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13. ABSTRACT *(Maximum 200 words)*

Tumors require generation of new blood vessels to sustain their growth. Angiogenesis is the process through which tumors develop new blood vessels. The scope of my studies was to investigate the role of Tissue Transglutamina se (TG) in angiogenesis and tumor biology. I discovered that TG was a wound healing enzyme which was active and promoted angiogenesis during healing cascade. Continuing my work in tumors (murine mammary breast carcinoma) we found that was expressed as part of host response and limited the growth of tumors by initiating a fibrotic response against them. Also, the tumor cells transfected with TG had growth and metastasis inhibition and prolonged the survival of the mice. In addition, our work has established that hypoxia is not the critical inducing factor for angiogenesis and may perhaps play a more vital role in vascular regression. We faced considerable problems with the fibrin gel chambers and have since refined them and work is now in progress using them to dissect the mechanisms of TG"s effect onangiogenesis and tumor biology. I also successfully defended my dissertation and was awarded PhD in June,1999. I have been appointed Research Associate and am continuing the work outlined in the Defense Breast cancer grant.

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Introduction

Malignant tumors require the development of new blood vessels (angiogenesis) for progressive growth. Angiogenesis is a complex process involving degradation of the basement membrane, proliferation, migration, and remodeling of the tissues by vascular cells. The goal of my dissertation was to explore the different modalities through which tumor angiogenesis progresses and study the role of the enzyme tissue Transglutaminase (TG), a crosslinking enzyme, in tumor growth, angiogenesis and metastasis. In the light of recent advances concerning role of anti-angiogenic therapy for Breast and other carcinoma, this work would enhance our understanding and provide valuable insights to the complicated process of tumor growth and angiogenesis. Also, we wanted to investigate the role of host response in inhibiting tumor growth as a new way of restricting tumor progression.

Body:

The goal of my dissertation was to investigate the role of a crosslinking enzyme tissue transglutaminase (TG) in tumor growth and angiogenesis. Since tumors are considered wounds that do not heal, we also studied the role of this enzyme in normal wound healing. In the process of that investigation, we also looked at the relationship of hypoxia to angiogenesis during wound healing. This has provided insights to the primary stimulus of angiogenesis, which could have implications in how we approach anti-angiogenic therapy in the future. We investigated TG's role in tumor growth and metastasis from two different angles i.e. host and tumor. As a host response, TG restricts tumor growth through fibrosis. Meanwhile, as a tumor protein, it has different functions altogether. Now, I will enumerate the research accomplishments as stated in statement of work.

Technical Objective 1; Task 4 to 8:

We had problems in getting consistent results from fibrin gel chambers. The gel liquefied in the chamber that led to non-evaluation of chambers. This also resulted in excessive use of materials including fibrinogen. Since then, we have since altered the design of these chambers reducing the amount of materials used and added Factor XIII to stabilize the fibrin for longer time periods. We have a new protocol approved by the Duke IACUC and are proceeding with those experiments. Although, we have addressed the same issues with a different approach.

We investigated the role of TG during wound healing and have since completed the manuscript that was accepted in FASEB Journal (Appendix 1). This study details role of TG during wound healing including the cellular expression,

molecular form and activity pattern, the relationship of TG to injury cytokines and its ability to induce angiogenesis.

We also investigated the role of hypoxia in induction of angiogenesis and repair and have the manuscript accepted in Annals of Surgery (Appendix 2). We showed that hypoxia may not be the primary stimulus of angiogenesis and repair and coagulation system has a much larger role to play in this aspect. Since TG is part of this first coagulation response, it gave us more impetus to study in detail its role in wound healing and angiogenesis.

Technical Objective 2; Task 1 to 2:

We have since divided the approach to technical objective 2 in two spheres i.e. TG as part of host response and TG as part of tumor tissue.

We have completed the study of TG's role as part of the host response against tumor growth in a rat mammary adenocarcinoma R3230 Ac. The manuscript is in final review with Laboratory Investigation (Appendix 3). We have shown that TG is part of the host response and acts to inhibit tumor growth by inducing a fibrotic reaction with help of TGF β.

We also investigated role of TG as part of tumor cells. We transfected murine breast carcinoma 4T1 with TG tagged with green fluorescent protein (GFP) and the details of the preliminary results are given in Appendix 4.

Dissertation:

I successfully defended my dissertation in June, 1999. I am still continuing the work related to the Defense grant as a Research Associate at Duke University Medical Center.

Key Research Accomplishments:

- TG is a wound healing enzyme and promotes angiogenesis.
- Hypoxia is not the critical factor in induction of angiogenesis and repair and is involved in vascular regression.
- TG is expressed as a host response to tumor growth and acts to limit tumor growth through fibrosis.
- TG enhances establishment of metastasis in murine breast carcinoma.
- TG limits tumor growth and metastasis in primary breast tumor mass.

Reportable Outcomes:

1. **ZA Haroon**, KG Peters, CS Greenberg and MW Dewhirst. Ch:01 Angiogenesis and Oxygen transport in the solid tumors in *Antiangiogenic Agents* (BA Teicher, ed) Humana, Totowa, NJ (*1999*). (Appendix 5)
2. **ZA Haroon**, JM Hettasch, TS Lai, RL McCauley, MW Dewhirst and CS Greenberg. Tissue Transglutaminase is expressed and active during Rat Dermal Wound Healing and Angiogenesis. *Accepted in FASEB Journal.*
3. **ZA Haroon**, JA Raleigh, CS Greenberg and MW Dewhirst. Early wound healing exhibits cytokine surge without evidence of Hypoxia. *Accepted in Annals of Surgery.*
4. **ZA Haroon**, TS Lai, JM Hettasch, RA Lindberg, MW Dewhirst and CS Greenberg. Tissue Transglutaminase is expressed as a host response and inhibits tumor growth. *Submitted to Laboratory Investigation.*
5. **ZA Haroon**, T Wannenburg, CS Greenburg, and DC Sane. Immunohistochemical detection of Transglutaminase, a Tissue stabilizing enzyme in athersclerotic plaques. *AHA, Dallas, Texas, Novemeber, 1998.*
6. TS Lai, A Hausladen, TF Slaughter, JP Eu, KA Peoples, JM Hettasch, **ZA Haroon**, JS Stamler and CS Greenberg. Tissue Transglutaminase binds and releases Nitric Oxide- Role in regulating thrombogenesis of blood vessels. *40th Annual ASH, Miami, Florida, December, 1998.*
7. **ZA Haroon**, JA Raleigh, CS Greenberg and MW Dewhirst. Early wound healing exhibits cytokine surge without evidence of Hypoxia. *ASBMB, SanFrancisco, CA, May 1999.*
8. **ZA Haroon.** Hypoxia and Tissue Transglutaminase – A wound healing perspective. *Dissertation, June , 1999.* PhD awarded.
9. Research Associate at Duke University Medical Center. Job.

Conclusions:

The work to date has established TG as a wound healing enzyme that can also induce angiogenesis. TG's expression and activity is related to major injury cytokines such as TGF β and VEGF. This has provided much needed insights into the role of this enzyme in tumor biology also since tumors utilize wound healing mechanisms to propagate themselves.

This work was the lead to our investigations in TG's role in tumor biology and we have found that TG both as a host response and as part of primary tumor mass acts to inhibit tumor growth and metastasis. This is vital information that after further research could potentially be exploited to eradicate breast cancer.

Also, we established that hypoxia is not one of the critical factors for induction of angiogenesis and repair. This study can have major impact on the importance of initial coagulation response as the true source of angiogenesis trigger.

Appendix 1

Tissue Transglutaminase is Expressed, Active, and Directly Involved in Rat Dermal Wound Healing and Angiogenesis

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ABSTRACT

Tissue transglutaminase (TG) is an enzyme that stabilizes the structure of tissues by covalently ligating extracellular matrix molecules. The expression and localization TG is not well established during wound healing. In this study, we performed punch biopsy wounds on anesthetized rats and monitored the wound healing process by histologic and immunohistochemical methods. The TG antigen and activity are expressed at sites of neovascularization in the provisional fibrin matrix within 24 hrs of wounding. Endothelial cells, macrophages and skeletal muscle cells expressed TG throughout the healing process. The TG antigen within the wound was active *in vivo* based on the detection of isopeptide bonds. The TG antigen increased 4-5 times by day 3 post wounding and was proteolytically degraded. TG expression occurred in association with TGF β, TNF α, IL-6, and VEGF production in the wound. Recombinant TG increased vessel length density (a measure of angiogenesis) when applied topically in rat dorsal skin flap window chambers. In conclusion, we have established that TG is an important tissue stabilizing enzyme that is active during wound healing and can function to promote angiogenesis.

Keywords Tissue Repair, Extracellular matrix, Crosslinking, Endothelial cells and TGF β.

INTRODUCTION

Wound healing is a complex and intricate process initiated in response to injury that restores the function and integrity of damaged tissues. Wound healing involves continuous cell-cell and cell-matrix interactions that allow the process to proceed in overlapping stages i.e. inflammation, proliferation and remodeling (1). Defects in any phase of wound healing can contribute to the pathology of many diseases (psoriasis, rheumatoid arthritis etc) and tumor growth (2). The tissue transglutaminase (TG) is a calcium dependent enzyme that covalently crosslinks a wide variety of extracellular matrix (ECM) proteins producing a protease resistant matrix and is reported to be expressed at sites of inflammation (3). The TG therefore appears to play a role in tissue repair and be involved in several phases of wound healing.

The TG catalyzes the formation of ε-(γ-glutamyl) lysine bonds (isopeptide bond) between peptide bound glutamine residues and the primary amine group of various amines (4). These isopeptide bonds are stable and more resistant to proteolytic degradation than non-covalent linkages. The covalent crosslinking reaction increases the resistance of proteins to chemical, enzymatic and physical disruption (3). The list of proteins that are TG substrates is extensive and includes extracellular adhesive proteins such as fibronectin (5), collagen (6), fibrinogen (7), fibrin (8), laminin/nidogen (9), osteopontin (10) and vitronectin (11) to name a few. Recent data has implicated TG in several intra- and extracellular processes that are critical for wound healing including apoptosis (12), osteogenesis (13), cellular signaling (14), and cell adhesion (15).

Bowness et al (16, 17) reported TG activity was present at sites of wound healing and that inhibition of TG by putrescine caused decreased breaking strength and increased solubility of the

repairing wound tissue (18). However, the cellular expression, distribution and metabolic fate of the TG at sites of wound healing in relationship to the expression of cytokines and migration of inflammatory cells was not investigated. The goal of this study was to identify the distribution of TG antigen, activity and as well as the metabolic fate of TG during wound healing. In addition, the expression of various cytokines and inflammatory cells associated with the expression of TG was analyzed. Lastly, a direct effect of TG on angiogenesis was studied in an *in vivo* window chamber model. These studies and their implications add direct significance to the role of TG in wound healing and angiogenesis.

METHODS

Animal Protocols: The Duke Institutional Animal Care and Use Committee approved all animal protocols.

Wounds: Fisher 344 female rats from Charles River Laboratories (Raleigh, NC), were anesthetized with intraperitoneal injections of pentobarbital (40 mg/kg) and ketamine (70 mg/kg), then shaved and depilated Nair, Carter-Wallace (NewYork, NY). Eighteen 5 mm biopsy punch wounds were made on the dorsal skin. The normal skin served as unwounded skin controls. Wounds were harvested at days 1 through 9 while animals were anesthetized. Days 0, 1, 3, 5, 7 and 9 post-wounding were used in western blots and days 0, 1, 2, 4, 6 and 8 were utilized for immunohistochemistry. Two rats were sacrificed for each timepoint by intravenous pentobarbital overdose and the experiments were done in triplicate. Tissues were either snap frozen in liquid nitrogen for western blots and kept at -80 C or fixed in 10% neutral buffered formalin for paraffin embedding for immunohistochemistry.

Window Chambers: Dorsal skin flap window chambers were used as described by Papenfuss (19). Briefly, Fisher 344 rats were anesthetized and the skin over the back was depilated and

surgically prepared. The skin flap was pulled dorsally away from the back. Two opposing one-cm diameter windows were created on each side of the flap by surgically resecting the epidermis. One to two fascial planes were left, which contained a few preformed vessels. The resultant subcutaneous window was protected by glass coverslips and was held away from the body of the animal by an anodized aluminum superstructure. This wounded tissue window created a visual field through which the process of wound healing could be observed non-invasively. Recombinant human TG (20) (500 μl of 40μg/ml or 40μM) was applied topically on the day of surgery and days 1 and 2 post surgery. Normal saline was used as a control. Rats were sacrificed at day 10. Intravital microscopy was used to document the level of neovascularization at days 1 and 10. Quantitation of angiogenesis was provided by measuring vessel length density (21).

Immunohistochemistry: Immunohistochemistry was carried out using procedures described by Hsu et al (22). Briefly, paraffin embedded tissues were sectioned (5 microns) and antigen retrieval was performed using citrate buffer from Biogenex (San Ramon, CA). Tissues were treated with primary antibody against Tissue Transglutaminase (TG100 & CUB 7402, 1:10, non-reactive to Factor XIIIa, both monoclonals were epitope mapped to a region between 447-538 aminoacids, unpublished data) and VEGF 3 (1:100) Neomarkers (Fremont, CA), ED1-macrophage marker (MCA341, 1:100) Serotec (Oxford, UK), Isopeptide (23, 24) (814 MAM, 1:75) CovalAB (Oullins, France), Pan-specific TGF beta (AB-100-NA, 1:100, recognizes the active forms of TGF β1, 2 and 5) and rat anti-TNF α (AB-510-NA, 1:100) R&D (Minneapolis, MN), Interleukin-6 (R-19, 1:100) Santa Cruz (Santa Cruz, CA) and Mast cell tryptase (M7052, 1:100) DAKO (Carpenteria, CA). Secondary and tertiary antibodies were provided in a kit (314KLD) Innovex, (Richmond,CA) and the location of the reaction was visualized with 3, 3'-diaminobenzidine tetrahydrochloride Sigma (St. Louis, MO). Slides were counterstained with

hematoxylin and mounted with coverslips. Controls for the immunohistochemistry were treated with normal mouse serum (NMUS) or mouse IgG (TG100, CUB 7402, 814 MAM, M7052, VEGF 3 & MCA 341), rabbit IgG (AB-100-NA) and goat IgG (IL-6, AB-510-NA) and were negative in any reactivity. Masson's trichrome and Hematoxylin and Eosin (H&E) were carried out as described by Sheehan (25).

Western Blot: Wounds from days 0, 1, 3, 5, 7 and 9 were homogenized in 2 ml cold lysis buffer containing the proteolytic inhibitor cocktail (#1697498) Boehringer Mannheim, (Mannheim, Germany) followed by sonification. The blots were performed with four different wound sets. They were then centrifuged and supernatant was removed and protein content was determined using Bio-Rad. Gel electrophoresis of the extracted tissue samples (50 ug/ml) was performed on an 8.5% polyacrlyamide gel using the buffer system of Laemmeli. Following electrophoresis, the proteins were transferred to nitrocellulose (0.2um). After the transfer was complete, the nitrocellulose membrane was blocked for 1 hr with 5% nonfat milk dissolved in 20mM Tris-HCl, pH 7.4, 150mM NaCl, 0.5% Tween 20. The TG antigen was detected by incubation for 1 hr using a monoclonal antibody for TG (TG 100, CUB 7402) Neomarkers (Fremont, CA) diluted 1:1000, followed by incubation for 1 hr with sheep anti-mouse IgG conjugated to horseradish peroxidase. The TG antigen was visualized using chemiluminescence reagents (ECL, Amersham) and a 30-sec exposure to autoradiography film. The amount of protein on the blot was estimated with a densitometer. The data in Figure 5A shows one sample blot and Figure 5B has the cumulative data for the four blots in a graphical presentation.

RESULTS

Light Microscopy Findings

At the earliest time point at day 1 (Figure 1A), provisional matrix with inflammatory cells (Figure 2A) and dilated blood vessels could be observed underneath a newly formed epithelial layer. Re-epithelialization was completed by day 2 post wounding. Maturation of granulation tissue was detected by day 4 (Figure 1B) and characterized by the presence of new blood vessels, inflammatory cells (Figure 2B & C) and collagen fibers organized into a dense connective tissue. Skeletal muscle cells which previously formed a boundary between the normal and wounded tissue at earlier time points, had moved to the base of the wound and closed the gap created by the injury (Figure 1B). By day 6 (Figure 1C), granulation tissue started to contract and increased in density. At day 8 (Figure 1D) the healing was in its final stages with remnants of granulation tissue left at the base of the wound. The injury site was filled with dense collagen tissue with very few blood vessels. The widely recognized three stages of wound healing (1) and their time course in our model of healing are depicted at the bottom of Figure 1.

Expression and Localization of TG

The normal rat skin consistently expressed TG antigen in the blood vessels that reside in the dermis and subcutaneous tissue (Figure 3A). Some sebaceous glands and basal keratinocytes also showed expression but in a sporadic fashion. The TG antigen was detected in the new blood vessels that invaded the provisional fibrin matrix and the dilated blood vessels at day 1 post injury (Figure 3B, C & D). The TG antigen staining was particularly intense in macrophages adjacent to the re-epithelialization border and in the provisional matrix (Figure 3B & C). Mast cells (Figure 2D) were present in highest density on day 1 post wounding and exhibited TG expression. The keratinocytes involved in re-epithelialization expressed TG antigen (Figure 3B). TG reactive skeletal muscle cells, macrophages and blood vessels formed a distinct boundary between the normal and injured tissue (Figure 3C). TG was also detected in the provisional fibrin

matrix (Figure 3D). By day 2, re-epithelialization was completed and TG expression was reduced in the epithelial layer and limited to the dermo-epidermal junction. The provisional fibrin matrix was slowly replaced by granulation tissue by day 2 and the wound began to accumulate collagen and TG antigen reactivity. TG immunoreactivity in the collagen also increased as the number of endothelial cells, macrophages and skeletal muscle cells increased in the wound.

The TG antigen (Figure 3E) and macrophage (Figure 2B) staining was absent from the keratinocyte layer at day 4. Macrophages, endothelial cells in the neovessels and more mature vessels continued to stain for the TG antigen (Figure 3E). The TG reactive skeletal muscle cells started to move underneath the wounded tissue to bridge the gap created by injury (Figure 3E). TG was still present in the granulation tissue matrix at day 4 (Figure 3F). As the granulation tissue continued to contract and the tissue was further remodeled (day 6), the highly reactive TG at the edge of the wound vanished and the TG staining became localized to the blood vessels and base of the wound (Figure 3G). By day 8, the early granulation tissue was replaced with a dense collagen rich scar. The TG antigen was predominantly expressed in blood vessels, skeletal muscles and macrophages at the base of the wound (Figure 3H). A few remaining vessels in the scar tissue also exhibited TG staining (Figure 3H).

Localization of TG activity

The isopeptide bond created by TG crosslinking was detected surrounding blood vessels, newly generating epithelial layer and fibrin by day 1 of wounding (Figure 3I). The intensity of immunoreactivity of the isopeptide bond increased within the ECM of the granulation tissue, basement membrane of blood vessels and also along the epithelial region at day 4 (Figure 3J). In contrast to the reduction of staining for TG and the redistribution of the TG antigen to the base of

the wound, the isopeptide bond antigen continued to be detectable but at a reduced level during the formation of the dense scar tissue at day 8.

Expression of Cytokines (TGF β, TNFα, IL-6 & VEGF)

TGF β was maximally expressed at the wound surface, granulation tissue and wound border over the entire time course examined (Figure 4A). The intensity of staining was greatest in regions where TG antigen reactivity was the highest. The one exception to this finding was in the mature epithelial layer at day 4 where TGF β antigen was expressed but TG was absent.

IL-6 stained intensely in the skeletal muscle cells of the dermis throughout the study (Figure 4B) with less intense staining in the macrophages, fibroblasts and endothelial cells during the early wound healing (days 1 through 4). IL-6 co-expressed with TG in the skeletal muscle cells at all time points examined. TNFα stained predominantly macrophages in the wounded tissue and co-expressed with TG in those cells throughout the healing process (Figure 4C). Endothelial cells and skeletal muscle cells also exhibited partial expression of this cytokine.

The endothelial cells and macrophages expressed VEGF antigen at day 1 post wounding (Figure 4D) which correlated with TG expression. VEGF expression in endothelial cells and macrophages even though TG continued to be expressed at high levels at day 4. By day 8, TG and VEGF co-expressed in remnant blood vessels in the scar tissue.

A summary of the immunohistochemical data is provided in Table 1.

Western blot analysis of TG antigen in wounded tissue

SDS-PAGE and quantitative immunoblotting demonstrated that the total TG antigen increased 4-5 fold by day 3 (Figure 5A & B). Quantitative analysis of the antigen (n=4) revealed that more than 95% of the protein was proteolytically degraded to 55, 50 and 20 kda fragments by day 1 post wounding. The extent of proteolysis and the amount of TG antigen reached maximum

the TG gene could be induced by these cytokines and function to aid in tissue repair. These findings also indicate that complicated and dynamic interactions exist between the cytokines and TG.

