Angiogenesis in Wound Healing

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During wound healing, angiogenic capillary sprouts invade the fibrin/fibronectin-rich wound clot and within a few days organize into a microvascular network throughout the granulation tissue. As collagen accumulates in the granulation tissue to produce scar, the density of blood vessels diminishes. A dynamic interaction occurs among endothelial cells, angiogenic cytokines, such as FGF, VEGF, TGF-β, angiopoietin, and mast cell tryptase, and the extracellular matrix (ECM) environment. Specific endothelial cell ECM receptors are critical for these morphogenetic changes in blood vessels during wound repair. In particular, αvβ3, the integrin receptor for fibrin and fibronectin, appears to be required for wound angiogenesis: αvβ3 is expressed on the tips of angiogenic capillary sprouts invading the wound clot, and functional inhibitors of αvβ3 transiently inhibit granulation tissue formation. Recent investigations have shown that the wound ECM can regulate angiogenesis in part by modulating integrin receptor expression. mRNA levels of αvβ3 in human dermal microvascular endothelial cells either plated on fibronectin or overlaid by fibrin gel were higher than in cells plated on collagen or overlaid by collagen gel. Wound angiogenesis also appears to be regulated by endothelial cell interaction with the specific three-dimensional ECM environment in the wound space. In an *in vitro* model of human sprout angiogenesis, three-dimensional fibrin gel, simulating early wound clot, but not collagen gel, simulating late granulation tissue, supported capillary sprout formation. Understanding the molecular mechanisms that regulate wound angiogenesis, particularly how ECM modulates ECM receptor and angiogenic factor requirements, may provide new approaches for treating chronic wounds. ***Key words: endothelium/extracellular matrix/fibrinogen/integrin. Journal of Investigative Dermatology Symposium Proceedings 5:40–46, 2000***

Thirty-five million cutaneous wounds that require major intervention occur yearly in the U.S.A. alone. Some experts have estimated that the total number of chronic wounds exceeds 2 million and perhaps up to 5 million annually in the U.S.A. alone (1998). The social and financial tolls of chronic wounds are extremely high.

The most common cause of acute wounds is thermal injury, with an estimated 2.5 million burns each year in the U.S.A. (1982). Other significant acute cutaneous wounds are caused by trauma, excision of extensive skin cancer, and medical conditions such as deep fungal and bacterial infections, vasculitis, scleroderma, pemphigus, toxic epidermal necrolysis to name a few. Categories of chronic wounds include arterial ulcers, diabetic ulcers, pressure ulcers, and venous ulcers. It is estimated that the prevalence of leg ulcers alone is between 0.5%–1.5% with an annual cost of nearly $1 billion (Phillips and Dover, 1991).

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Abbreviations: HDMEC, human dermal microvascular endothelial cells; PDGF, platelet-derived growth factor; VEGF, vascular endothelial cell growth factor.

Principal goals in wound management are to achieve rapid wound closure and a functional and aesthetic scar. Over the past two decades extraordinary advances in cellular and molecular biology have greatly expanded our comprehension of the basic biologic processes involved in wound repair and tissue regeneration (Clark, 1996a). Ultimately these strides in basic knowledge will lead to advancements in wound care resulting in accelerated rates of ulcer and normal wound repair. Furthermore, as tumor stroma generation is similar to wound healing (Dvorak, 1986), increased knowledge of wound repair may lead to unexpected advances in tumor therapy. Clearly today's scientific breakthroughs in molecular and cell biology will lead to tomorrow's therapeutic successes in wound care and tissue engineering (Singer and Clark, 1999).

During the early phase of cutaneous wound repair, new stroma, often called granulation tissue, begins to form approximately 4 d after injury. The name derives from the granular appearance of newly forming tissue when it is incised and visually examined. Numerous new capillaries endow the neostroma with its granular appearance. Macrophages, fibroblasts, and blood vessels move into the wound space as a unit (Hunt, 1980), which correlates well with the proposed biologic interdependence of these cells during tissue repair. Macrophages provide a continuing source of cytokines necessary to stimulate fibroplasia and angiogenesis, fibroblasts construct new extracellular matrix necessary to support cell ingrowth, and blood vessels carry oxygen and nutrients necessary to sustain cell metabolism. The quantity and quality of granulation

