells. Recently, elevated levels of VEGF/vascular permeability factor mRNA were found in tumor cells and "occasional stromal cells" immediately adjacent to necrotic zones in adenocarcinoma of the gastrointestinal tract (20). In addition to considerations of stromal influence, a further clarification of the role of microenvironmental induction of angiogenic factors in tumors (versus the role of persistent factor expression resulting from the switching of tumor cells to an angiogenic phenotype) would be of considerable value in the elucidation of the mechanisms of angiogenesis in tumors, and in the design of effective anticancer strategies aimed at reversing tumor angiogenesis.

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