There is an important interaction between TGF β and TG that could amplify production of granulation tissue. TG is bound to cell surface complexes comprised of plasminogen, uPAR and the mannose-6-phosphate receptor (29). The function of this complex is to facilitate the conversion of latent TGF β to its active form (29). Our immunohistochemistry results show maximal staining for active TGF β in areas demonstrating high TG immunoreactivity when the provisional fibrin matrix is being replaced by newly synthesized connective tissue. This interaction appears to be operational in the second phase of wound healing. The increased activation of TGF β by TG could lead to the expression of TG gene early within the fibrin clot and at sites of re-epithelialization since the TG gene itself is induced by TGF β. TG and TGF β effectively complete a positive amplification loop whereby the TG increases TGF β activation, which then induces more TGF β activity to further amplify TGF β activation and TG expression (Figure 7). The end product of this process is the replacement of fibrin matrix with granulation tissue.

The early expression of TG by endothelial cells and macrophages invading the fibrin clot observed during wound healing appeared to stabilize the fibrin, since isopeptide bonds were detected in both the fibrin and the newly synthesized loose granulation tissue. Isopeptide bonds could be generated by other isoforms of TG including Factor XIIIa and epidermal transglutaminase. We cannot quantitate to what extent factor XIIIa or TG is responsible for the generation of isopeptide bonds. However, the distribution of factor XIIIa in wounds is different from that of TG antigen reported in this study (30). Earlier studies in patients with Factor XIIIa

deficiency have shown that there were wound healing defects in only 20% (31), suggesting an alternate pathway of fibrin stabilization in the tissues of these patients. Factor XIIIa requires thrombin for activation while TG is synthesized in an active form (32). The factor XIIIa molecule may be responsible for the isopeptide bonds formed within the fibrin clot. Since TG does not require thrombin activation, it can catalyze the stabilization of newly formed ECM as thrombin is removed from the wounded tissue. The TG in human blood vessels catalyzes the α-α crosslinks of fibrin and fibrinogen in human atherosclerotic plaques (33) demonstrating this enzyme is active during human disease process. The stabilization of the matrix by TG could regulate assembly of the granulation tissue and the neovascularization that is essential for an effective healing response.

The transient expression of TG in the epithelial layer suggested a role of TG in re-epithelialization and keratinization, as reported by others (34). Raghunath et al found that TG was expressed at the dermo-epidermal junction in wounds. They suggested that TG might play a role in attaching the epithelial layer to the dermo-epidermal junction. TG might be responsible for the earlier re-epithelialization episode and epidermal transglutaminase takes over later to stabilize the mature epithelial layer as TG expression diminishes. Deficiency of epidermal transglutaminase results in icthyosis, not a total breakdown of the skin barrier, which suggest there are other transglutaminases that can maintain epidermal integrity (35).

TG could promote wound contraction by crosslinking ECM molecules at the edge of wound. Cohen et al suggested that the plasma Factor XIII transglutaminases expressed by platelets contributed to the contraction of the fibrin clot (36). The TG, by ligating protein molecules throughout the wound, could lead to more effective wound contraction. The TG

expression by skeletal muscle cells and location of TG activity at these sites could also firmly anchor granulation tissue with the existing tissues to promote wound closure.

Migration of endothelial and inflammatory cells into fibrin forms an indispensable part of the healing process. Major pro-inflammatory and angiogenic cytokines such as TGF β, TNF α and VEGF exert their influence by promoting migration of cells to the injured site and the scaffolding function of fibrin has been shown to be an essential part of VEGF mediated migration of endothelial cells (37). During migration, the stability of the provisional fibrin matrix is of utmost importance and TG's ability to stabilize the matrix that resists degradation may be vital for orchestrating tissue repair.

Results obtained by adding recombinant TG to the sites of skin wound healing caused an increase in the vessel length density, a measure of neovascularization in the rat skin. The TG could be an important mediator of angiogenesis by regulating important events in vascular assembly either directly by its covalent modification of proteins or indirectly by modifying TGF β function. TGF β function is essential for normal vascular development (38) since defects in the TGF β binding protein, endoglin, leads to the congenital vascular malformation syndrome of hereditary hemorrhagic telangiectasia (39). In ongoing experiments we have found that TG placed in a fibrin chamber enhances angiogenesis (unpublished data). Additional studies are in progress to define the mechanisms responsible for TG mediated enhancement of angiogenesis in fibrin.

The proteolytic degradation of the TG may provide a method to regulate the duration and extent of the crosslink reaction. By degrading the TG, the positive amplification loop between TG expression and TGF β activation would be disrupted and allow for tissue remodeling to occur

in the granulation tissue. Extracts from the wound could degrade recombinant TG demonstrated that there was proteolytic processing of the TG (unpublished data).

In conclusion, we have established that TG antigen and activity are expressed and function within the wound at sites of neovascularization and granulation tissue formation. TG appears to undergo regulation with the cytokines, and directly promotes angiogenesis.

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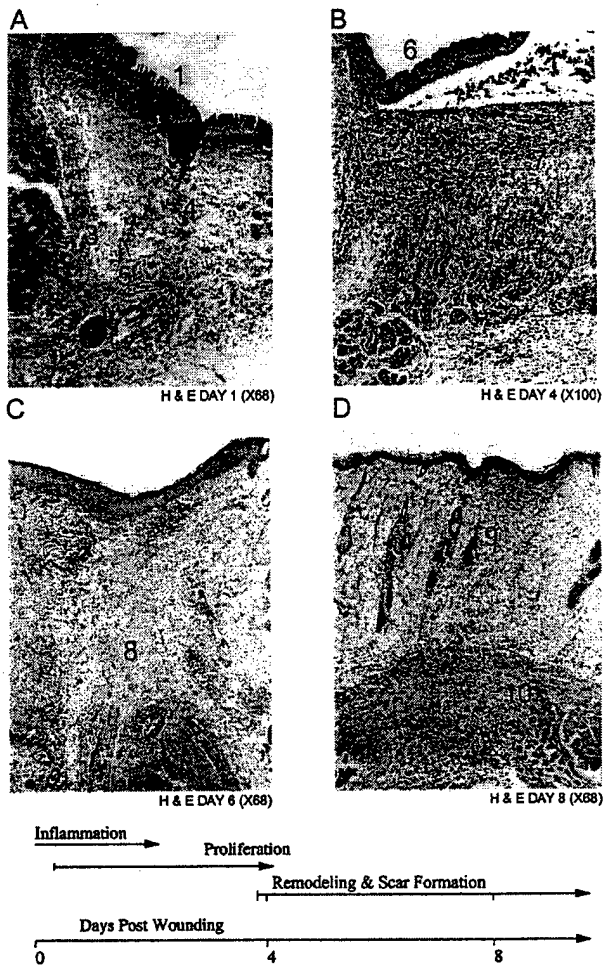


Figure 1: Light microscopic histology with Hematoxylin and eosin (H&E) of normal rat dermal wound healing. New epithelium (A1) is being laid down as early as day 1 post wounding (A). Neovessels (A4) and dilated existing vessels (A5) can be visualized in provisional fibrin matrix. Skeletal muscle cells (A2) form a border zone (A3) between normal and wounded tissue. By day 4 (B), epithelial layer (B6) is complete and granulation tissue (B7) has matured. The granulation tissue starts to contract (C8) by day 6 (C). Scar tissue is visible by day 8 (D) and the remnants of granulation tissue (D10) moved down to the base of the wound. At the bottom of the figure I s a time course illustration of normal rat dermal wound healing divided in the three stages of wound healing.

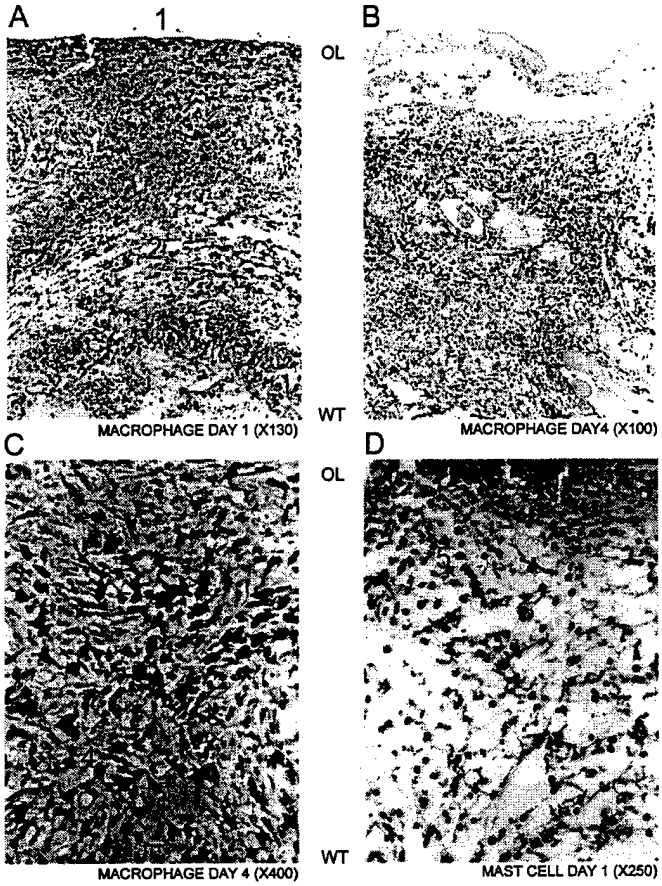


Figure 2: Expression of macrophages and mast cells is shown here. Macrophages line the re-epithelialization border (A1) and form the predominant part of provisional fibrin matrix (A2) at day 1 (A). By day 4 (B), macrophages have spread through out the mature granulation tissue (B3) and could be visualized in high density (C4). Mast cells (D5) stained predominantly at day 1 only. Orientation of the figures is shown as outer layer (OL) and wounded tissue (WT).

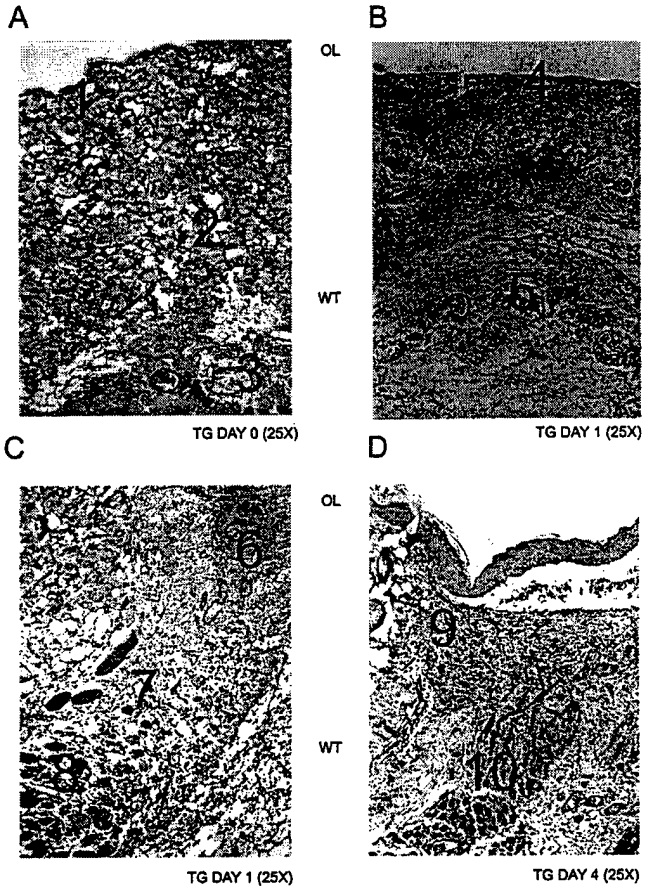
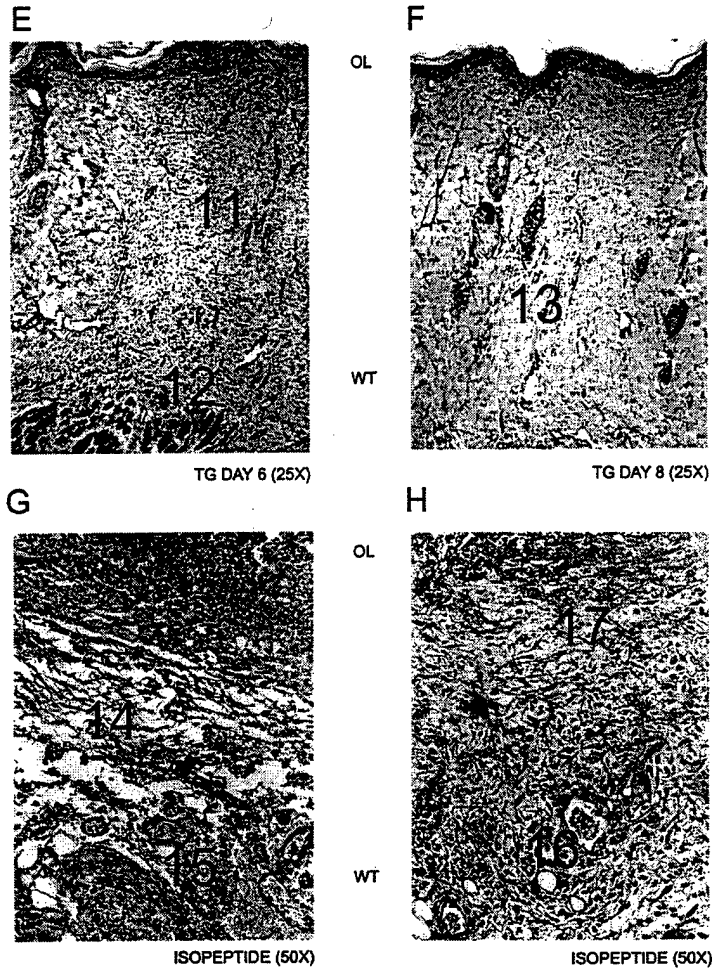


Figure 3: Immunohistochemical evidence of TG expression and activity during wound healing. Normal rat skin stained for TG is shown in 3A. Epithelial layer (A1) shows very little expression while blood vessels in the dermis (A2) and subcutaneous fascia (A3) show TG staining. Re-epithelialization zone consisting of keratinocytes and macrophages (B4) along with neovessels (B5) in provisional fibrin matrix exhibit TG staining. The border zone between normal (left) and wounded (right) tissue at day 1 (C) shows TG reactive endothelial cells (C6), macrophages (C7) and skeletal muscle cells (C8). By day 4 post wounding (D), expression is limited to basal keratinocytes in the epithelial layer, endothelial cells (D9), skeletal muscle cells (D11) and matrix.



TG continues to be detected in the blood vessels (E12) in granulation tissue and skeletal muscle cells (E13) at the base of the wound at day 6. Expression of TG is limited to the scattered blood vessels in the scar tissue (F14) with major portion of immunoreactivity localized to the base of the wound. Isopeptide bond detection in provisional fibrin matrix (G) and granulation tissue (H) at days 1 and 4 post wounding respectively. Isopeptide bonds could be observed in the provisional fibrin matrix (G15), basement membrane of the blood vessels (G16) at day 1. Isopeptide bonds continue to be detected in the blood vessels (H17) and granulation tissue (H18) at day 4. Orientation of the figures is shown as outer layer (OL) and wounded tissue (WT).

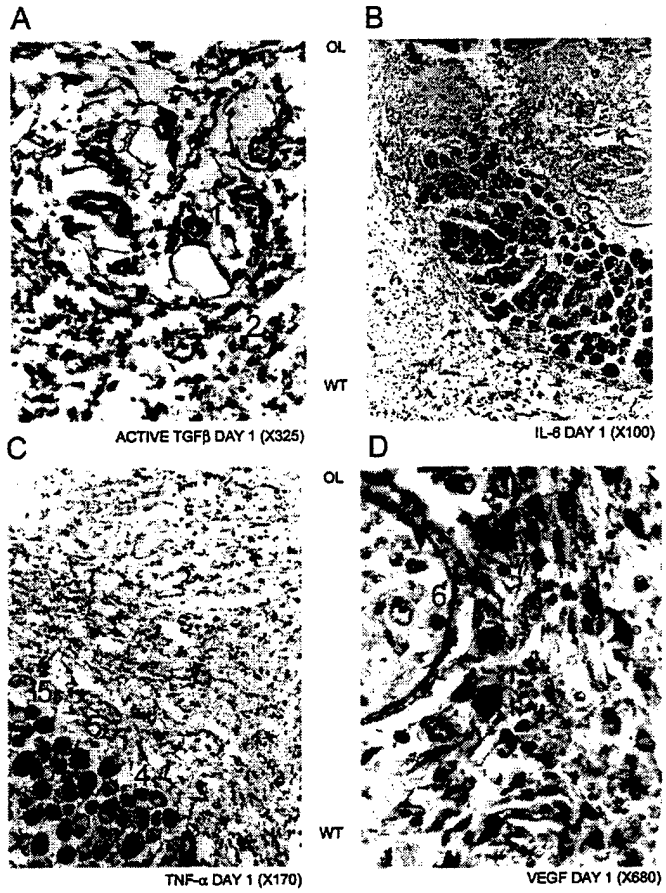
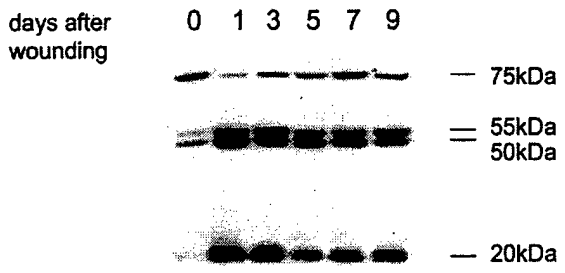


Figure 4:Cytokine expression during wound healing. Active TGF β (A) can be seen staining for endothelial cells (A1) and macrophages (A2) at day 1 post wounding. IL-6 (B) exhibited intense staining in the skeletal muscle cells (B3) at day 1. TNF α (C) is shown here to be immunoreactive in macrophages (C4) and sporadically in endothelial cells (C5) and skeletal muscle cells. VEGF (D) was intensely staining for endothelial cells (D6) and macrophages (D7) at day 1 post wounding. Orientation of the figures is shown as outer layer (OL) and wounded tissue (WT).

A



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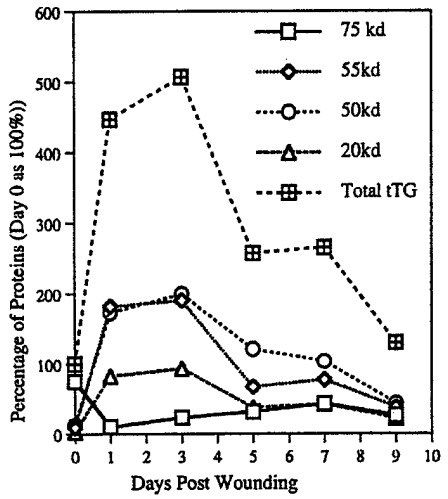

Figure 5: Western blot analysis of wounds (A). Normal skin shows expression of full length TG (75kda) with small amounts of 55 and 50 kda fragments. Although the total TG expression goes up significantly (days 1 and 3), almost all of TG is present in form of 55, 50 and 20 kda fragments. Quantitative analysis of western blots (B) (n=4). Plot shows TG and its fragments at days 0 through 9 taking total TG at day 0 (normal skin) as 100%. Total TG increases 4-5 times on day 1 and 3 and slowly falls down to basal level by day 9. Full length TG constitutes more than 80% of total TG at day 9, but is reduced to less than 5% by day 1 and recovers only to about 25% at day 9. The 55 kda, 50 kda and 20 kda form a minimal portion of total TG at day 0. The amount of fragmented TG increases dramatically by day 1 and 55 and 50 kda forms account for as much as 40% each of total TG. All fragments start to decline by day 5 and make up only about 25% each at day 9 of total TG.

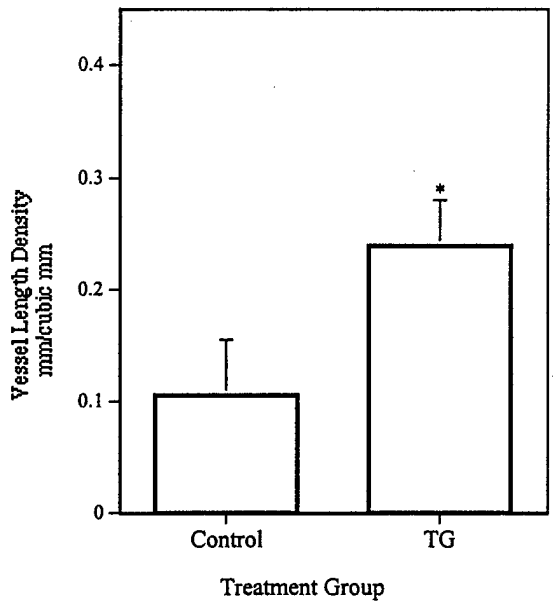

Figure 6:Vessel length density (mm^2) of rat dorsal skin flap chambers treated with saline or recombinant wild type TG. Chambers treated with wild type TG showed a significant (*$p=0.05$, two-tailed Student's t-test, $n=6$)* doubling over controls illustrating a pro-angiogenic effect on healing process.