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tissue depends on the presence of biologic modifiers, the activity level of target cells, and the extracellular matrix environment (Juliano and Haskill, 1993; Clark, 1996b). Biologic modifiers include lipid mediators, metabolic products including those derived from oxygen, as well as proteins and peptides. Peptides with potent mitogenic activities are usually referred to as growth factors. Low levels of some growth factors circulate in the plasma; however, activated platelets release substantial amounts of preformed growth factors into wounded areas. Arrival of peripheral blood monocytes and their subsequent activation to macrophages ensures continual synthesis and release of growth factors. In addition, injured and activated parenchymal cells can synthesize and secrete growth factors. The provisional extracellular matrix also promotes granulation tissue formation. Once fibroblasts and endothelial cells express the proper integrin receptors, they invade the fibrin/fibronectin-rich clot in the wound space.

New blood vessel formation is a critical component of wound healing. In the form of developing capillary sprouts, endothelial cells digest and penetrate the underlying vascular basement membrane, invade the ECM stroma, and form tube-like structures that continue to extend, branch, and create networks, pushed by endothelial cell proliferation from the rear and pulled by chemotaxis from the front. These events require a dynamic temporally and spatially regulated interaction between endothelial cells, angiogenesis factors, and surrounding ECM proteins (Clark, 1996b; Madri *et al*, 1996).

THE SOLUBLE FACTORS OF WOUND ANGIOGENESIS

The soluble factors that can stimulate angiogenesis in wound repair are gradually being elucidated (Roesel and Nanney, 1995); however, the factors that do stimulate wound angiogenesis are less clear. Angiogenic activity can be recovered from activated macrophages as well as the epidermis and soft tissue wounds. Twelve years ago acidic fibroblast growth factor (aFGF) or basic fibroblast growth factor (bFGF) appeared to be responsible for most of these activities (Folkman and Klagsbrun, 1987). In the interim, other molecules have also been shown to have angiogenic activity, including vascular endothelial growth factor (VEGF) (Keck *et al*, 1989), TGF-β (Yang and Moses, 1990), angiogenin (Vallee and Riordan, 1997), angiopoietin (Suri *et al*, 1996), and human mast cell tryptase (Blair *et al*, 1997).

aFGF and bFGF were the first members of the large FGF family to be discovered and are now designated FGF-1 and FGF-2, respectively (Abraham and Klagsbrun, 1996). These two growth factors have potent angiogenic activity by rabbit cornea and chorioallantoic membrane assays (Folkman and Klagsbrun, 1987). Neither FGF-1 nor FGF-2, however, have a transmembrane sequence and therefore cannot be secreted. Nevertheless, at least some forms of cell injury can cause FGF-1 release (Jackson *et al*, 1995). Perhaps these two factors are released from disrupted parenchymal cells at a wound site resulting in the initial stimulus for angiogenesis.

Although TGF-β promotes angiogenesis *in vivo* (Roberts *et al*, 1986; Yang and Moses, 1990), it inhibits the growth and proliferation of endothelial cell monolayers *in vitro* (Baird and Durkin, 1986; Frater-Schroder *et al*, 1986; Heimark *et al*, 1986). This apparent discrepancy between *in vivo* and *in vitro* activities may be attributable, in part, to the capacity of TGF-β *in vivo* to recruit and stimulate macrophages that then produce other active angiogenesis factors (Weisman *et al*, 1988). An alternative, but not preclusive, explanation is that TGF-β is a growth inhibitor for cultured endothelial cell monolayers, but a mitogen for cultured endothelial cells that have formed capillary-like tubes (Iruela-Arispe and Sage, 1993). In fact, the types of TGF-β receptors on endothelial cells are altered when cultured endothelial cells form tubes (Sankar *et al*, 1996). Likewise cultured monolayer endothelial cells make PDGF-BB but have no receptor (PDGFR-β) for this ligand. In contrast, once the cultured cells form tubes, they express PDGFR-β and respond to the ligand that they no longer produce (Battegay *et al*, 1994).