□

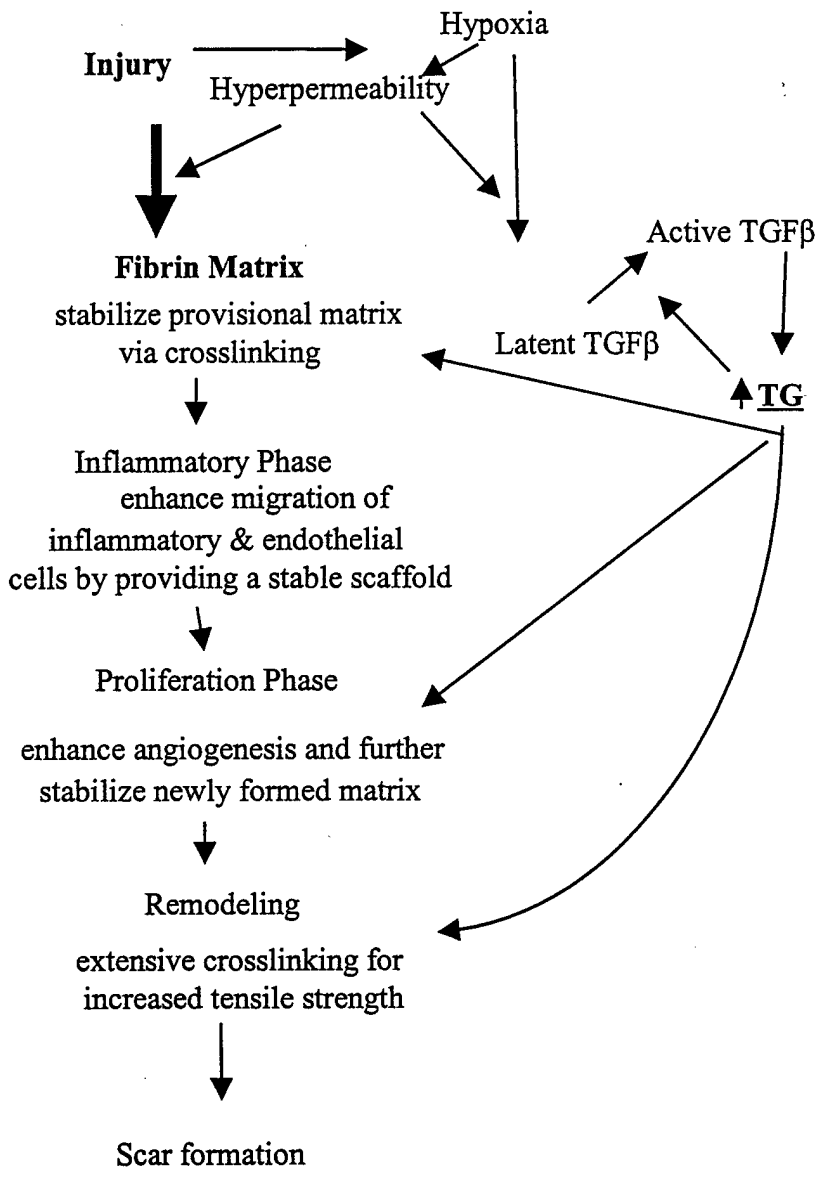

Figure 7:Schematic illustration showing TG's role and possible mechanisms of action during wound healing.

Appendix 2

Early Wound Healing Exhibits Cytokine Surge Without Evidence of Hypoxia

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Running Title:

Cytokine Expression and Hypoxia *In Vivo*

Structured Abstract

Objective: The object of our study was to ascertain the spatial and temporal relationship of wound hypoxia to: 1) the cell types involved 2) expression of selected angiogenic cytokines, 3) the proliferative status of cells in the wound site, and 4) angiogenesis.

Summary Background Data: Hypoxia is considered to drive the angiogenic response by upregulating angiogenic cytokines observed during wound healing. But this correlation has not been shown on a cell-to-cell basis *in vivo* due to limitations in measuring tissue pO_2 at the cellular level.

Methods: Using punch biopsy wounds in rats as a wound healing model, the distributions of VEGF, TGF β, TNF α and pimonidazole adducts (as a hypoxia marker) were followed immunohistochemically during the healing process.

Results: We report the absence of hypoxia at day 1 post wounding even though angiogenesis and maximal expression of cytokines was observed in the wounds. Hypoxia was maximum in the granulation tissue stage at day 4 and correlated with increased cellularity and cellular proliferation. Hypoxia started to decrease after day 4 and was limited to the remnant blood vessels and epithelial layer in the scar tissue.

Conclusions: Our data suggests that induction of angiogenic cytokines early during wound healing perhaps be due to other triggering mechanisms than hypoxia. Alternatively, the unique pattern of development and decline of cellular hypoxia as wound cellularity and proliferation regress suggest its involvement in initiating vascular regression during the later stages of healing.

INTRODUCTION

Tissue hypoxia is considered a major signal that initiates and regulates angiogenic processes such as wound healing and tumor growth [1-3]. Hypoxia has been shown (*in vitro*) to induce a number of major cytokines such as VEGF [4], TGF β [5], TNF α [6] and Interleukin-8 [7] from a wide variety of cells involved in tissue repair including fibroblasts, endothelial cells and macrophages. During wound healing, tissue pO_2 is considered to be low at the center of the wound but it increases as the wound heals [8-10]. Wound induced hypoxia, as suggested by the *in vitro* data, is thought to be a major determinant of all phases of wound healing by regulating cellular proliferation, cell migration, and extracellular matrix protein synthesis through the induction of cytokines and diverse intracellular signaling pathways. *In vivo* studies have demonstrated decreased pO2 in wounds [9-11], suggesting that conditions favorable to hypoxic stimulation of cytokine production exist [12, 13]. These studies of wound tissue pO_2 have provided useful information on the kinetics of change in oxygenation after wounding. However, the methods have measured overall average tissue pO2, whereas it is known that the diffusion distance of oxygen is on the order of a few tens of microns [14, 15]. To further elucidate the relationship between wound hypoxia, cytokine expression, and cellular responses to wounding, we utilized immunohistochemical methods that allowed us to evaluate these responses on a cell to cell basis. The object of our study was to ascertain the spatial and temporal relationship of wound hypoxia to: 1) the cell types involved 2) expression of selected angiogenic cytokines, 3) the proliferative status of cells in the wound site, and 4) angiogenesis.

Pimonidazole hydrochloride belongs to a group of compounds known as 2-nitroimidazole hypoxia markers that form protein adducts under conditions of low oxygen tension (i.e., $\leq$ 10 mmHg) by the action of cellular nitroreductases [16, 17]. The introduction of immunochemical

reagents that recognize marker adducts [18] allowed for the non-radioactive detection of tissue hypoxia [19-21]. Although originally developed for use in animal tumors [22, 23] and human tumors [24-26], pimonidazole hydrochloride has also been applied to the study of hypoxia and hypoxia-associated pathophysiological changes in normal rat liver and kidney [27-30]. Of particular interest to the present study is the binding of pimonidazole to suprabasal cells in epithelial structures including skin (e.g., [31]). This binding is consistent with radiobiological data that indicates that the skin is hypoxic [32]. Importantly, the binding of pimonidazole to skin epithelium serves as a useful positive control in the present study of wound healing. Another advantage of the immunohistochemical hypoxia marker approach is that it measures cellular events with spatial resolution at the cell level without physically disturbing the tissue during the accumulation of the hypoxia signal. This is essential to the present study and no other assay can do this in vivo. Validation of the immunohistochemical technique has been carried out in rodent tumors and human tumor xenografts tumors where correlations between pimonidazole adduct formation, oxygen electrode measurements and radiation response have been demonstrated [23, 33]. In a discussion of the scope and limitation of pimonidazole as a hypoxia marker [23] it was noted that the bioreductive activation of pimonidazole was not dependent on specialized enzymes nor does the concentration of P450 cytochrome enzymes in the perivascular region of rat livers, for example, override the oxygen dependence of pimonidazole activation. A cell type of specific interest to the present study is the macrophage and its bioreductive properties vis a vis the oxygen dependent activation of 2-nitroimidazole binding. Although relatively little appears to be known about this topic, Olive has shown that host macrophages in rodent tumors behave as tumor cells with respect to binding hypoxia markers in the sense that binding occured only in those macrophages that were in hypoxic regions of the tumors [34].

We report the absence of pimonidazole adduct formation in the wound site and surrounding normal skin at day 1 post wounding, indicating the absence of tissue regions with $pO_2 \leq 10$ mmHg. Hypoxia marker intensity was maximal at day 4 after wounding, which coincided with the greatest cellularity and proliferation. Hypoxia diminished as the cellularity of the wounds regressed and mature scar tissue was generated by day 8. In contrast, hypoxia inducible cytokines such as VEGF, TGF β and TNF α exhibited maximal immunoreactivity at day 1, a time point where we saw no evidence for hypoxia. These results suggest there are likely to be signals present other than hypoxia that initiate tissue repair and angiogenesis during the early time points after wounding. Hypoxia reached its maximal intensity in granulation tissue at a critical juncture in the healing process when apoptosis and remodeling was being initiated. This pattern of development of hypoxia suggests that it may play a role in triggering the apoptosis and remodeling of the granulation tissue as opposed to providing the initial stimulus for pro-angiogenic cytokine production in the early phases of wound healing.

METHODS

Animal Protocols: The Duke Institutional Animal Care and Use Committee approved all animal protocols.

Wounds: Fisher 344 female rats from Charles River Laboratories (Raleigh, NC), were anesthetized with intraperitoneal injections of pentobarbital (40 mg/kg) and ketamine (70 mg/kg), then shaved and depilated using Nair, Carter-Wallace (NewYork, NY). Sixteen 5 mm punch biopsy wounds were made in the dorsal skin immediately following depilation. The normal rat skin served as unwounded skin controls. Wounds were harvested at days 1, 2, 4, 6 and 8 while animals were anesthetized. Two rats were sacrificed for each time-point by intravenous pentobarbital overdose and the experiments were done in triplicate. Tissues were

either snap frozen using OCT in liquid nitrogen and kept at -80 C or fixed in 10% neutral buffered formalin for paraffin embedding for immunohistochemistry.

Immunohistochemistry: Immunohistochemistry was carried out using procedures described by Hsu et al [35]. Briefly, paraffin embedded tissues were sectioned (5 microns) and antigen retrieval was performed using citrate buffer from Biogenex (San Ramon, CA). Tissues were treated with primary antibody against Tissue Transglutaminase (1:10; TG100; non-reactive to Factor XIIIa, unpublished data) and VEGF 3 (1:100) Neomarkers, Fremont, CA), ED1-macrophage marker (MCA341; 1:100) Serotec (Oxford, UK), Pan-specific TGF beta (AB-100-NA, 1:100) and rat anti-TNF α (AB-510-NA, 1:100) R&D (Minneapolis, MN), Ki-67 (1:100, NCL-Ki67p) Novocastra Laboratories (Newcastle Upon Tyne, UK) and Rabbit polyclonal antisera to Pimonidazole protein adducts (1:10,000, supplied by JA Raleigh. Secondary and tertiary antibodies were provided in a kit (314KLD) Innovex, (Richmond, CA) and the location of the reaction was visualized with 3, 3'-diaminobenzidine tetrahydrochloride Sigma (St. Louis, MO). Slides were counterstained with hematoxylin and mounted with coverslips. Controls for the immunohistochemistry were treated with normal mouse serum (NMUS) or mouse IgG, rabbit IgG and goat IgG and were negative in any reactivity. Masson's trichrome and hematoxylin and eosin (H&E) staining were carried out as described by Sheehan [36]. The immunostaining was evaluated in a blinded fashion. The stained sections were scored on the basis of the strength of the brown product (DAB) present. The designations used were no staining = -, light or weak staining = +, strong staining = ++, and intense staining covering most of the cellular tissue and matrix = +++.

Pimonidazole and Hoechst 33342 administration: Pimonidazole hydrochloride (Hydroxyprobe-1; Natural Pharmacia International Inc., Research Triangle Park, NC). Pimonidazole

hydrochloride (concentration of 1.0 mg/100 ml in 0.9% saline) was administered at 70 mg/kg IP 3 hours prior to sacrifice. The tissues were then harvested and processed for immunohistochemistry. Hoechst 33342 (bis-benzimide, Hoechst 33342, Sigma, St. Louis, MO) was made at a concentration of 5 mg/ml in 0.9% NaCl and perfused through the tail vein of anesthetized rats over 45 sec to give a total dose of 12 mg/kg for days 0 and 1 post wounding only. Wounds were harvested within 5 minutes of Hoechst 33342 administration and were immediately embedded in OCT and frozen. Two rats were used for each time point. Five micron sections were made within 2 hours and images were visualized with MPS Intravital Microscopy System (Carl Zeiss, Hanover, MD) attached to color camera ZVS 3C75DE (Optronics Engg., Goleta, CA). The images were captured with NIH image software via a CG7 frame grabber (Scion Corp., Frederick, MD).

RESULTS

Light Microscopy Findings

At the earliest time point at day 1 (Figure 1A), provisional matrix with dilated blood vessels (Figure 2A) and inflammatory cells (Figure 2C) could be observed underneath a newly generating epithelial layer. Re-epithelialization was completed by day 2 post wounding. Maturation of granulation tissue was seen by day 4 (Figure 1B) characterized by the presence of new blood vessels (Figure 2B), inflammatory cells (Figure 2D) and collagen fibers organized into a dense connective tissue. Skeletal muscle cells which previously formed a boundary between the normal and wounded tissue at earlier time points, had moved to the base of the wound and closed the gap created by the injury (Figure 1 B). By day 6 (Figure 1C), granulation tissue started to contract and increased in density. At day 8 (Figure 1D) the healing was in its final stages with remnants of granulation tissue left at the base of the wound. The injury site was

filled with dense collagen tissue with very few blood vessels.

Hypoxia distribution during Wound Healing

Hypoxia was detected in the epithelial layer, sebaceous glands and hair follicles in unwounded skin (Figure 3A). We observed very little immunoreactivity in wounds examined on day 1 for the hypoxia marker (Figure 3B). The distribution of hypoxia in adjacent normal skin structures was ordinarily diminished at this point as compared with unwounded skin. By day 2, the wounds started to exhibit some staining for hypoxia marker in the newly laid epithelial layer and the border zone between the normal and wounded tissue, a region which was infiltrated by inflammatory and endothelial cells (Figure 3C). Immunoreactivity for hypoxia reached its highest intensity on day 4 post wounding (Figure 3 D & E). The keratinocytes in the epithelial layer, endothelial and inflammatory cells in the granulation tissue and the border zone stained intensely for hypoxia. As the healing process progressed to day collagen became more organized and the tissue was less cellular. Hypoxia marker staining intensity was diminished in the organized collagen and more localized to the base of the wound, coinciding with residual cellularity. At 8 days post wounding, hypoxia marker staining was confined to the limited cellular content of the scar tissue such as blood vessels and macrophages and to the granulation tissue at the base of the wound (Figure 3F). The intensity of hypoxia immunoreactivity in the epithelial layer was equivalent to that seen in unwounded skin.

Expression of Cytokines

VEGF (Figure 4A), TGF β and TNF α expression showed the same distribution as hypoxia marker binding in normal rat skin i.e. epithelial layer, sebaceous glands and hair follicles. On day 1 post wounding, VEGF (Figure 4B), TGF β (Figure 4C) and TNF α (Figure 4D) immunoreactivity was observed in the inflammatory and endothelial cells. VEGF and TGF

β were found in the provisional fibrin matrix as well (Figure 4 B & C). VEGF antigen started to decline after day 2 and detection was limited to endothelial cells in scar tissue and granulation tissue at the base of wound by day 8 in a pattern similar to the distribution of hypoxia. TGF β antigen remained at a high level and was detected throughout the healing process in the matrix, inflammatory and endothelial cells. TNF α expression continued to be similar to that of hypoxia marker in the healing process.

Hoechst 33342 and Proliferation marker Ki67 distribution in wounds

Since we observed no hypoxia marker staining on day 1 post wounding, we wondered whether this might be caused by lack of marker drug delivery (perfusion). We used Hoechst 33342 dye extravasation in the wounds as a means to determine if perfusion was adequate. The Hoechst dye permeated the epithelial layer, the sebaceous glands, hair follicles and the dermis of the normal skin (Figure 5A). We found that this dye diffused throughout the wound tissue on days 1 (Figure 5B).

Since proliferating cells consume oxygen at rates 3-5 times higher than cells in G_0 [37], we hypothesized that the high intensity of hypoxia in granulation tissue may be due to increased consumption. We utilized Ki67 as proliferation marker and it was detected as early as day 1 post injury in the endothelial and epithelial cells (Figure 6A). The immunoreactivity for Ki67 was highest at day 4 (Figure 6B) and started to decline in intensity by day 6 and was reduced to the remnants of granulation tissue at the base of wound by day 8.

Summary of the time course of these events is shown in Table 1.

DISCUSSION

This is the first study to report the distribution of hypoxia at cellular level and its association with cytokine expression, endothelial and inflammatory cells during wound healing.

There are two interesting and unexpected findings of this study: A) the absence of hypoxia in the provisional fibrin matrix at day 1, a time when cytokine expression is elevated and B) maximum hypoxia in granulation tissue at day 4 post wounding at a time when cellular proliferation is at its peak, which is followed by decreased wound cellularity and wound contraction. These results suggest that hypoxia probably does not play a role in the initial onset of cytokine expression that occurs at 1 day post wounding. Alternatively, it probably plays a role in maintenance of the angiogenic response in the granulation tissue from day 2 forward. Also, the apparent relationship between peak levels of hypoxia and the onset of decreasing cellularity seems to indicate more prominent role of hypoxia in initiation of remodeling during the healing process.

The absence of hypoxia in dermal wounds early during healing is readily explainable. Once breakdown of the epithelial barrier occurs, diffusion of air ($pO_2 \geq 120$ mmHg) into the open wound and the provisional fibrin matrix happens as long as scab formation and/or re-epithelialization has not taken place. Interestingly, earlier investigators found similar results with wound fluid chambers fitted with oxygen electrodes [8]. They reported high pO_2 levels (20-30 mmHg) during the first two days of healing and gradual decline in pO_2 levels down to lowest (5-8 mm Hg) at 5-7 days post wounding. They believed that the elevated pO2 level seen in the first two days post wounding was an artifact caused by oxygen contamination from the air into the wound chamber. We argue that this is not an artifact. We propose that in normal dermal healing, oxygen will diffuse into the wound site by passing through the disrupted epithelial barrier from the surrounding air. Our strongest argument to support this hypothesis is based on a comparison of hypoxia marker binding in unwounded skin and in normal skin adjacent to the wound site at 1 day post wounding. In unwounded skin strong marker binding was seen in epithelial layer, hair follicles and sebaceous glands. This pattern of binding was absent in

normal skin surrounding the wound site at day 1. One could argue that the wound itself might have created enough vascular destruction to prevent adequate perfusion. If this were true, it is possible that the hypoxia marker drug failed to reach to the target tissues. However, the strong perivascular staining with the Hoescht dye demonstrated that the tissue was adequately perfused and that the hypoxia marker would have easily reached the target tissues. Based on this evidence, we conclude that hypoxia is not responsible for the initial triggering of the repair and angiogenesis cascade. Recently, Howdieshell et al have shown that wound fluid obtained from abdominal wounds was normoxic although they detected high levels of cytokine (VEGF and TGF β) expression [38].

There are alternate pathways that could initiate tissue repair and angiogenesis during the early stages of wound healing which are based on the coagulation system [39, 40]. Platelets are known to comprise first wave of exogenous cells to appear at an injured site [41]. In addition to initiating hemostasis and coagulation at the site of injury, they also contain a significant number of cytokines, extracellular matrix proteins and enzymes that are released from storage granules after activation. These cytokines include VEGF [42], TGF β [43], TGF α [44] and PDGF [45]. Such cytokines could promote tissue repair and angiogenesis once they are released. It is known that platelet depletion leads to defective wound healing [46]. This is further evidence that they play a role in regulation of wound healing. Our observation that hypoxia is absent during early wound healing even though angiogenic cytokine levels are elevated supports the role of platelets as being involved at this early time point. Thus, regulation of cytokines during early wound healing may be a function of the coagulation system rather than being hypoxia driven.

The other major event during early wound healing is the generation of thrombin and formation of a provisional fibrin matrix. The provisional fibrin matrix provides the essential

scaffold for the endothelial and inflammatory cells to move into the wounded tissue. Fibrin and its degradation products have diverse biological effects for cells invading the provisional matrix and can induce various cytokines such as IL-8 [47], tissue factor [48] and adhesion molecules (ICAM) [49] in those cells. This provides a second pathway for the production of cytokines to initiate repair and angiogenesis during early wound healing as tissue factor itself can stimulate VEGF [50] in cells. Thus, there seems to be a wide array of pathways and mechanisms that may be responsible for induction of cytokines during wound healing as an alternative to hypoxia.

The results on day 4 post wounding are also very intriguing. Extensive hypoxia marker distribution correlated very well with the proliferation marker Ki67, suggesting that increased oxygen consumption rates in the granulation tissue were responsible for maximal hypoxia staining. Apoptosis of endothelial cells and myofibroblasts has been shown to be responsible for the decrease in cellularity following the surge of proliferation during healing episodes [51, 52]. This wave of apoptosis has been associated with CD95 [53] and p53 [54] related pathways. The signals that trigger these events are not well understood but it is well established that activation of p53 dependent apoptotic pathways is triggered by hypoxia [55]. Additional evidence implicating hypoxia in the regulation of apoptosis suggests that hypoxia may provide the essential signaling pathways for initiating apoptosis during healing [56].

The unique pattern of emergence of hypoxia marker reactivity at the beginning of remodeling of the wounded tissue and its gradual decline in the remodeled tissues suggests the development of hypoxia may be to stimulate endothelial cell and myofibroblast apoptosis. Hypoxia might very well hold the key to the puzzle of remodeling initiation as its progression indicates a diminished capacity for nutrient and oxygen delivery at a time that coincides with the trimming of excess cell mass by apoptosis. This is an exciting possibility that needs further investigation.

Chang et al studied the oxygenation of human wounds and observed values in the range of 60mmHg in unwounded skin [9]. Oxygenation dropped to 40mmHg in the first few days post wounding and showed a gradual decline to 30 mmHg at 4-5 days post wounding. The authors suggested that the decline in pO_2 in the early post wounding period would be sufficient to stimulate wound healing. Since our method of hypoxia measurement only detects pO2 values less than 10 mmHg, and we saw little evidence for hypoxia at day one post wounding, there appears to be a discrepancy between this earlier study and ours. The pO_2 measurements were performed by analyzing the pO_2 of fluid inside implanted silastic catheters. By virtue of its size, this type of device provides an estimate of the average tissue pO2 and such measurements are likely to be influenced strongly by vascular pO2 in regions immediately adjacent to the catheter. However, values of 30 mmHg are not really hypoxic, if one considers the oxygen tension distribution when measured on a more microscopic scale. Using modern polarographic methods with electrodes that have minimal self consumption artifact, pO_2 in normal tissues such as brain [57], uterus [58], skeletal muscles and skin [59] drops below 15 mmHg. Some tissues, such as retina [60] and liver [61] exist under chronically hypoxic conditions (pO_2 values $\leq$10 mmHg). One could argue that a drop from 60 to 40 mmHg might be sufficient to stimulate angiogenesis and wound healing, but there is no direct evidence from the literature to support this conjecture. There have been a number of *in vitro* studies published where the production and release of pro-angiogenic cytokines has been evaluated. In general such studies involved a dramatic change in pO2 (room air to 10 mmHg) over several hours. Under such conditions, cytokines such as VEGF [62], bFGF [63], TNF α [6] and TGF β [12] have been shown to be upregulated. However, these conditions are not comparable to the 30% drop reported by Chang. Studies are underway in our laboratory that address this concern by maintaining the cells at physiological levels of oxygen (pO_2 of 15-30

mmHg) for a few cell cycles and then exposing them to hypoxia.

To summarize (Figure 7), we have shown that there is no hypoxia during the early events of wound healing but that it is maximal in the most cellular phases of granulation. We found expression of pro-angiogenic cytokines in the provisional fibrin matrix during early wound healing, suggesting other trigger mechanisms for induction of these cytokines at this time point. However, the unique pattern of development and decline of hypoxia as wound cellularity and proliferation regress suggest its involvement in initiating apoptosis during the later stages of the healing process.

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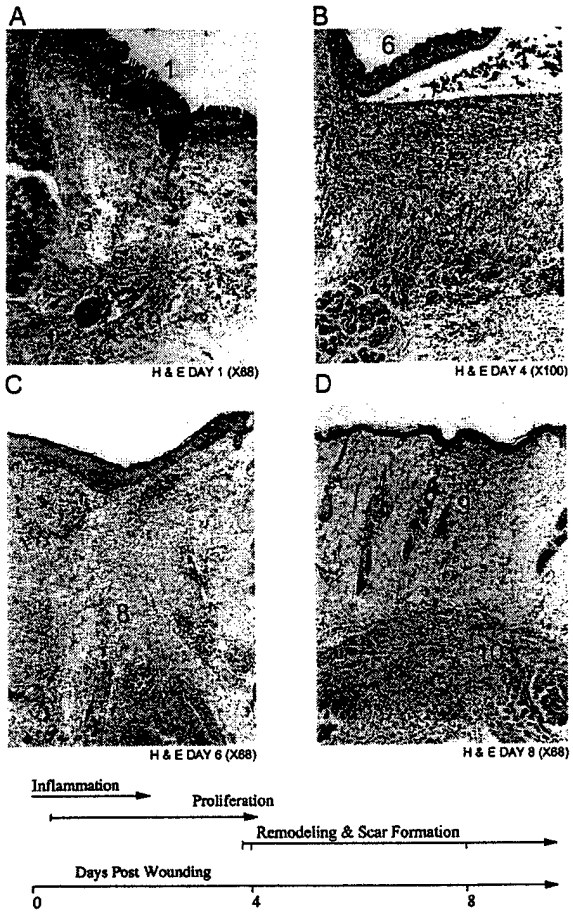


Figure 1: Light microscopic histology with hematoxylin and eosin (H&E) of normal rat dermal wound healing. New epithelium (A1) is being laid down as early as day 1 post wounding (A). Neovessels (A4) and dilated existing vessels (A5) can be visualized in provisional fibrin matrix. Skeletal muscle cells (A2) form a border zone (A3) between normal and wounded tissue. By day 4 (B), epithelial layer (B6) is complete and granulation tissue (B7) has matured. The granulation tissue starts to contract (C8) by day 6 (C). Scar tissue is visible by day 8 (D) and the remnants of granulation tissue (D10) moved down to the base of the wound.

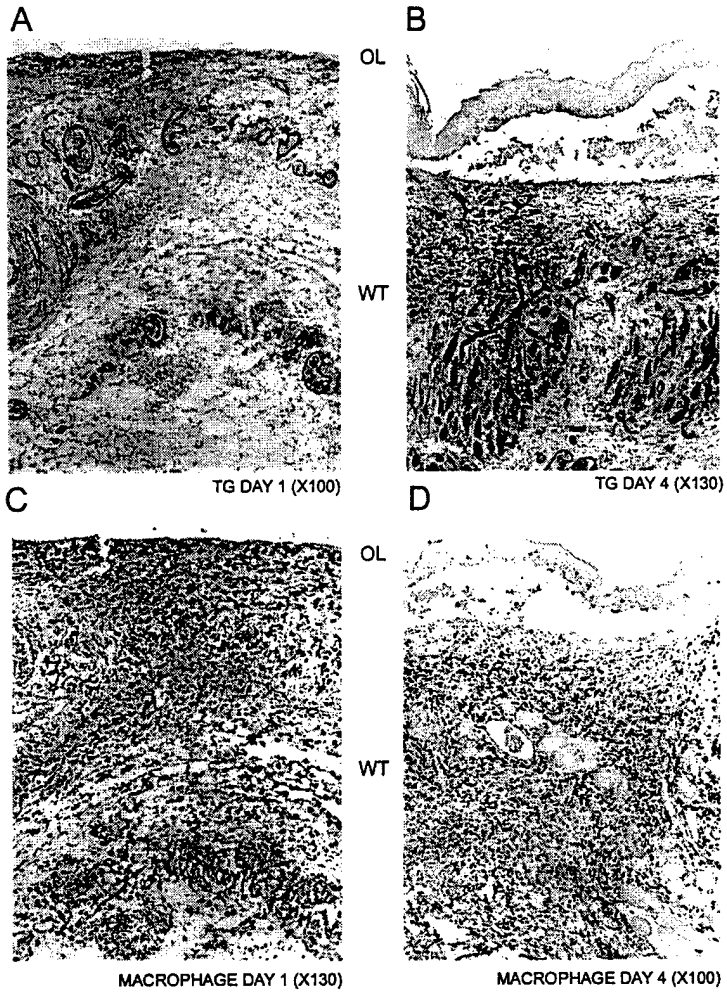


Figure 2: Localization of blood vessels and macrophages is shown here. Tissue transglutaminase was utilized as a blood vessel marker. Abundant blood vessels can be identified at day 1 (A) in the provisional fibrin matrix and in the granulation tissue at day 4 (B). Macrophages, as identified by ED1 marker, invade the provisional fibrin matrix (C) by day 1 and are spread through out the granulation tissue (D). Orientation of the figures is shown as outer layer (OL) and wounded tissue (WT).

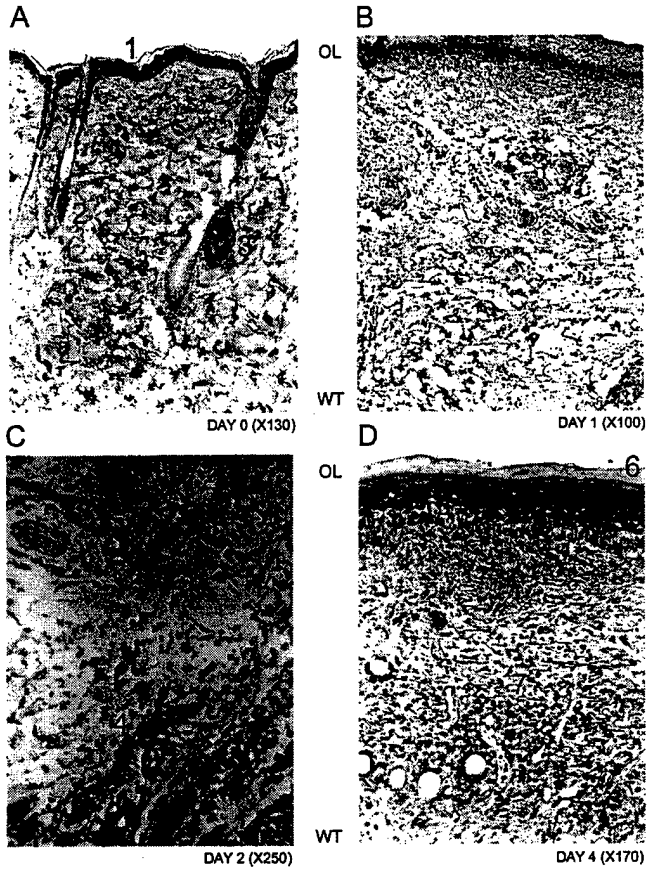
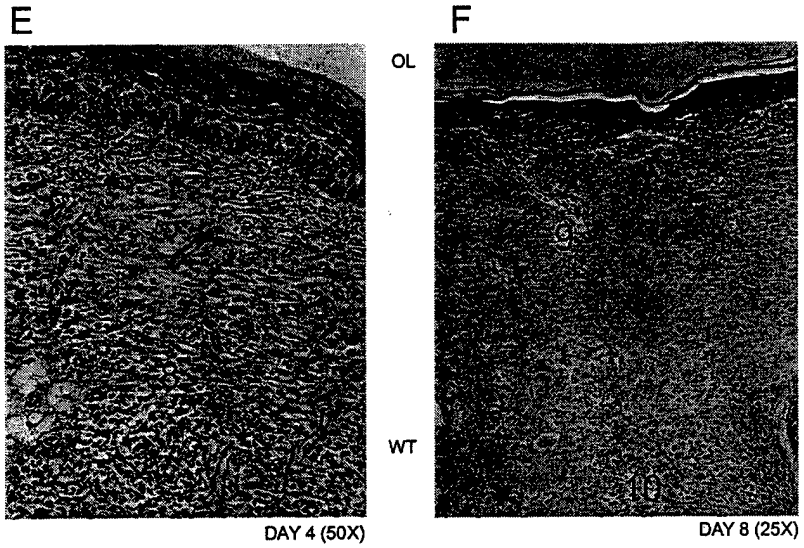


Figure 3: Immunohistochemical evidence of hypoxia during wound healing. In normal rat skin (A), the pimonidazole adducts were detected in epithelial layer (A1),hair follicles (A2) and sebaceous glands (A3). At day 1 (B), we did not detect any reactivity for the hypoxia marker in the wounded tissue. On day 2 (C), macrophages (C4) and endothelial cells (C5) started to exhibit immunoreactivity at the border of normal and wounded tissue. By day 4 (D&E), maximal hypoxia reactivity was observed. The epithelial layer (D6) and granulation tissue both were highly reactive. Macrophages (D7) and endothelial cells (D8) in small blood vessels could be seen with evidence of pimo-adduct formation. By day 8 (F), the immunoreactivity was limited to epithelial layer, remnant blood vessels (F9) macrophages in the scar tissue. Orientation of the figures is shown as outer layer(OL) and wounded tissue (WT).



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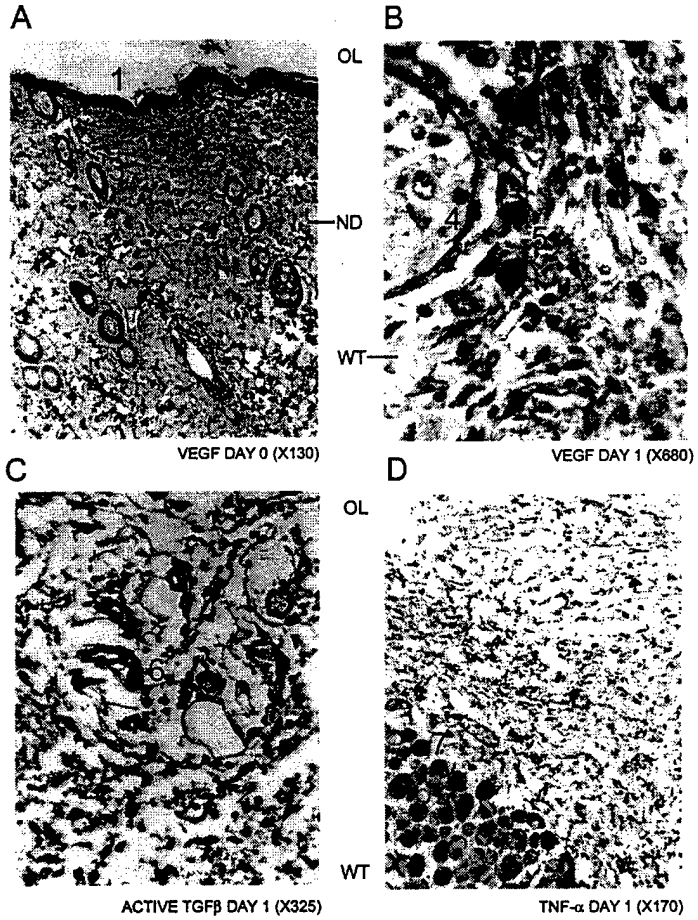

Figure 4: Cytokine expression during wound healing. VEGF expression could be seen in epithelial layer (A1), sebaceous glands (A2) and hair follicles (A3) in normal rat skin (A). By day 1, VEGF expression was present in endothelial cells (B4) and macrophages (B5). Active TGF β expression at day 1 (C) could also be localized to endothelial cells (C6) and macrophages. TNF α (D) was predominantly detected in endothelial cells, macrophages and skeletal muscle cells (D7). Orientation of the figures is shown as outer layer (OL) and wounded tissue (WT).

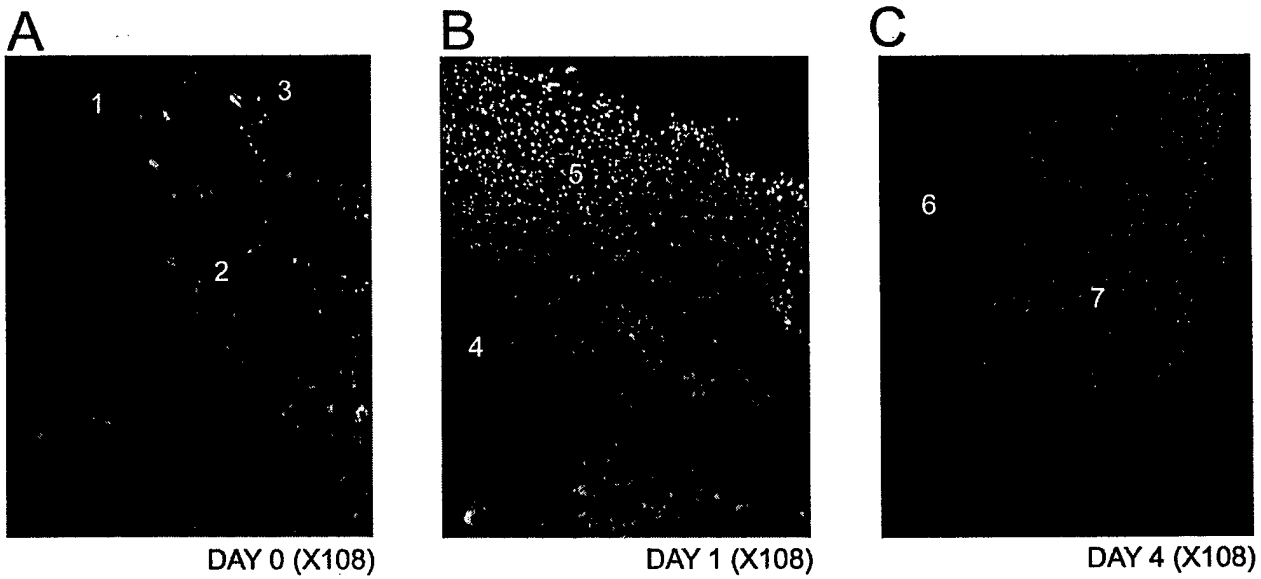

Figure 5: Hoechst Dye staining for the normal rat skin and wounded tissue. In normal rat skin (A), Hoechst dye stain was present in the epithelial layer (A1), the sebaceous glands (A2) and hair follicles (A3). At day 1 (B), the wounded tissue was very well perfused and the newly generating epithelial layer (B4) and provisional fibrin matrix (B5) exhibited the nuclear stain.

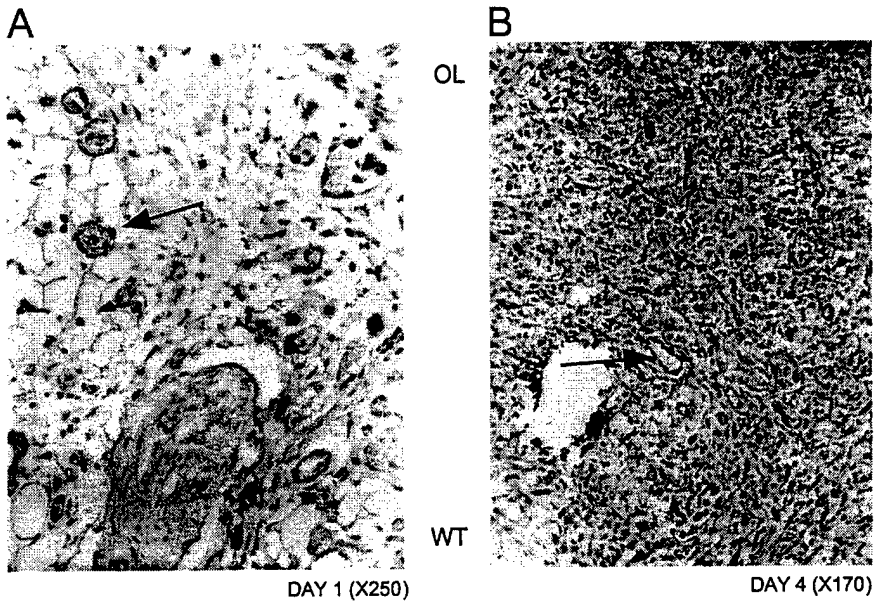

Figure 6: Ki-67 proliferation marker expression for the wounded tissue. At day 1 (A), endothelial cells (arrow) can be identified as picking up the proliferation marker. The wounded tissue at day 4 (B) has very high levels of Ki-67 marker expression, indicating high proliferation rate in the granulation tissue. Endothelial cells in a blood vessel are shown to be expressing the Ki-67 marker (arrow). Orientation of the figures is shown as outer layer (OL) and wounded tissue (WT).

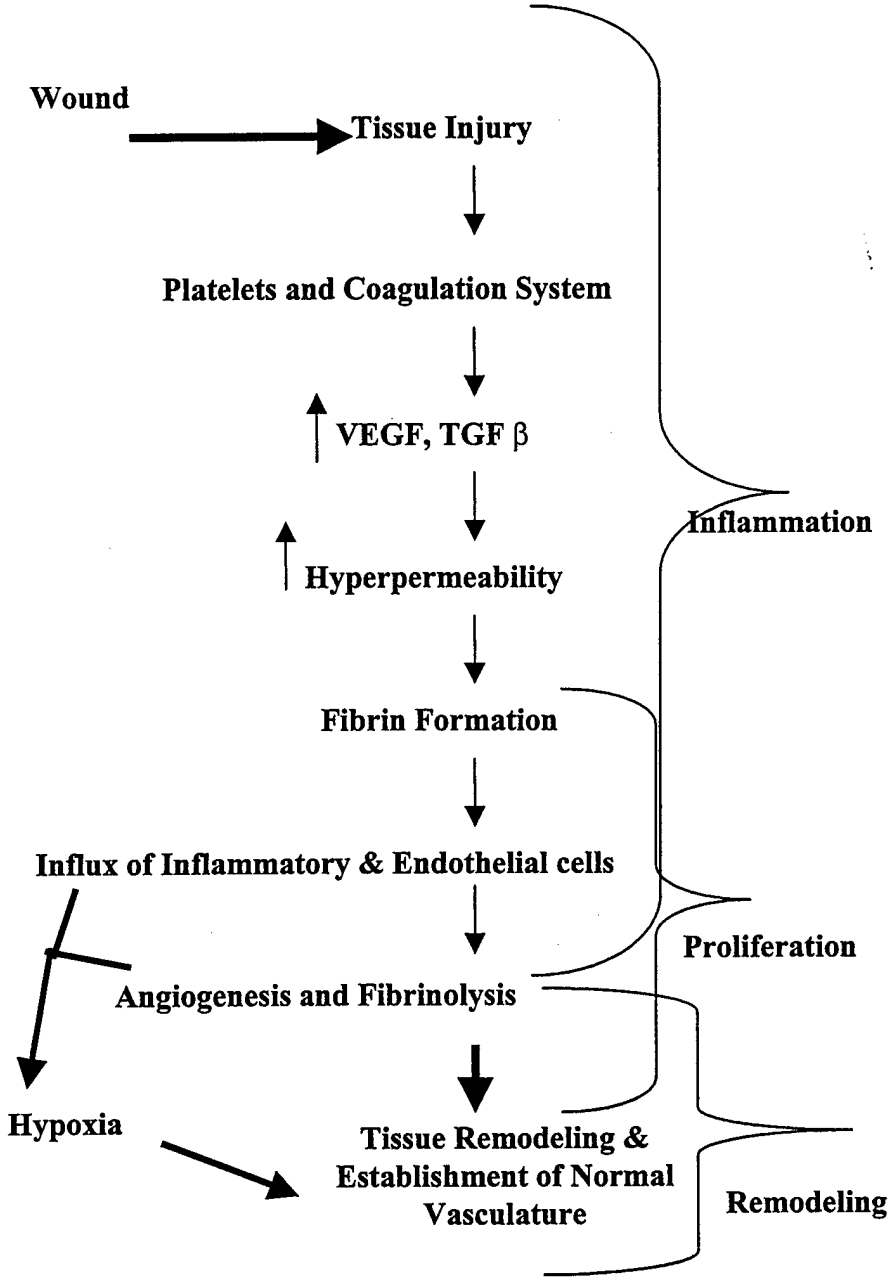

Figure 7: Schematic illustration showing the proposed role of hypoxia during wound healing cascade.