VEGF, a member of the PDGF family of growth factors, has potent angiogenesis, as well as vasopermeability, activity which led to its initial designation as vasopermeability factor (VPF) (Dvorak *et al*, 1995). This factor is produced in large quantities by the epidermis during wound healing (Brown *et al*, 1992). Low oxygen tension, as occurs in tissue hypoxia, is a major inducer of this growth factor (Shweiki *et al*, 1992; Detmar *et al*, 1997) and its receptor (Brogi *et al*, 1996). Thus, cell disruption and hypoxia, hallmarks of tissue injury, appear to be strong initial inducers of potent angiogenesis factors at the wound site. Recent data suggest that bFGF may set the stage for angiogenesis during the first 3 d of wound repair, whereas VEGF may be critical for angiogenesis during granulation tissue formation from day 4 through 7 (Nissen *et al*, 1998). Several additional members of the VEGF family have been found recently (VEGF-B, VEGF-C, and VEGF-D) (Veikkola and Alitalo, 1999). Although their general role in angiogenesis processes is quickly being elucidated, their specific function in wound angiogenesis is not yet clear.

The angiopoietins have recently joined the members of the VEGF family as the only known growth factors largely specific for vascular endothelium. The angiopoietins include a naturally occurring agonist, angiopoietin-1, as well as a naturally occurring antagonist, angiopoietin-2, both of which act by means of the Tie2 receptor. Two new angiopoietins, angiopoietin-3 in mouse and angiopoietin-4 in human, have recently been identified but their function in angiogenesis is unknown (Valenzuela *et al*, 1999). Neither bind the Tie2 receptor.

Recently one of us collaborated in research that demonstrated that mast cell tryptase is an additional angiogenesis factor (Blair *et al*, 1997). The frequent presence of mast cells near capillary sprouting sites suggests an association between mast cells and angiogenesis. Coculture of human mast cells (HMC) with human dermal microvascular endothelial cells (HDMEC) led to a dose-dependent increase in the network area of vascular tube growth. Moreover, the extent of neovascularization was enhanced greatly when HMC were degranulated in the presence of HDMEC. Further examination using antagonists to various mast cell products revealed a diminished response (73%–88% decrease) in the area of vascular tube formation if specific inhibitors of tryptase were present. Tryptase (3 microg per ml) directly added to HDMEC caused a significant augmentation of capillary growth, which was suppressed by specific tryptase inhibitors. Tryptase also directly induced cell proliferation of HDMEC in a dose-dependent fashion (2 pM–2 nM). These results are consistent with the concept that mast cells act at sites of new vessel formation by secreting tryptase, which then functions as a potent and previously unrecognized angiogenesis factor.

ANGIOGENESIS AND THE WOUND ECM

The ECM of a healing wound undergoes rapid changes as the fibrin clot is replaced by fibronectin and hyaluronan and subsequently by types I and III collagen (Clark, 1996b). These transitions from fibrin-rich provisional matrix to a second-order provisional matrix to a collagenous scar are highly orchestrated and tightly regulated both spatially and temporally. As fibroblasts invade the fibrin clot it is lysed and fibronectin and hyaluronan are deposited, forming early granulation tissue. This process initially occurs in the periphery of the clot and later more centrally as the granulation tissue grows into the wound space. At any given time, the ECM at the wound margin differs qualitatively and quantitatively from the ECM situated centrally.

Orchestration and regulation of the rapid new tissue development observed in wound healing indubitably depends not only on the cells and cytokines present but also on the ECM microenvironment. The complex interaction and feedback control of cells/cytokines/matrix has been termed ''dynamic reciprocity'' (Bissell *et al*, 1982). For example, previous studies from our laboratory have

demonstrated that three-dimensional ECM proteins regulate ECM receptor expression of normal human dermal fibroblasts (Xu and Clark, 1996). Thus, ECM proteins control fibroblast expression of ECM receptors, which regulate fibroblast interaction with and alteration of the ECM.

Therefore, besides the growth factors and chemotactic factors, an appropriate ECM is also necessary for wound angiogenesis (Madri *et al*, 1996). Dilated and hypertrophied blood vessels adjacent to the wound transiently (from 3 to 5 d after injury) deposit increased amounts of fibronectin within their vascular walls (Clark *et al*, 1982a, c), whereas fibrin and fibronectin leak from the blood into the perivascular stroma (Clark, unpublished data). At day 4 post-injury capillary sprouts emanate from these "mother" vessels and invade the wound clot (McClain *et al*, 1996). Remarkably, neovascular invasion of the wound fibrin clot precedes fibroblast invasion and lysis of the clot (**Fig 1**) (Clark *et al*, 1996; Singer and Clark, 1999). At day 4 the spatial distance between these two tissue cell invasion zones is approximately 100 μm. Thus, the capillary tips of angiogenic blood vessels are surrounded by plasma-derived fibrin and fibronectin, not wound fibroblast-derived ECM composed of fibronectin and hyaluronan.