Table 1: A summary of the immunohistochemical data described in the text.

Characteristic	Time Post Wounding (days)					
	Control	01	02	04	06	08
Pimonidazole Immunoreactivity	*Ep, †Sg & ‡Hf	-	+	+++	++	+
Cytokines						
VEGF	Same as Pimonidazole	+++	+++	++	++	+
TGF β		+++	+++	+++	++	++
TNF α		+++	+++	+++	++	+
Cell Proliferation	-	+	++	+++	+	-
Angiogenesis	-	+ +	+++	+++	++	+

Intensity of immunoreactivity is graded as - none, + weak, ++ strong, +++ intense
*Ep=Epidermis †Sg=Sebaceous glands ‡Hf=Hair follicle

Appendix 3

Tissue Transglutaminase is Expressed as a Host Response to Tumor Invasion and Inhibits Tumor Growth

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Zishan A Haroon

ABSTRACT

A stable extracellular matrix (ECM) constitutes an important part of host response mechanism against tumor growth and invasion. Tissue transglutaminase (TG), a calcium dependent enzyme, can crosslink all major ECM proteins to form a stable ECM since these crosslinks are resistant to proteolytic and mechanical damage. TG can also enhance stability and strength of the ECM by its ability to facilitate the activation of Transforming Growth Factor β. We hypothesized that TG's ECM promoting abilities form an important part of host response mechanism against tumor growth. Increased expression of TG was observed in the ECM of the host tumor interface of subcutaneously implanted rat mammary adenocarcinoma R3230 Ac. TG expression was also detected in the endothelial cells and macrophages throughout the tumor and adjacent host tissues. We also detected the crosslink product in the tumor showing that TG was active. Western blots showed TG was degraded into 3 fragments of 55, 50 and 20 kDa forms. When recombinant wild type TG was applied to R3230 Ac implanted in rat dorsal skin flap window chamber, it caused significant growth delay at day 7 as compared to recombinant inactive TG controls. Collagen was detected in increased amounts in TG treated tumors suggesting augmentation of production and stability of the ECM. We conclude that TG forms a distinct part of host response system against and acts to inhibit tumor growth.

Keywords: Host response, Tissue Transglutaminase, Transforming Growth Factor β, Fibrosis, Extracellular matrix.

INTRODUCTION

The extracellular matrix (ECM) of the tumor plays an important role in the regulation of tumor growth, metastasis and angiogenesis (Dano et al., 1999; Horton, 1995; Lukashev and Werb, 1998; Pepper et al., 1996; Taipale and Keski-Oja, 1997; Wernert, 1997). The ECM modulates tumor growth by 1) binding and storing cytokines, 2) providing a stable matrix for attachment and migration of cells, and 3) interacting with cell surface receptors that provide cell signals that control cell cycle, cell proliferation and apoptosis pathways. Tumors are known to elicit a wound healing response from the host tissues resulting in formation of a granulation tissue at the advancing margins (Brown et al., 1989; Dvorak, 1986; Senger et al., 1994). The newly produced and remodeled ECM can either stimulate or inhibit tumor growth. The molecular mechanisms regulating the outcome between tumor and host factors in remodeling the ECM are just beginning to be defined.

Tissue transglutaminase (TG) is a calcium dependent enzyme that covalently crosslinks a wide variety of ECM proteins producing a protease resistant matrix (Greenberg et al., 1991) (Aeschlimann et al., 1996). TG expression has been shown in wounded tissues and at sites of inflammation and has been implicated in numerous interactions that stabilize ECM (Bowness and Tarr, 1997; Bowness et al., 1988; Weinberg et al., 1991). TG regulates the conversion of latent to active Transforming Growth Factor β (TGF β) (Kojima et al., 1993), a cytokine that can modify epithelial growth, enhance synthesis of various ECM proteins and inhibitors of metalloproteases (Clark and Coker, 1998; Pepper, 1997; Taipale et al., 1998). TG could also crosslink elafin (Nara et al., 1994) and PAI-2 (Jensen et al., 1993), potent inhibitors of elastase and plasmin respectively, to ECM molecules. Fibroblasts transfected with TG have been shown

to have a distinctive spread morphology and increased resistance to protease digestion (Gentile et al., 1992). These TG activities, as part of the host response mechanisms, could alter the adhesive and invasive properties of malignant cells by modulating and influencing matrix production and stability of the host tissues. Thus, TG appears to be an important host factor that could play a role in tumor biology.

In an earlier study, we observed that TG antigen expression was associated with the neovasculature and the host's ECM surrounding human mammary tumors (Hettasch et al., 1996). The central hypothesis of our study was to ascertain if TG in host tissue was involved in stabilizing the ECM around tumors. If stabilization occurred, we believed that addition of exogenous TG could inhibit tumor growth in a murine mammary carcinoma model. We found that TG antigen was localized to neovasculature and the ECM of rat R3230 Ac mammary tumor. TG expression was most intense at the interface between tumor and normal host tissue. The TG antigen expression pattern suggested TG was playing a role in regulating tumor growth. We transplanted R3230 Ac in the window chamber and subsequently recombinant TG was applied to the fascial surface of the window. We found that TG stimulated ECM formation and inhibited tumor growth.

RESULTS

Light Microscopy: Subcutaneous tumor implants exhibited two distinct zones (Figure 1A) in cross sections stained with MT, arbitrarily assigned as tumor core region (TCR) and host-tumor interface (HTI). The advancing margins at the HTI tissue showed extensive inflammatory reaction. There was also an increased amount of granulation tissue deposition consisting of endothelial cells, macrophages and neutrophils interspersed among tumor and skeletal muscle

cells in the HTI. The mammary tumor cells appeared granular and formed glandular clusters separated by thin bands of matrix and blood vessels in the TCR.

Localization, Expression and Activity of TG: TG was expressed throughout the tumor and surrounding host tissue (Figure 1B), although being more intense in HTI. TG antigen staining was present in the thin matrix encapsulating the tumor islands in the TCR (Figure 1C). The blood vessels present in the matrix in TCR also showed consistent expression of TG antigen. The few macrophages that were detected in this matrix exhibited TG expression. The tumor cells did not show any reactivity for the TG antigen except in some instances where the tumor cells were adjacent to the matrix.

The host tissue initiated an intense wound healing reaction to the tumor resulting in granulation tissue formation all along the edges of the tumor tissue. HTI region was characterized by intense TG antigen staining that formed a TG positive barrier between the normal and tumor tissues (Figure 1D). TG antigen was expressed by endothelial cells, macrophages, skeletal muscle cells and was also deposited in the ECM in the HTI region (Figure 1E).

The presence of isopeptide bonds in tissues is a direct evidence for TG activity. The basement membranes of blood vessels and ECM displayed consistent staining for isopeptide bonds, closely approximating TG distribution in the TCR and HTI (Figure 1F).

Detection of Macrophages and Active TGF β: Since TG is expressed by macrophages and is directly involved in activation of TGF β, we investigated the distribution of macrophages and TGF β in relation to TG antigen staining. We found a high density of macrophages in the HTI region (Figure 2A) and these cells also stained positive for TG antigen. The TCR staining pattern was largely devoid of macrophages. The few macrophages present stained positive for

TG and were in the matrix surrounding necrotic areas. Active TGF β staining was co-localized primarily in the endothelial cells with TG antigen reactivity in the HTI (Figure 2B) and TCR.

Western Blots of the R3230 Ac Tumors: SDS-PAGE and quantitative immunoblotting demonstrated 4 distinct bands for TG antigen (Figure 3A & B). Quantitative analysis of the antigen revealed that more than 95% of the protein was rapidly degraded to 55, 50 and 20 kDa fragments from the full length wild type TG of 75kDa.

Administration of Recombinant TG on R3230 Ac Implanted Dorsal Skin Flap Window Chamber: The R3230 Ac tumors treated with recombinant wild type TG showed significant growth inhibition at day 7 post implantation as compared with the control tumors treated with recombinant inactive TG (Figure 4). The TG treated tumors exhibited increased fibrosis in the surrounding matrix and around the tumor islands in both the TCR and HTI as indicated by intense staining of collagen fibers (Figure 2C-F). A few TG treated tumors escaped growth inhibition by day 10 (2/6) and these tumors reached the size of controls.

DISCUSSION:

The host response to tissue injury caused by a tumor is characterized by complex and intricate mechanisms initiated to limit the growth and spread of tumors (Tabor, 1997). Host response mechanisms include increased activity of tumor suppressor genes, induction of cytokines that promote wound healing, stimulation of the immune system to resist foreign invasion and production of a stable ECM. A stable ECM is intrinsically anti-angiogenic and growth inhibitory by virtue of being more protease resistant and mechanically resistant to disruption by the expanding tumor mass (Dano et al., 1999; Lochter et al., 1998; Werb et al., 1999). The formation of a stable ECM may be an effective barrier to growth and metastasis of tumors by restricting infiltration by tumor cells and growth of new blood vessels. The central hypothesis of

this study was that host TG could enhance the formation of a stable ECM and modify tumor growth. The distribution of the TG antigen and isopeptide bonds in rat mammary tumor model and inhibition of tumor growth by administration of recombinant TG supports this hypothesis. TG appears to be a part of host injury response machinery that works to inhibit growth of tumors by maintaining the integrity of the host tissues.

TG can crosslink several major constituents of the ECM including fibronectin (Martinez et al., 1994), collagen (Mosher and Schad, 1979), fibrin (Greenberg et al., 1987), fibrinogen (Martinez et al., 1989), vitronectin (Sane et al., 1988) and laminin/nidogen (Aeschlimann and Paulsson, 1991). TG can crosslink these matrix proteins with each other to form a protease resistant barrier that would be a formidable obstacle to growth of blood vessels and limit tumor progression as part of host response mechanism. We identified both expression and activity of TG in the matrix surrounding the tumor. However, despite the host response, tumor progression was not halted perhaps in part by the ability of the tumor to produce factors that inactivate the TG. The majority of the TG antigen in the tumor tissue was degraded suggesting that tumor associated proteases could cleave and inactivate the TG. TG when exposed to extracellular levels of calcium ions is protease sensitive and readily degraded by several serine proteases (Lai et al., 1998). The tumors' production of proteases may overwhelm the ability of TG to inhibit growth by crosslinking the host's ECM. The fact that a single administration of recombinant TG to the tissue surrounding a tumor implanted in window chambers resulted in the enhanced production of ECM and an inhibition of tumor growth indicates that TG expression by the host has the ability to restrict tumor growth. However, the tumor appears to have a potent mechanism that can overwhelm the host response and allow the tumor to expand.

During malignant transformation, the acquisition of high protease activity by the tumor cells is essential for breakdown of basement membrane and matrix that accompany tumor cell migration, proliferation, expansion and the development of a tumor blood supply that promotes metastasis (Benaud et al., 1998; Dano et al., 1999; Lochter et al., 1998). Anti-proteases such as maspin were recently recognized as tumor suppressor genes in their ability to restrict the effectiveness of this proteolytic cascade (Sager et al., 1997). TG is known to crosslink anti-proteases such as PAI-2 (Jensen et al., 1993) and Elafin (Nara et al., 1994) to ECM making them readily available for interaction with proteases. The anti-tumor effect observed in tumor implanted window chambers could be due in part to the elevated anti-protease response mediated by TG.

We also observed a distinct fibrotic reaction in the tumors treated with TG and this could be due to the ability of the TG to enhance the rate of synthesis of ECM molecules. The cytokine TGF β was identified as possessing distinct anti-tumor activity that may be due to its effect on the host production of a stable ECM (Clark and Coker, 1998; Taipale et al., 1998). TG in conjunction with urokinase Plasminogen activator receptor, mannose-6-phosphate receptor and plasminogen can function at the cell surface to activate latent TGF β (Kojima et al., 1993). The ability of TG to generate TGF β imparts another mechanism that can assist the host's injury response system to limit tumor growth. TGF β stimulates matrix production, increases expression of Tissue inhibitors of metalloproteinases and other protease inhibitors that would allow the newly synthesized matrix molecules to accumulate (Clark and Coker, 1998). The increased levels of collagen observed in the recombinant active TG treated indicate that TG can promote this reaction in the host tissues. We also detected the co-expression of the TG antigen and active TGF β at sites within the TCR and HTI regions of the mammary tumor.

The ability of the tumor to overwhelm the host's ability to repair damaged tissues is essential for its survival (Dvorak, 1986; Nagy et al., 1989). In this study we provide direct evidence that the induction of the TG gene occurs in response to the expanding tumor. Furthermore the host tissues can use active TG to produce a stable ECM that restricts tumor expansion and limits angiogenesis. The TG-matrix stabilizing mechanism has limited capacity since the tumor tissues appear to degrade and inactivate TG by the expression of proteases. Future studies will investigate how over expression of TG at sites of tumor growth can be enhanced to restrict the growth and metastasis of tumor cells.

MATERIALS & METHODS:

Animal Protocols: The Duke Institutional Animal Care and Use Committee approved all animal protocols.

Tumor Implantation: 0.1 mm^3 pieces of rat R3230 Ac tumors were surgically implanted in the subcutaneous tissue of the thigh from a donor rat. These tumors were grown to an average size of $1cm^3$ and then surgically removed from the rat. The tumor was cut in half and the tissue was either snap frozen in liquid nitrogen for western blots or fixed in 10% neutral buffered formalin and paraffin embedded for immunohistochemistry.

Window Chambers: Dorsal skin flap window chambers were used as described by Papenfuss (Papenfuss et al., 1979). Briefly, Fisher 344 rats were anesthetized and the skin over the back was depilated and surgically prepared. The skin flap was pulled dorsally away from the back. Two opposing one-cm diameter windows were created on each side of the flap by surgically resecting the epidermis. One to two fascial planes were left, which contained a few preformed vessels. A 0.1 mm^3 piece of mammary adenocarcinoma (R3230 Ac) from a donor animal was transplanted near vessels with active flow. The resultant tissue window was protected by glass

coverslips and held away from the body of the animal by an anodized aluminum superstructure. This provided protection against desiccation and infection as the tumor grew. This tumor tissue window created a visual field through which the process of tumor growth could be observed non-invasively. Recombinant human TG (Lai et al., 1996) (0.5 ml of 39.0μM solution) was applied topically on the day of surgery. Recombinant human inactive TG (mutation Cys277Ala) was used as a control. Rats were sacrificed at day 10. Photographs were taken at days 0, 3, 7 and 10 post surgery to document the growth of the tumor implants. Quantitation of tumor growth was provided by measuring tumor size at the above mentioned time point. The tumor size was measured as a fraction of the window size through computer image analysis.

Immunohistochemistry: Immunohistochemistry was carried out using procedures described by Hsu et al (Hsu et al., 1981). Briefly, paraffin embedded tissues were sectioned (5 microns) and antigen retrieval was performed using citrate buffer from Biogenex (San Ramon, CA). Tissues were treated with primary antibody against Tissue Transglutaminase (TG100, 1:10, endothelial cell marker, non-reactive to Factor XIIIa, unpublished data) Neomarkers (Fremont, CA), ED1-macrophage marker (MCA341, 1:100) Serotec (Oxford, UK), Isopeptide (814 MAM, 1:75, non-reactive to glutamine and lysine, (Roch, 1991)) CovalAB (Oullins, France), Pan-specific TGF beta (AB-100-NA, 1:100, recognizes the active forms of TGF β1, 2 and 5) R&D (Minneapolis, MN). Secondary and tertiary antibodies were provided in a kit (314KLD) Innovex, (Richmond,CA) and the location of the reaction was visualized with 3, 3'-diaminobenzidine tetrahydrochloride Sigma (St. Louis, MO). Slides were counterstained with hematoxylin and mounted with coverslips. Controls for the immunohistochemistry were treated with normal mouse serum or mouse IgG and rabbit IgG and were negative in any reactivity. Masson's trichrome (MT) was carried out as described by Sheehan (Sheehan, 1980).

Western Blot: Subcutaneous implanted tumors were homogenized in 2 ml cold lysis buffer containing the proteolytic inhibitor cocktail (#1697498) Boehringer Mannheim (Mannheim, Germany) followed by sonification. The blots were performed with four different tumor tissues. They were then centrifuged and supernatant was removed and protein content was determined using Bio-Rad (Hercules, CA). Gel electrophoresis of the extracted tissue samples (50 ug/ml) was performed on an 8.5% polyacrlyamide gel using the buffer system of Laemmeli. Following electrophoresis, the proteins were transferred to nitrocellulose (0.2um). After the transfer was complete, the nitrocellulose membrane was blocked for 1 hr with 5% nonfat milk dissolved in 20mM Tris-HCl, pH 7.4, 150mM NaCl, 0.5% Tween 20. The TG antigen was detected by incubation for 1 hr using a monoclonal antibody for TG (TG 100, CUB 7402) Neomarkers (Fremont, CA) diluted 1:1000, followed by incubation for 1 hr with sheep anti-mouse IgG conjugated to horseradish peroxidase. The TG antigen was visualized using chemiluminescence reagents (ECL, Amersham) and a 30-sec exposure to autoradiography film. The amount of protein on the blot was estimated with a densitometer. The data in Figure 3A shows one sample blot and Figure 3B has the cumulative data for the four blots in a graphical presentation.

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FIGURE LEGEND:

Figure 1:

A MT staining of the rat mammary adenocarcinoma R3230 Ac (A) showing the basic histology and the two distinct regions of the tumor. Skeletal muscle cells (A1), blood vessels, macrophages and collagen deposits (A2) can be observed in the HTI. In TCR, glandular cell masses (A3) pack the interior of the tumor with some areas of necrosis. TG expression in the blood vessels and ECM was intense in the HTI forming a boundary between the tumor and host tissue. In TCR, TG expression was markedly less, predominantly in blood vessels (B5). At higher magnification in TCR, TG could be seen in endothelial cells (C6) and also in the matrix between tumor islands. At higher magnifications in HTI (D & E), ECM (D8 & E 11), blood vessels (D9) and neovessels (E 10) in the inflammed tissue exhibited TG expression. Isopeptide in HTI (F) was detected in the basement membrane (F12) and ECM (F13).

Figure 2:

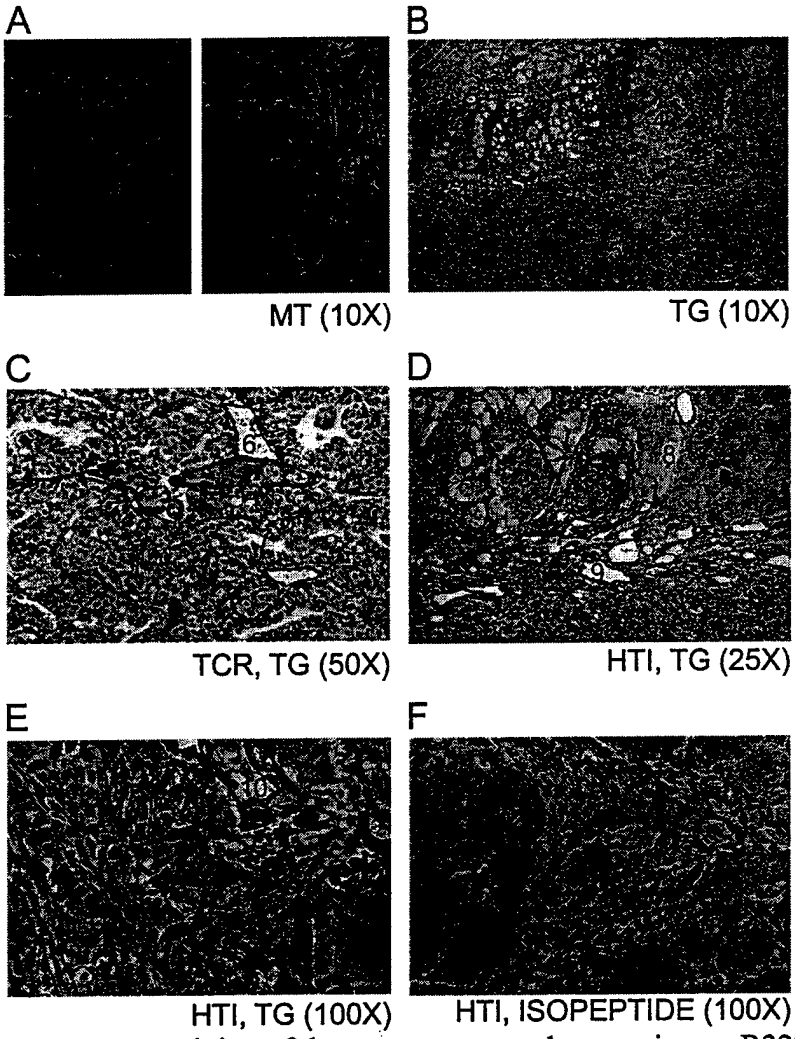
Macrophage distribution (A1) was intense in HTI, being dispersed widely throughout the inflammatory interface. TGF β (B) was detected in the blood vessels (B2) and ECM (B3) in the HTI. A rat dorsal skin flap window chamber implanted with R3230 Ac and treated with recombinant inactive TG (C) at day 10 (tumor boundary marked by arrows). A MT stain (D) for this tumor showed little collagen deposition (D4, green strands) in the ECM. A R3230 Ac implanted in window chamber and treated with recombinant active TG (E) shows marked inhibition at day 10 post surgery. Increased amounts of collagen (F5) was detected by MT in the ECM of TG treated tumor.

Figure 3:

Western blot of R3230 Ac tumor (A). Lane 1 shows the recombinant human TG at 80 kDa. Rat wild type TG showed at 75 kDa. Three fragmented forms of TG were detected at 55, 50 and 20 kDa. Graphical representation (B) of the cumulative data (n=4) shows 55kDa form as the dominant isoforms of TG, while full length TG accounted for only 9% of the total protein.

Figure 4:

Recombinant active TG treated R3230 Ac tumors showed significant growth inhibition (p=0.04, *student's two tail t-test*) against recombinant inactive TG treated controls at day 7. (Error bars ± SEM, n=6).



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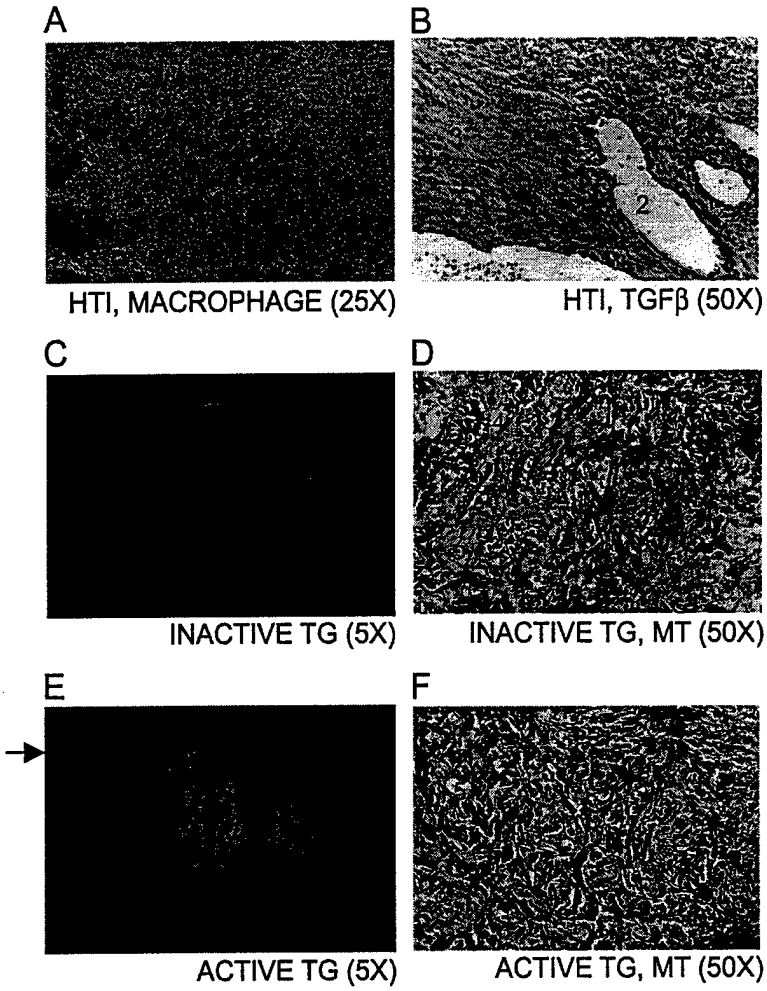

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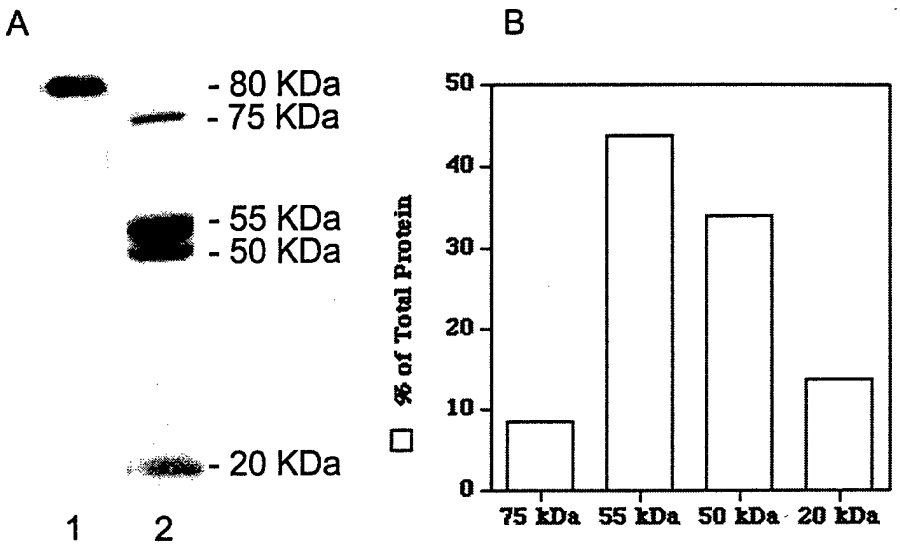

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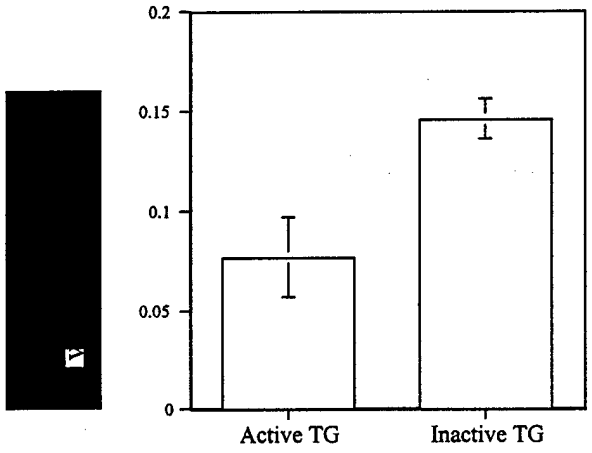

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Appendix 4

TG Transfected 4T1 Mammary Carcinoma Shows Paradoxical Effects on Metastasis Depending on Site of Implantation

Earlier work involving TG transfection in tumor cell lines have showed that TG transfected tumor cell lines growth inhibition (1). It has also been reported that the amount of TG expression in tumor cell lines is inversely proportional to their metastatic potential. Our goal was to assess the effect of TG transfection on metastasis and tumor growth in a highly metastatic mammary carcinoma cell line at different transplantation sites.

Methods:

Transplantations: Nude mice were given a tail vein (Intravenous IV) injection of 10^5 cells transfected with either GFP-TG or GFP alone as control. The mice were sacrificed when they showed physical signs of distress/tumor load i.e. $\geq$ 20% weight loss, labored breathing and slow activity. For ectopic transplantation, 10^6 cells transfected with GFP-TG or GFP alone were first mixed in matrigel and then transplanted in the subcutaneous fascia in the hind leg. Mice were sacrificed every week to follow the process of tumor growth and metastasis at the histological level

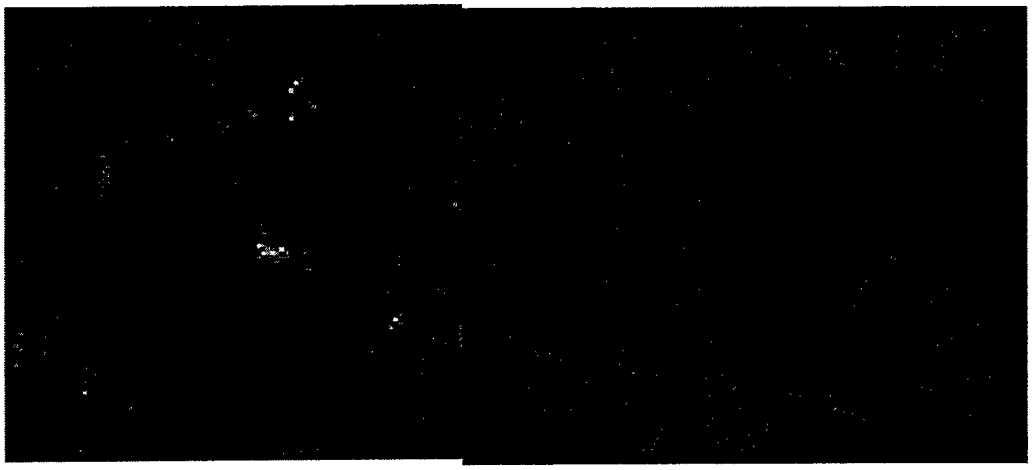
also. Tumors size was measured on a weekly basis. In all experiments, lung and tumor tissue was preserved for immunohistochemical analysis.

Transfections: Prepare cells, 50-80% full (0.1-0.5 million cells per well 24 hours before transfection). Prepare reagent A in tube 1, TG Plasmid DNA-1-3ug, OPTI-MEM-100ul. Prepare reagent B in tube 2, LIPOFECTAMINE-10ul, OPTI-MEM-100ul. Combine B with A in tube 1, mix gently. Incubate for about 1 hour at room temperature. When mixture A+B is ready, rinse cells once with PBS, then twice with OPTI-MEM. Add 0.8ml OPTI-MEM in tube 1, mix gently. Add the whole mixture (A+B+OPTI-MEM) into 35mm-well and cover the cells evenly. Incubate in normal condition for 5-10 hours. In time, add 1ml OPTI-MEM and 200ul FBS per well. Incubate in normal condition for 12-24 hours. Replace medium with fresh complete growth medium (10% FBS, 1%Antibiotic) and add G418.

Results and Discussion:

Tail-vein (IV) Injections: GFP-TG transfected 4T1 cells showed enhanced metastasis as compared to GFP alone. On gross examination, the GFP-TG lungs appeared riddled with mets as compared to GFP alone. The lifetime of the GFP-TG mice was also reduced to more than 60% as compared to GFP alone mice since almost all the GFP-TG mice died at week 2 and GFP alone were doing well at week 3 post injection. On histological examination, GFP-TG mets more numerous and seemed to creep along the septa of the lung and give a matted appearance (Figure 1).

Control



TG

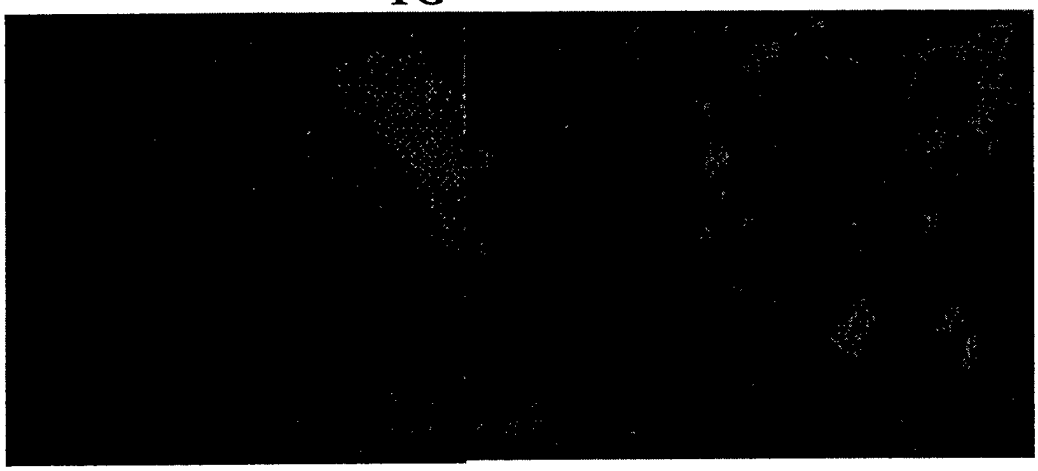


Figure 1: 4T1 transfected with either GFP (control) or GFP-TG (TG) were given IV (tail vein) to the animals. Control lungs do not exhibit surface mets and have less than 25% lung involved with mets on histological examination at week 2. Meanwhile, GFP-TG lung are covered in mets and the whole lung is replaced with the tumor cells.

Figure 2

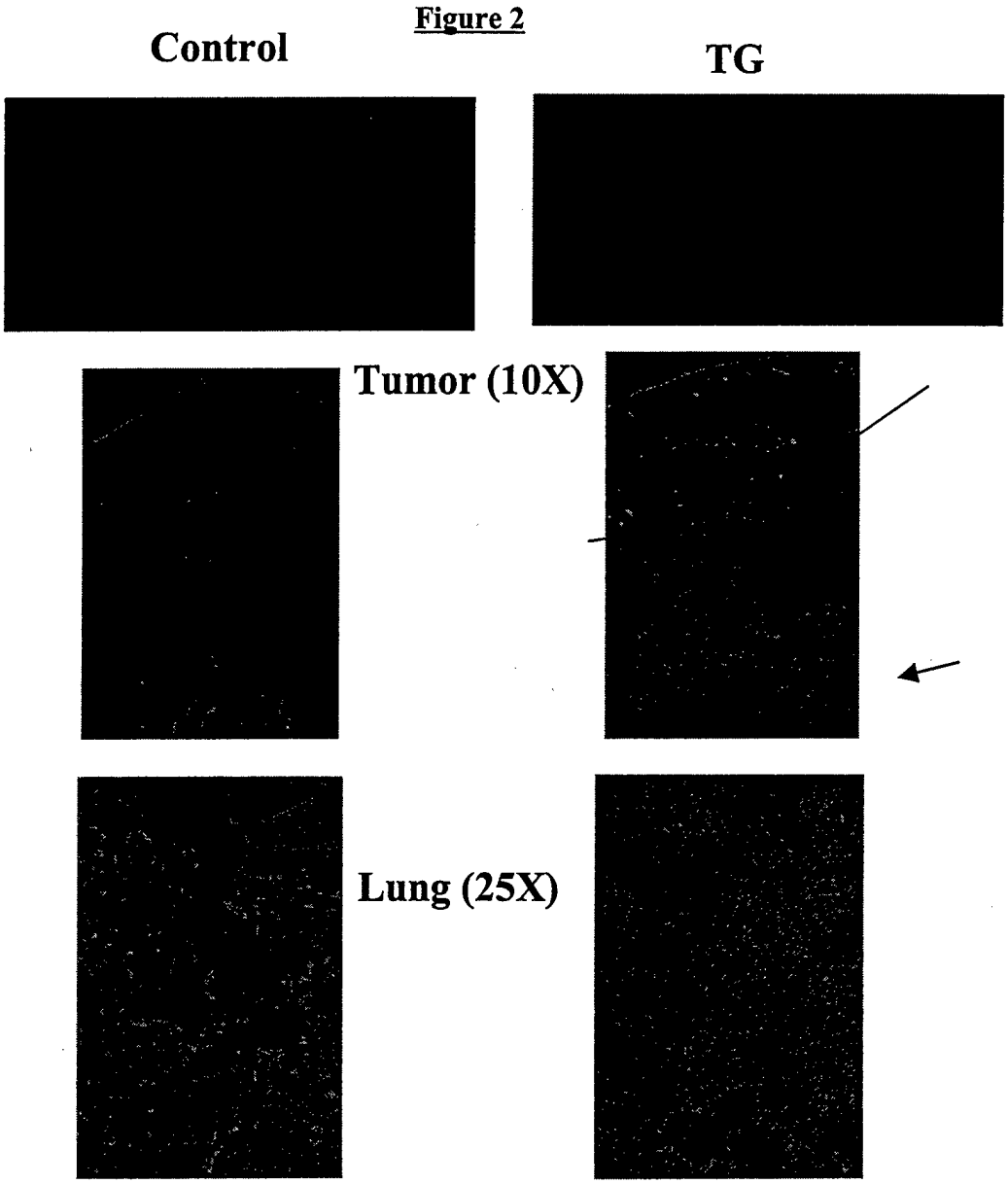


Figure 2: Results of the ectopic implantation of 4T1 cells. Panel on left side shows GFP transfected (control) tumors and lungs. Tumors show multiple satellites and early metastasis. GFP-TG (TG) transfected tumors grow in perfect spheres and gain 3-4 times less size than GFP alone. They also show minimal metastasis.

Table 1: A summary of Metastasis data for TG transfected 4T1 cells
(-) none, (±) ≤10%, (+) ≥10% , (++) >20%, (+++) ≥50%,
(++++) >80% of the lungs surface area

Metastasis to Lungs

Time Weeks	IV(Tail Vein)		Ectopic (Sub Q)	
	GFP-TG	GFP	GFP-TG	GFP
1	++	+	-	+
2	++++ Dead	++	±	++
3	X	+++ Dead	+	+++ Dead
4	X	X	Alive	X

Ectopic Transplantation: GFP-TG tumors grew at a much slower pace than GFP alone. They also exhibited intense central necrosis and only the outer margin had live tumor cells. GFP-TG tumors grew in perfect spheres as compared to multiple tumor masses in case of GFP alone. On an average, tumor size of GFP alone was 3-4 times larger than GFP-TG. GFP tumors developed mets by week 2 and all of the animals died by week 3. On the other hand, GFP-TG bearing mice were alive at week 4 and there were very few mets in the lungs of these animals (Figure 2).

This pattern of these results may seem paradoxical at first but is perhaps due to a similar mechanism of action. TG transfected cells have been shown to have a high degree of adhesiveness and they also spread very effectively (2, 3). This property can impart positive attribute for the metastasizing cells since the most difficult part of metastasis is attachment at distant sites. Any cell which has advantage here will be better adapted to grow. Meanwhile, this enhanced adhesiveness can cause a primary tumor mass to grow in a tightly packed manner that makes the first part of metastasis very difficult i.e. detachment and access to blood vessels. The idea of these tumors growing in a tight sphere which is highly crosslinked is further promoted by the fact that these tumor show extensive necrosis in the middle which may be due to the intense pressure imposed by such a structure.

Thus, it seems that TG's ability to cross link major ECM and cell membrane proteins can have diverse effects on tumor growth and metastasis. TG expression in primary tumor seems to limit the growth and metastasis potential of the tumors. On the other hand, mets with high expression of TG seem to have a distinct advantage in metastasis.

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Appendix 5

1 Angiogenesis and Oxygen Transport in Solid Tumors

Zishan A. Haroon, Kevin G. Peters, Charles S. Greenberg, and Mark W. Dewhirst

CONTENTS

INTRODUCTION
NORMAL ANGIOGENESIS
TUMOR ANGIOGENESIS
TUMOR PATHOPHYSIOLOGY LEADING TO HYPOXIA
NITRIC OXIDE

1. INTRODUCTION

Angiogenesis, the formation of new vessels from existing microvasculature, is a tremendously complex and intricate process, essential for embryogenesis and development of multicellular organisms *(1)*, but it occurs only rarely in adult tissues in a tightly controlled manner during normal wound healing and the female reproductive cycle (corpus luteum, placenta, and uterus) *(2)*. When these tight controls are breached, the result is unchecked angiogenesis, which has been implicated in the development and progression of a variety of diseases (Table 1). The prevalence of pathologic angiogenesis in human diseases, and the significant mortality associated with these disorders, underscore the importance and emergence of antiangiogenesis therapy as a major clinical tool. In the case of solid malignancies, the generation of proangiogenic substances is in part caused by the pathologic microenvironment that develops in response to uncoordinated vascular production.

A common consequence of the abnormal microvascular structure and function that exist in tumors is hypoxia, which is known to induce a number of factors involved in regulating angiogenesis. Thus, hypoxia may prove to be a common initial signal for tumor vessel formation. There are other microenvironmental factors that may also play a role in the process as well, including endogenous levels of nitric oxide (NO). This chapter presents an overview of the features of normal and pathologic angiogenesis, with an emphasis on the role of hypoxia and dysfunctional vasculature during the angiogenic process in tumors.