As the wound granulation tissue matures during the second week after injury, the neostroma accumulates increasing amounts of types I and III collagen (Welch *et al*, 1990; Clark *et al*, 1995). The density of blood vessels present in the granulation tissue bed diminishes as collagen accumulates (unpublished data). Such delineation of the precise ECM present around wound blood vessels and at the tip of capillary sprouts is necessary for constructing meaningful investigations of the dynamic interactions between endothelial cells and the surrounding ECM milieu during wound angiogenesis.

Using a microcarrier-based angiogenesis assay, Nehls and Herrmann (1996) demonstrated that fibrin structure played an important role in *bovine* pulmonary artery endothelial cell migration and capillary morphogenesis. They showed that the degree of rigidity of fibrin gel strongly influenced tube formation by bovine endothelial cells in response to bFGF or VEGF. They did not, however, compare fibrin with collagen gels. Takel *et al* (1995) reported that addition of fibrin into type I collagen gel significantly increased the length of the tubular structures formed by monolayer bovine capillary endothelial cells cultured on the gel by about 180% compared with type I collagen alone. This assay, however, appears to more closely simulate vasculogenesis as occurs during embryogenesis rather than sprout angiogenesis as occurs in wound healing (Risau, 1997).

We have established an *in vitro* system of human microvascular sprout angiogenesis by modifying the original assay described by Nehls (Nehls and Drenckhahn, 1995). HDMEC are cultured on microcarrier beads and embedded in a three-dimensional extracellular matrix (3-D ECM) (Feng *et al*, 1999b). When the ECM was a fibrin gel and an angiogenesis factor, such as VEGF or bFGF, was added to the culture construct, capillary-like sprouts developed within 24 h and capillary networks developed by 5 d. Such an *in vitro* environment, in fact, recapitulates angiogenesis invading a wound clot. The presence of lumina in these sprouts was confirmed by confocal microscopy. If a collagen gel was used for the 3-D ECM instead of fibrin, VEGF and bFGF induced endothelial cells to invade the matrix as individual cells, without formation of tubes. If fibrin, however, was added to the collagen matrix, capillary-like tubes sprouted from the beads. The fibrin/collagen 3-D ECM *in vitro* environment, in fact, simulates tumor stroma. From these data we conclude that the presence of fibrin in the ECM, as well as VEGF and bFGF, appears necessary to actively promote human sprout angiogenesis.

ECM RECEPTORS DURING WOUND ANGIOGENESIS

Presumably cell surface receptors that recognize fibrin and other provisional matrix molecules are required for periwound blood vessel hypertrophy and wound bed invasive angiogenesis during the early phase of granulation tissue formation. Endothelial cells express