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Table 1
List of Major Diseases in Which Angiogenesis Plays a Role in Pathogenesis

Inflammatory diseases:
Arthritis, chronic inflammations, inflammatory bowel diseases, psoriasis
Neoplasms:
Breast, bladder, colon, glioblastoma, hemangioblastoma, lung, melanoma, neuroblastoma, pancreas, renal, uterine–cervix
Ocular diseases:
Age-related macular degeneration, proliferative retinopathy (diabetic)

2. NORMAL ANGIOGENESIS

Normal angiogenesis is a multistep, tightly orchestrated process that occurs predominantly during physiologic events involving tissue repair and/or remodeling (wound healing, placental development, and so on) *(3)*. Tissue repair and remodeling involves continuous feedback and interaction between endothelial cells and the extracellular matrix (ECM) in a process that has been termed "dynamic reciprocity" by Clark *(4)*. Similarly, vascular remodeling is accomplished by targeted apoptosis and proliferation, deposition of matrix and its stabilization, and organization by enzymatic crosslinking and proteolysis.

Normal angiogenesis involves an initial localized breakdown of the basement membrane in the parent vessel that is mediated by proteases *(3,5*; Fig. 1]. Endothelial cells then migrate into the perivascular space and adjoining matrix, and form a capillary sprout. These sprouts elongate by further endothelial migration at the tip, and proliferation at the base, to replace the migrated cells. Subsequently, remodeling occurs, and these cords anastamose to form a loop, basement membrane is laid out, and a patent vessel is formed.

2.1. Molecular Mediators of Angiogenesis

Although many angiogenic substances have been identified, polypeptide growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) seem to be the most potent ones (Table 2). It is interesting to note that the aforementioned growth factors signal through receptor tyrosine kinases *(6)*. VEGF, also known as vascular permeability factor, is a multifunctional cytokine that is upregulated in response to hypoxia, and is considered one of the most potent proangiogenic molecules *(7)*.

Apart from activating endothelial cells to proliferate and express matrix metalloproteinases, plasminogen activators, and tissue factor, the most prominent effect of VEGF is induction of vascular hyperpermeability. It has been consistently observed that hyperpermeability to plasma proteins is associated with both pathological and physiological angiogenesis *(8)*. This association may have two very important implications for angiogenesis: First, it leads to the formation of a provisional fibrin matrix, which provides the primary scaffold for assembly of elements necessary for neovascularization *(8)*. The provisional fibrin matrix probably provides a more fluid matrix that is supportive of the angiogenic process. Second, another suggestion for hyperpermeability's role in angiogenesis has been proposed by Folkman *(9)*, which relates to the observation that confluent endothelial cells are refractory to mitogenic stimuli. The vasodilation and hyperpermeability that precede angiogenesis may subject endothelial cells to stretch and decrease confluence, which increases reactivity to proangiogenic mitogens.

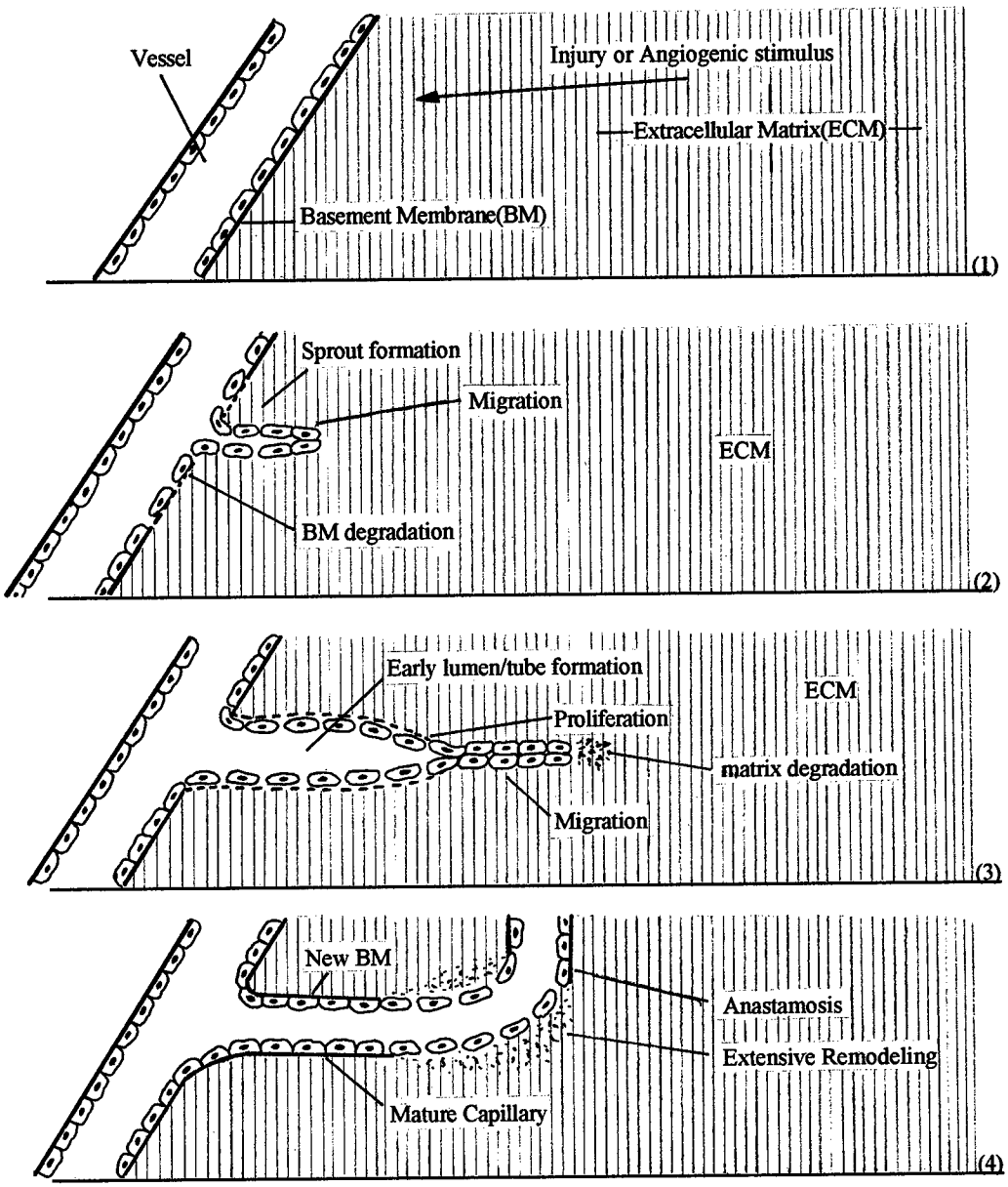


Fig. 1. A general mechanism of normal angiogenesis process. (1) Injury or tissue repair activates the endothelial cells. (2) Results in basement membrane degradation by proteases, and initial sprout formation by migration of endothelial cells. (3) Endothelial cells continue to migrate at the tip, with controlled matrix degradation. Proliferation occurs proximal to migration with formation of the primitive tube. (4) Extensive remodeling occurs all along the new capillary; new basement membrane is laid out to form a mature capillary as it anastamoses with other sprouts.

Normal angiogenesis involves complex interactions among endothelial cells, inflammatory cells, and ECM. These precisely controlled interactions involve ECM proteolysis during basement membrane degradation, invasion of the provisional fibrin matrix, and remodeling of the matrix and vessels *(10)*. These proteolytic activities also activate and/

Table 2
Prominent Molecules That Have Been Shown to Possess Proangiogenic Properties

Angiogenin
Fibroblast growth factors (acidic and basic)
Heparin
Hepatocyte growth factor (scatter factor)
Insulin-like growth factors
Interleukin-8
Platelet-activating factor
Platelet-derived endothelial cell growth factor
Platelet derived growth factor-BB
Transforming growth factor-α
Transforming growth factor-β
Tumor necrosis factor-α
Vascular endothelial growth factor

Table 3
Role of Balanced Proteolysis in Angiogenesis

Migratory path formation and remodeling:
Basement membrane degradation
Controlled ECM degradation involved during migration/invasion of endothelial and inflammatory cells into matrix
Anastamoses and capillary lumen/tube formation
Release of cytokines:
Release of bound basic FGF and VEGF
Activation of TGF-β from latent to active form
Degradation products with angiogenesis modulating capability:
Angiostatin (plasminogen)
Collagen derived peptides
Endostatin (collagen XVIII)
Fibrin and fibronectin fragments
16-kDa fragment of prolactin

or release important angiogenic cytokines, such as transforming growth factor-β (TGF-β), VEGF, and basic fibroblast growth factor (bFGF) *(11)*. In addition, biologically active degradation products of ECM, such as angiostatin from plasminogen, are generated, which regulate angiogenesis *(12)*. A balance of proteases and antiproteases, in a tightly regulated temporospatial pattern, is required for proper neovessel formation and remodeling/maturation (Table 3). Cytokines that regulate proteolytic activity during normal angiogenesis include bFGF, VEGF, TGF-β, hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1) *(13)*.

Although endothelial cells provide the foundation for neovasculature, angiogenesis also involves complex interactions with fibroblasts and inflammatory cells, such as macrophages and mast cells. The role these inflammatory cells play during angiogenesis is further strengthened by the fact that many proinflammatory cytokines, such as interleukin-8 (IL-8) and TNF-α, induce angiogenesis *(14,15)*. Macrophages *(16)* and mast cells *(17)*

are involved during induction and propagation of the angiogenic cascade, and mediate their effects through secretion of cytokines and growth factors (including VEGF), release of proteases, and activation of fibroblasts. Fibroblasts are chiefly responsible for production of ECM and release of matrix metalloproteinases (MMPs) for selective degradation and organization of the ECM *(4)*.

Migration of endothelial and inflammatory cells forms an indispensable part of the angiogenic cascade. Migration is a multistep process that begins with a strong directional stimulus to migrate, and is followed by coordinate expression of receptors and matrix molecules to facilitate movement. TGF-β, VEGF, bFGF, and PDGF provide the chemotactic signals *(18)*, and chemokines provide chemokinetic signals *(14)* for both inflammatory and endothelial cells in induction of angiogenesis. The best-characterized receptors that are involved in migration during angiogenesis are integrins β_1, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ *(19)*. Although the evidence is still sketchy, it is becoming apparent that particular integrins are upregulated, and are required for the angiogenic effects of specific cytokines, such as $\alpha_v\beta_3$ for bFGF and $\alpha_v\beta_5$ for VEGF *(20)*.

The final steps in physiologic angiogenesis include transforming loosely associated endothelial cells and ECM into mature and patent vessels with intact basement membranes. Current evidence suggests that vessel maturation may be driven by novel molecular mediators, such as angiopoietins and their Tie family of receptors.

Angiopoietin-1 and -2 have recently been described as ligands for an endothelial-cell-specific tyrosine kinase receptor, Tie-2 *(21)*. Tie 2 receptors are essential for embryonic angiogenesis, since knock-outs of this receptor are embryonic lethal, with profound defects in assembly of microvessels, and it is proposed that the activity of the Tie-2 receptor is located downstream from VEGF on the angiogenic cascade *(22)*. The presence of the Tie-2 receptor on endothelial cells during angiogenesis in a variety of settings *(23)*, combined with evidence for constitutive angiopoietin 1 expression by vascular smooth muscle cells and/or pericytes, suggest that they may be involved in a regulated feedback system to modulate and steer the vessel maturation and organization process *(21)*. Meanwhile, angiopoietin-2 acts as a natural antagonist to Angiopoietin-1 and Tie-2, to provide a critical balance during induction of angiogenesis, vascular remodeling, and maturation *(21)*. Thus, angiopoietins and Tie-2 demonstrate a complex and intertwined relationship among themselves and their environment, and also exhibit the ability to regulate the angiogenic process.

3. TUMOR ANGIOGENESIS

Although developmental (embryonic) and disease-associated (pathologic) angiogenesis share many mechanistic features, as first suggested by Haddow *(24)* and later by Dvorak *(25)*, they probably differ regarding to regulatory controls *(26)*. As described above, tissue repair is normally a self-limiting process that occurs in response to hypoxia generated at the site of tissue injury. In contrast, progressive tumor growth creates ongoing hypoxia and acidosis, which do not recede as they would after injury (Fig. 2). Moreover, tumor vessels fail to mature into a normally functioning vasculature. In this manner, a positive feedback loop is created as continued tumor proliferation, handicapped by a disorganized vasculature, again outstrips its supply, which leads to continuing hypoxemia and angiogenesis (Fig. 3).

It is of interest to note that production of several of the proangiogenic compounds are regulated by hypoxia, including VEGF, bFGF, TGF-β, TNF-α, and IL-8 (Table 4).

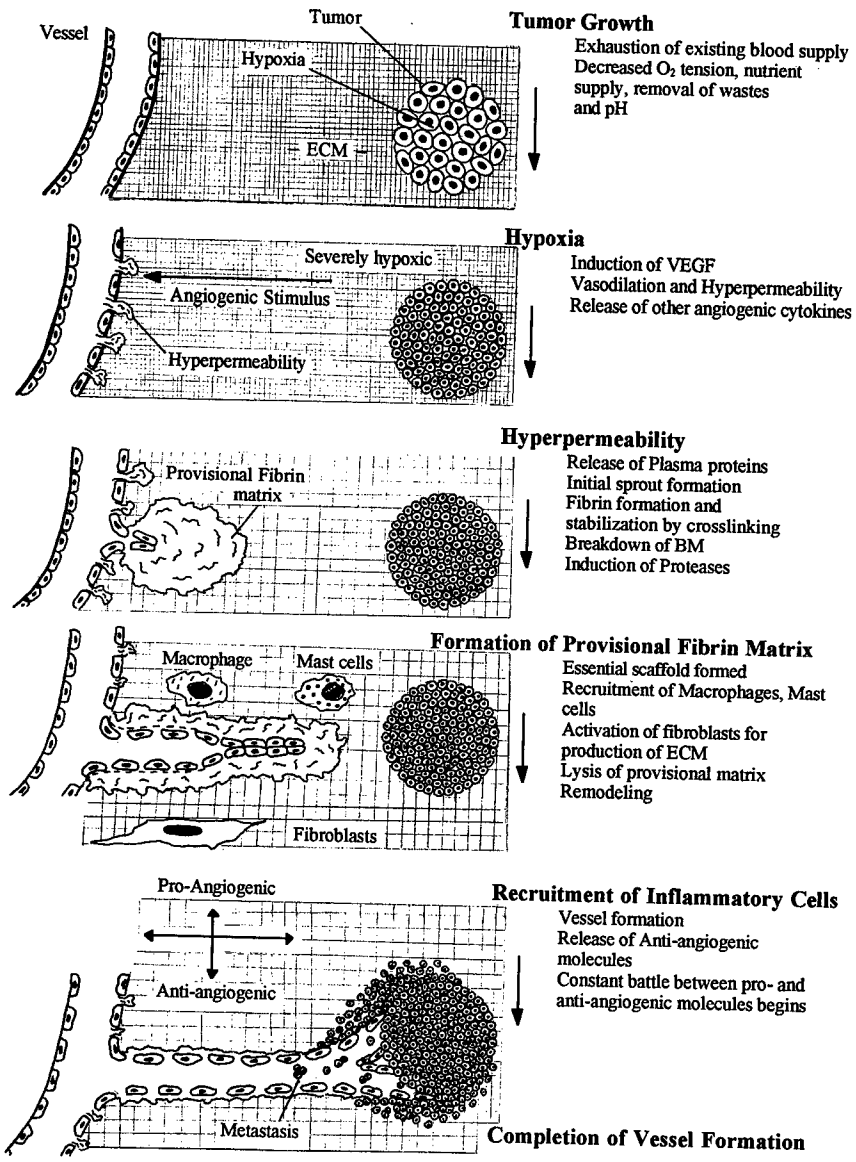


Fig. 2. A flow chart showing the current understanding of the process of tumor angiogenesis.

Furthermore, the presence of tumor hypoxia has been tied to more aggressive phenotypes in murine tumor models, as well as in human tumors. For example, in human cervix cancer and soft tissue sarcomas, presence of hypoxia prior to the start of therapy has been tied to a greater likelihood for distant metastases *(36,37)*. Additionally, the presence of tumor regions with high vascular density predicts for poorer overall survival in carcinoma of the breast, and in prostate cancer *(38)*. Taken together, these data strongly suggest that hypoxia upregulates angiogenesis, which in turn provides vascular access for metastasis.

Oncogenic transformations of tumor cells may also play a direct role in induction and propagation of angiogenesis, through production of angiogenic factors. It has been shown

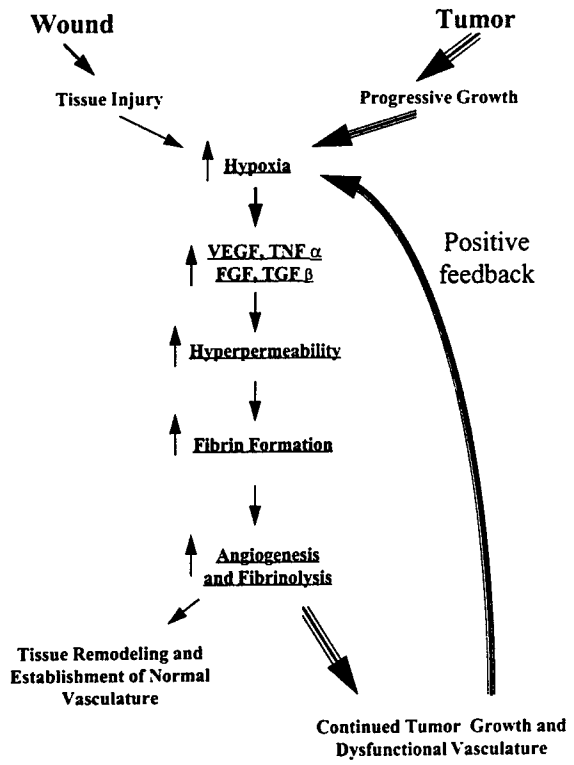


Fig. 3. Both normal tissue repair/remodeling mechanisms (wound healing) and tumors share common pathways to stimulate angiogenesis. Tissue repair, being a self-limited process, leads to regeneration of normal vasculature; tumor-induced angiogenesis produces a dysfunctional vasculature, which, coupled with progressive growth of tumor cells, results in continuing hypoxemia and angiogenesis.

Table 4
List of Proangiogenic Cytokines and Receptors Whose Expression Is Modulated by Hypoxia

Cytokine or receptor	*Hypoxia level*		*Gene expression*
	PO_2	*Time*	
Angiopoietin 1	NS	18 h	↓ *(27)*
aFGF, bFGF	2.0%	24 h	↑ *(28)*
Flk-1/KDR	2.0/2.0%	24/24 h	↓/↑ *(29[a],30[b])*
Flt-1	2.0%	24 h	↑ *(30)*
Interleukin-8	2.0%	24 h	↑ *(31)*
PDGF-A, PDGF-B	1.0%	16 h	↑ *(32)*
TGF-β	0.0%	24 h	↑ *(33)*
TNF-α	1.0%	24 h	↑ *(34)*
VEGF	1.0%	06 h	↑ *(35)*

↑, increase, ↓, decrease, NS, not specified.
*a*Reoxygenation performed in the experiments.
*b*Conflicting data has been reported in the papers.

Table 5
Short List of Molecules That Exhibit Potent Antiangiogenesis Activity

Angiostatin
Endostatin
Interferon-α
Metalloproteinase inhibitors
Platelet factor 4
Somatostatin
Thrombospondin

that VEGF expression is induced by mutant H- or K-*ras* oncogenes, as well as v-*src* and v-*raf,* in transformed fibroblasts and epithelial cells *(39)*. Other angiogenic growth factors, such as TGF-β and TGF–α, have also been shown to be upregulated by mutant ras *(39)*. These effects may be mediated through a ras-raf-MAP kinase signal transduction pathway, which results in activation of promoter regions of genes of angiogenic growth factors. Several of these proangiogenic cytokines have been shown to be produced by the same tumor *(40)*. This evidence points to a potential pitfall in approaching antiangiogenesis therapy directed against a singular angiogenic cytokine.

Aggressive neovascularization also leads to production and generation of numerous antiangiogenic compounds (Table 5). These substances are vital in regulating the process of angiogenesis, and are discussed in detail in later chapters.

4. TUMOR PATHOPHYSIOLOGY LEADING TO HYPOXIA

It is generally believed that hypoxia in tumors develops in two ways. The first form, referred to as chronic hypoxia, has classically been thought to result from long diffusion distances between tumor vessels *(41)*. The second form, known as perfusion limited, or acute, hypoxia, has been attributed to transient blockages in, or collapse of, tumor vessels *(42)*. This chapter will distinguish between these two forms of hypoxia in the following manner: Cells that are chronically hypoxic exist in an environment where the PO_2 is <10 mmHg for many hours at a time. Such cells may never experience a normoxic condition once they become hypoxic. Cells that are acutely hypoxic exist in an environment where the PO_2 is <10 mmHg for many minutes at a time. These cells probably experience many cycles of hypoxia and reoxygenation. Mechanistic understanding of the relative importance of the factors leading to both forms of hypoxia may lead to additional lines of investigation regarding microenvironmental control of gene expression in tumors.

4.1. Origins of Chronic Hypoxia

The balance between how much oxygen is delivered and how much is consumed leads to the oxygen concentration that is found in tissue. Several factors contribute to the development of chronic hypoxia, including longitudinal gradients of PO_2, irregular vascular geometry and low vascular density, altered blood viscosity, and oxygen consumption that is out of balance with supply. Of these factors, oxygen consumption has the most dynamic effect on tissue PO_2.

4.1.1. Deficiencies in Tumor Oxygen Supply Longitudinal Gradients

It is now well recognized that tumor microvessels can be hypoxic, even when there is movement of red cells through the vessel *(43,44)*. The most likely origin of intravascular hypoxia comes from longitudinal or axial gradients in oxygen tension, which are defined as declines in oxygen tension along the vascular tree. Two features of longitudinal gradients lead to intravascular hypoxia in tumors. First, tumor-feeding arterioles are more deoxygenated than comparable arterioles in normal tissues. Dewhirst et al. *(45)* reported that the PO_2 of tumor-feeding arterioles averaged 60% that of comparable arterioles of normal granulating tissues, yielding an average PO_2 of 32 mmHg, when aortic blood gas PO_2 averaged near 100 mmHg. The relative deoxygenation of these arterioles is probably caused by one or more factors: tissue pH—there is a right shift of the hemoglobin saturation curve as the arteriole enters a region of tissue acidosis (a typical condition in tumors); higher oxygen consumption—it is likely that the arterioles pass through a region of high cell proliferation, where the oxygen consumption rate may be very high. It is well established that longitudinal gradients occur in normal tissues as well, but such conditions do not normally lead to vascular hypoxia, probably because of the normal abundance of arteriolar supply *(46)*.

It has been estimated recently that the majority of oxygen transport in normal tissues actually occurs at the level of the terminal arteriole, rather than in capillaries *(46)*. There are arteriolar supply vessels to tumors, but they typically do not enter the tumor parenchyma *per se,* unless the tumor happens to grow around them *(47)*. Thus, oxygen transport in tumors cannot occur via arterioles. This means that the majority of oxygen transport is delivered via capillaries or veins, and it is likely that the blood must travel unusually long distances in postarteriolar microvessels before exiting the tumor *(48)*. There are several sets of data to support this hypothesis. Using phosphorescence quench imaging, it has been shown that longitudinal gradients exist in skin window chamber tumors, where those microvessels nearest the arterioles are much better oxygenated than vessels most distant from the arterioles *(49)*. The most distant vessels appear to be hypoxic (PO_2 averaging <10 mmHg). Studies with oxygen microelectrodes *(43)* and phosporescence quench imaging studies of Helmlinger et al. *(44)* have independently demonstrated the presence of hypoxemic tumor microvessels, in the absence of vascular stasis. Collectively, these data substantiate the notion that vascular hypoxia in tumors occurs because of the relative lack of arterioles. Vascular hypoxia, of the type described here, does not occur in normal tissues.

4.1.2. Altered Blood Viscosity

The vascular hypoxia found in tumors was speculated to be responsible for altered blood viscosity, which could contribute to sluggish flow that is often found in tumors. Using a viscometer, suspension viscosity was found to be increased by 40% when PO_2 and pH were decreased below 10 mmHg and 6.8, respectively. This effect was caused by shrinking of red cells. When the calcium channel blocker, flunarizine (5–10 mg/L-1), was co-administered with red cell suspensions, viscosity returned to control levels *(50)*. When the effect of flunarizine was examined in vivo, the drug improved blood flow and oxygenation, selectively, in the center of tumors, where microvessels are more hypoxic *(51)*. In summary, the reduced vascular oxygenation observed in tumors leads to rheologic changes in red cells that increase viscosity and decrease tumor blood flow. This leads to a viscous cycle, as the resultant reduction in blood flow further exacerbates the vascular

hypoxemia. The use of agents, such as flunarizine, could break this cycle, and at least restore viscosity to normal limits.

4.1.3. Vascular Geometry

The classic theory, which originated with Thomlinson and Grey, is that chronic hypoxia develops because intervascular distances are too long in tumors *(41)*. However, the irregular geometry of vascular networks is also important. Dewhirst et al. *(52)* have performed simulations of oxygen transport in two microvascular networks. The simulations were done using microvessel flow velocities and hematocrits that are typical for the R3230Ac tumor line growing in skin window chambers. Oxygen consumption rates were assumed to be in the midrange of what has been measured for this tumor line. Two models were compared (actual geometry shown in Fig. 4A). The Greens' function model utilizes the actual microvascular geometry; the Krogh cylinder model assumes that all of the vessels are straight and parallel. In simulations, the Krogh cylinder model predicted no regions of tissue with PO_2 <10 mmHg; the Green's function model predicted substantial regions of hypoxia (Fig. 4B). These represent scenarios in which there is adequate vascular density, but the chaotic nature of the vascular geometry creates hypoxia *(47,53)*.

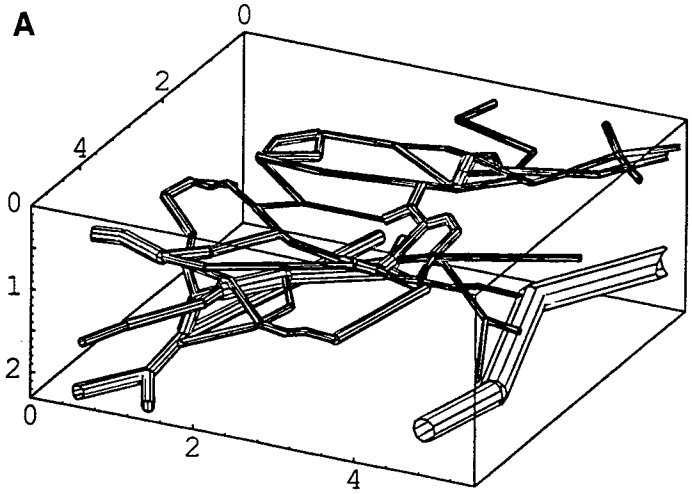
The presence of highly disordered vascular networks also leads to plasma channels. These are vessels that do not contain appreciable red cells, but do carry plasma. In the skin-fold chamber tumor model, it has been estimated that 8–9% of all microvessels have this characteristic *(54)*. These channels probably develop because of the abnormal branching angles and altered rheology of red cells. Both of these conditions will tend to favor concentration of red cells in vessels with the lowest flow resistance.

4.1.4. Oxygen Demand vs Oxygen Supply

Microregional measurements of oxygen consumption have been performed by fitting the profile of oxygen tension between vessels to a diffusion equation *(52)*. For the R3230 Ac tumor, oxygen consumption rates were observed in the range of what has been measured in murine and human tumor xenografts *(48,55)*, averaging 1.5 mL O_2/100 g/min (range 0.8–2.2 mL/100 g/min). The oxygen consumption rates that have been measured in tumors are in the midrange of what has been reported for normal tissues *(48)*. Thus, one cannot make the claim that tumor hypoxia is the result of oxygen consumption rates that are abnormally elevated.

The Green's function method described above has also been used to compare the relative importance of tissue perfusion, oxygen content, and oxygen consumption in controlling tumor oxygenation (Fig. 4C; *48,56)*. The object of the simulations was to

Fig. 4. Three-dimensional vascular architecture and its effects on oxygen transport. **(A)** Three-dimensional reconstruction of a microvascular network in a window-chamber tumor, as determined using confocal microscopy. **(B)** Comparison of the distribution of PO_2 values for actual geometry vs the same vascular density, but with evenly spaced, parallel vessels (Krogh cylinder simulation). The irregular geometry leads to hypoxia (PO_2 values < 10 mmHg), but no hypoxia is predicted for the Krogh cylinder simulation. **(C)** Relative effects of microvascular perfusion, oxygen content, and oxygen consumption rate on hypoxic fraction. This simulation indicates that the most sensitive parameter for reduction of hypoxia is manipulation of oxygen consumption rate. (Reproduced with permission from ref. *48*)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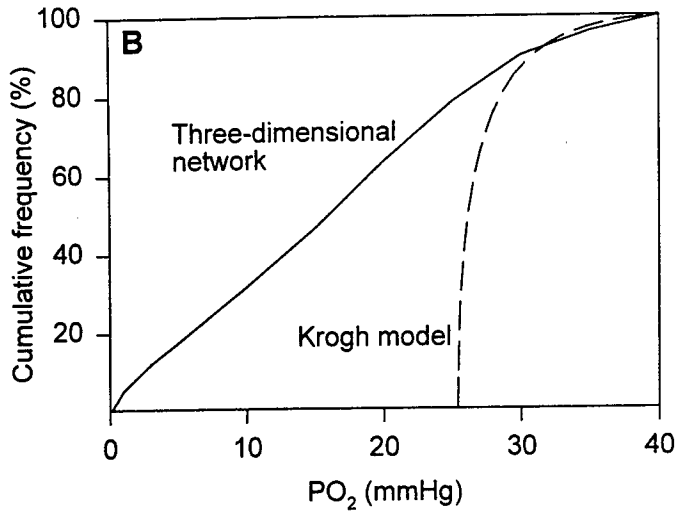
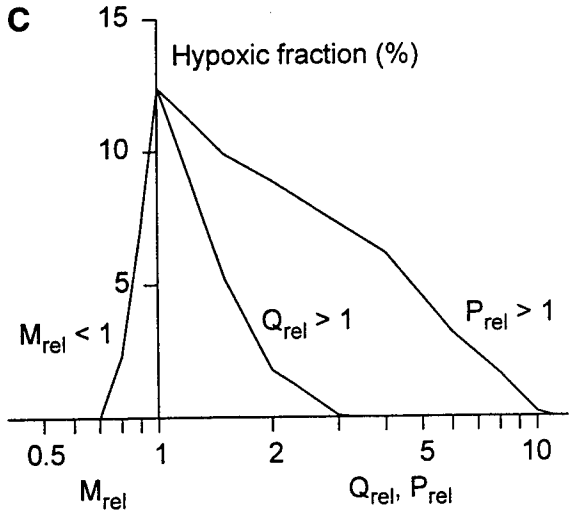

B
Cumulative frequency (%)
100
80
60
40
20
Three-dimensional network
Krogh model
0
10
20
30
40
PO2 (mmHg)


C
15
Hypoxic fraction (%)
10
5
Mrel < 1
Qrel > 1
Prel > 1
0.5
1
2
5
10
Mrel
Qrel, Prel

determine how much perfusion, oxygen content, or consumption rate would have to be changed, to eliminate hypoxia in the simulated region. Under two baseline conditions of oxygen consumption, the most efficient method to reduce hypoxia was via reduction in oxygen consumption rate. In one example, the relative efficiency of changing oxygen consumption vs oxygen content of blood was a factor of 30. To be specific, an 11-fold increase in oxygen content would be needed to eliminate hypoxia, compared with a 30% reduction in oxygen consumption rate. Thus, even though oxygen consumption is not greatly elevated, relative to many normal tissues, it still has the most significant impact on tissue oxygenation in tumors.

4.2. Acute Hypoxia

Until recently, the commonly held view has been that acute hypoxia results primarily from vascular stasis, and that this occurs from one of three causes: vascular collapse, leukocyte plugging, or impingement of tumor cells in the vascular lumen. Although these factors may play a role in vascular stasis, they are not the only cause for acute hypoxia. Using window chamber tumors to study the kinetics of tumor microvessel flow, total vascular stasis was observed in approx 5% of vessel segments, and the duration of stasis was <20 s *(57)*. These data are similar in magnitude to prior reports by Chaplin et al. using matched dye methods *(58)*. Based on these observations, it seems that total vascular stasis is not the most common source of transient hypoxia.

Flow instabilities are a rather common phenomenon, and there is strong evidence that instabilities in tumor perfusion, short of vascular stasis, can lead to transient hypoxia *(60)*. Trotter et al. performed a series of studies in murine tumors using, pairs of intravenously administered fluorescent dyes to monitor functional vessels *(59)*. They noted differences in staining intensity around groups of vessels, and theorized that this may be caused by fluctuations in blood flow rate within small networks of vessels. They also suggested that such behavior could lead to transient hypoxia, without a requirement of total vascular stasis. Temporal variations in perfusion have been seen in both experimental and human tumors, using laser Doppler flowmetry *(60,61)*. Similarly, the temperature of murine tumors has been shown to be temporally unstable, which must be related to variations in perfusion rate *(62)*. These data suggest that variations in blood flow rate occur in tumors, but until recently there was no direct proof that such variations in flow could lead to transient hypoxia.

This issue was also addressed by simultaneously monitoring microvessel red cell flux and PO_2 for periods up to 1 h, using window chamber tumors by Kimura et al. *(57)*. In most cases, the red cell flux in groups of microvessels was unstable, and the variations in red cell flux could be as much as several orders of magnitude, but more commonly is in the range of a factor of two. The Green's function method was used to simulate the effects of a twofold change in red cell flux on tissue PO_2 distribution. The simulation showed that as much as 25% of the tumor region could fluctuate above and below a hypoxic threshold, under these conditions. Considering the fact that the observed fluctuations were of at least this order of magnitude in nearly all vessels studied, the investigators concluded that transient hypoxia must be a common phenomenon, and that it is most frequently brought on by fluctuation in blood flow, rather than by total vascular stasis.

More recently, temporal instabilities of PO_2 and perfusion in hind limb tumors have been investigated *(63)*. To accomplish this task, 10-μm diameter recessed-tip electrodes and laser Doppler probes were introduced into tumors, and monitored continuously for

Table 6
Temporal Observations of Intermittent Hypoxia in R3230 Ac Flank Tumors *(63)*

Parameter	*PO_2 < 5 mmHg*
Fraction of experiments never hypoxic	5/13
Fraction of experiments always hypoxic	3/13
Median number of hypoxic events/h	3.9 (0.9–17.9)[a]
Median % time hypoxic	60.1 (16.2–84.7)[a]
Median duration of hypoxic episodes (min)	7.4 (0.9–44.7)[a]

[a]Numbers in parentheses indicate range of data.

periods ranging from 60 to 120 min. The observed fluctuations in PO_2 were similar in kinetics to those previously published for laser Doppler flowmetry by Chaplin and Hill *(60,61)*, and were consistent with Kimura and Braun *(57,63)* in the window chamber model (Table 6). In these studies, some measurements never dropped below a threshold value of 10 mmHg, and others remained below this threshold for periods up to 90 min of observation. The remaining experiments, however, demonstrated temporal variations above and below this threshold.

It has been reported that hypoxia might create negative selection pressure on cells with wild-type p53 suppressor gene, since activation of p53 leads to apoptosis *(64)*. Thus, cells with mutant forms of p53 would gain a survival advantage over cells with wild-type p53. The instabilities in tumor oxygenation perhaps suggest a more sinister effect of hypoxia *(57,63)*. If the process of oxygen transport is as unstable as these results would indicate, then it is possible that, once a tumor becomes vascularized, it is subjected to repeated cycles of ischemia-reperfusion injury. Since the primary bioactive product of this process is superoxide anion *(65)*, it is possible that mutagenic events leading to tumor progression could result. Recent evidence for the mutagenic effects of hypoxia reoxygenation injury comes from the work of Reynolds et al. *(66)*. In these studies, a phage shuttle vector reporter system was transfected into the tumorigenic cell line, LN12. In tissue culture under normoxic conditions the incidence of mutations averaged $<2 \times 10^{-5}$, even after more than 20 passages. However, the incidence increased by a factor of four after one episode of hypoxia/reoxygenation, and doubled again after another episode. The incidence was much higher when the tumor was grown in vivo, compared with in vitro culture, as well.

The causes of acute hypoxia are not well defined at this time. Certainly, all of the factors that contribute to chronic hypoxia will also contribute to acute hypoxia, since they create the baseline conditions that exist without blood flow fluctuations. Factors that may contribute to flow fluctuations include arteriolar vasomotion *(54)*, rapid vascular remodeling *(67)*, and other hemodynamic effects *(68,69)*.

It is clear from the above discussion that tumor hypoxia is a complex and dynamic process that is the result of several factors. Chronic hypoxia probably develops as a result of exaggerated longitudinal gradients, leading to intravascular hypoxia; irregular vascular geometry and/or long intervascular distances; irregular vascular branching patterns; and rheologic effects, leading to plasma channels and oxygen consumption rates that are out of balance with oxygen delivery. These features, combined with the dynamic nature of tumor blood flow, lead to an overall pattern of hypoxia that is both chronic and acute (Fig. 5).

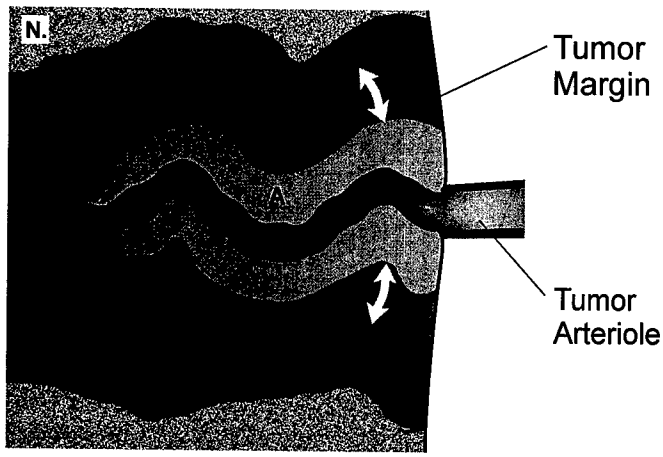


Fig. 5. Composite working model for interrelationships between chronic and acute hypoxia. Arrows indicate diffusion distance of oxygen that is influenced by instabilities in red cell flux. At the extremes, there are regions of vascular hypoxia where such fluctuations are of little consequence, since tissues immediately surrounding the vasculature would be hypoxic (C = chronically hypoxic). Conversely, there are regions near well-oxygenated vessels that are unaffected by changes in red cell flux (A = aerobic). It is likely, however, that large regions of tumor will lie between these extremes, and will be subjected to periodic fluctuations in PO_2 that lead to hypoxia reoxygenation injury. I = intermittently hypoxic; N indicates regions of necrosis.

It is of interest to note that many of the genes involved in regulation of angiogenesis are regulated by hypoxia (Table 4). However, there is very little information on whether such genes are also regulated by hypoxia reoxygenation injury, particularly with the kinetics shown in this chapter. Clearly this is an important area for future investigation.

5. NITRIC OXIDE

There is increasing evidence that tumors produce varying levels of NO *(70)*. A number of studies have been published that have investigated the effects of NO on angiogenesis. Consistently the story is emerging that NO definitely has an effect, but whether it up- or downregulates the process is controversial. It is important to note that the hyperpermeability of vascular endothelium that is stimulated by VEGF occurs via stimulation of NO synthesis *(71)*. Evidence for a proangiogenic effect of NO comes from the following observations: Exposure of glioblastoma and hepatocellular carcinoma cell lines to SNAP and NOR3 (both NO donor compounds) increased VEGF production, primarily by stabilizing mRNA levels *(72)*; use of NO donors leads to increased angiogenesis in the cornea pocket assay, when angiogenesis is stimulated by substance P *(73)*; use of NO donors stimulates proliferation of coronary postcapillary endothelial cells, in vitro *(74)*; and the human breast tumor line, DLD1, which was transfected with the NO synthase gene, grew more quickly and had better-vascularized tumors than the parent line *(75)*. There are also data that indicate that NO downregulates angiogenesis. Examples of such evidence include: VEGF production by arterial smooth muscle cells is downregulated by NO (in this case, the inhibition occurs by inhibition of AP1 binding to the VEGF promoter *[76]*); production of VEGF and its receptors are downregulated when NO is added to the perfusate of ex vivo perfused lungs, and angiogenesis is inhibited in the chick

corioallantoic membrane when exposed to exogenous NO, and is upregulated when NO synthase inhibitors are used *(77,78)*; primary tumor growth and metastasis frequency are lowered in the Lewis lung tumor model when animals are administered NO donor drugs *(79)*; proliferation and migration of endothelial cells is inhibited in vitro in the presence of NO donor drugs *(80,81)*.

The data that are available thus far suggest that NO has both positive and negative effects on angiogenesis. However, two sets of data in tumor models suggest that it plays a positive role in stimulating angiogenesis *(75,79)*. Clearly, the issue is very complicated, and additional work is needed to further elucidate the role that it plays in tumor angiogenesis.

6. SUMMARY

There is no question that there is a complex interrelationship between tumor hypoxia and tumor angiogenesis. A question that remains unanswered is whether the hypoxia in tumors is responsible for abnormal angiogenesis, or whether the abnormal angiogenesis is responsible for hypoxia. In all likelihood, dysfunction in both processes feed on each other to continue the process of tumor growth. At issue, then, is whether there is any benefit to be gained from trying to modulate tumor hypoxia as part of antiangiogenesis therapy. Presumably, if one were to improve tumor oxygenation by some means, there might be a reduction in the angiogenic stimulus, but, under such conditions, additional tumor cell growth might be favored. Alternatively, pharmacologic manipulation designed to increase tumor hypoxia may stimulate more angiogenesis, thus stimulating additional tumor growth. If either of these types of manipulations were combined with antiangiogenesis therapy, it is difficult to predict what the net result would be on tumor growth and metastasis. Alternatively, it is not clear what the physiologic consequences of antiangiogenesis therapy are on tumor oxygenation. Will the use of antiangiogenic agents exacerbate hypoxia, leading to altered cytokine expression, additional intermittent hypoxia, and tumor progression? Clearly additional studies are needed to begin to unravel these complex processes, since the answers may have important implications in how to implement these exciting new forms of therapy.

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