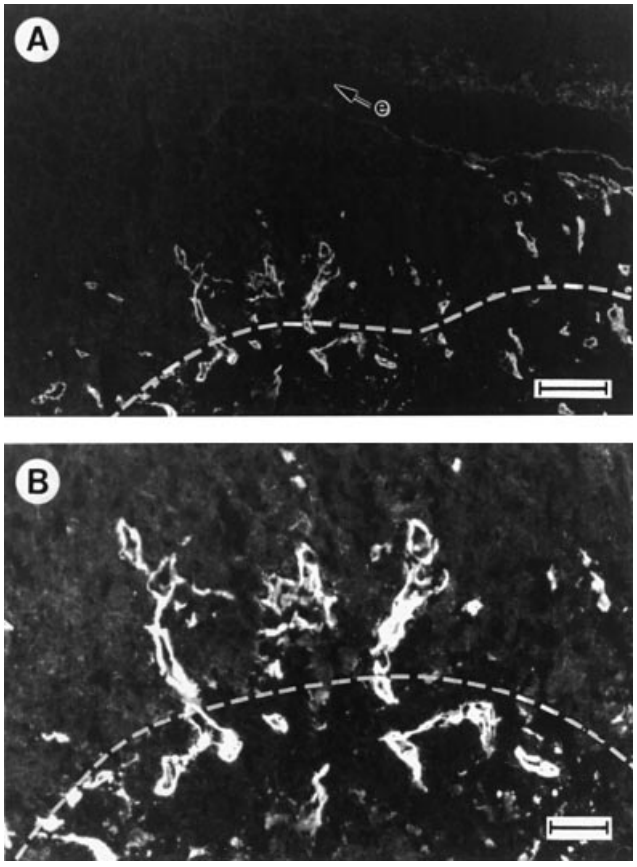

Figure 1. Capillary sprouts invade the wound fibrin clot during early granulation tissue formation. Full-thickness porcine wounds 5 d after extirpation were stained with polyclonal antibodies to laminin-1 by cryosection immunofluorescence technique. (*A*) The epidermis (e) migrates over the fibrin clot (*arrow* indicates the direction of migration) and capillaries defined by laminin invade the fibrin clot. The dotted line delineates the interface of the fibrin clot with the granulation tissue (Clark *et al,* 1982b). Collagen matrix bundles are not present in the granulation tissue at this early time; rather the granulation tissue is cell-rich (Welch *et al*, 1990). (*B*) A high-power view of the same section shown in (*A*). *Scale bar:* (*A*) 100 μm, (*B*) 20 μm. (Modified from figure in Clark *et al*, 1996.)

members of the integrin superfamily of cell surface receptors. These receptors are transmembrane, noncovalently linked heterodimeric glycoproteins consisting of one α chain and one β chain. Of the many integrin receptors that recognize one or more specific ECM molecules (Ruoslahti, 1991), only the αvβ3 receptor is capable of recognizing all the provisional matrix proteins including fibrin, fibronectin, and vitronectin (Cheresh *et al*, 1989; Charo *et al*, 1990). The αvβ3 receptor is heavily expressed on cultured human endothelial cells (Cheresh, 1987) and can mediate their attachment to fibrinogen, fibronectin, vitronectin, and von Willebrand factor (Cheresh *et al*, 1989). This receptor has also been shown to mediate endothelial cell migration *in vitro* (Leavesley *et al*, 1993). In addition, FGF induces increased levels of αvβ3 on cultured human dermal microvascular endothelial cells (Enenstein *et al*, 1992; Swerlick *et al*, 1993).

In support of the concept that αvβ3 may be critical for angiogenesis during wound repair, recent studies have revealed that stimulation of angiogenesis in the chick chorioallantoic membrane depends on the vascular integrin αvβ3 (Brooks *et al*, 1994). In addition, we have used a full-thickness cutaneous wound healing model in Yorkshire pigs to delineate the temporal relationships of αvβ3 integrin receptor expression with wound angiogenesis (Clark *et al*, 1996). At 3 d after injury (1 d prior to neovascular invasion of the fibrin/fibronectin-rich clot in the wound bed), αvβ3 receptor is localized on hypertrophied

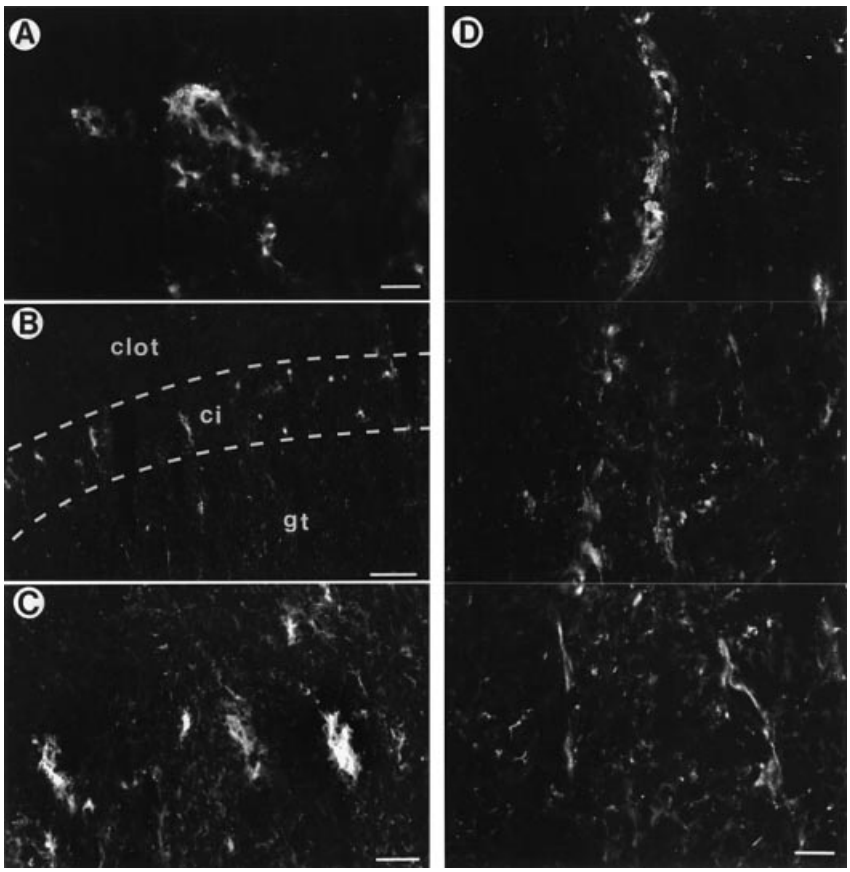

Figure 2. Capillary sprouts invading the wound fibrin clot express αvβ3 integrin. Full-thickness porcine wounds were stained for β3 with 7G2, a monoclonal antibody to the β3 integrin subunit (Gresham *et al*, 1989), by cryosection immunofluorescence technique. (*A*) Blood vessels at the base of a 3 d wound just prior to ingrowth. Some vessels are dilated and show bright uniform staining for β3 integrin subunit. (*B–D*) Four day wounds with capillary sprouts invading the fibrin matrix of the wound clot. (*B*) A low-power view shows that β3 expression is highest in the capillary sprouts invading the fibrin clot. The parallel dotted lines delineate the clot invasion zone (ci). gt, granulation tissue under the clot. (*C*) High power view of the clot invasion zone demonstrates bright β3 expression on capillary tips. (*D*) High power view of the clot invasion zone (top one-third) and underlying granulation tissue (lower two-thirds) demonstrates that β3 staining is most uniform and intense in clot invasion zone. *Scale bars:* (*A*, *C*, *D*) 20 μm, (*B*) 100 μm. (Modified from figure in Clark *et al*, 1996.)

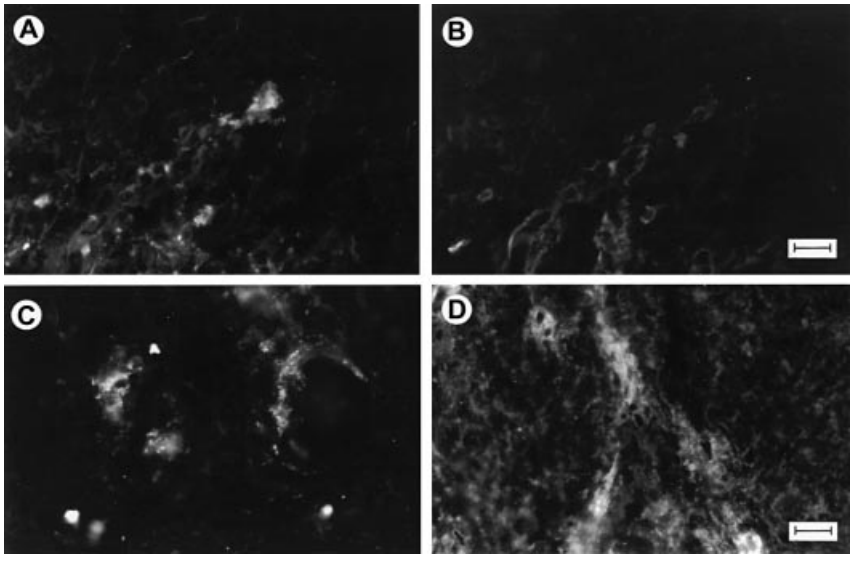

Figure 3. αvβ3 is highly expressed on the tips of capillary sprouts invading the wound clot. Five day full-thickness porcine wounds were stained for αvβ3 integrin (*A*) and laminin-1 (*B*), and for β3 (*C*) and β1 (*D*) integrin subunits by immunofluorescence techniques. (*A*) The tip of a newly forming capillary is brightly stained for αvβ3 whereas the remainder of the vessel is stained only dimly. (*B*) Laminin staining (double-label of the same section in *A*) is prominent in the more mature portions of the new capillary, but weak at the capillary tip. (*C*) Tips of capillaries stain intensely for αvβ3 (*D*) The entire capillary network stains brightly for β1 integrin subunit. Immunofluorescence techniques on cyrosections of wound specimens were used to delineate αvβ3 with 23C6, a monoclonal antibody to the αvβ3 complex (Horton *et al*, 1985), laminin-1 with polyclonal rabbit anti-laminin antibodies, β3 with 7G2, a monoclonal antibody to the β3 subunit (Gresham *et al*, 1989), and β1 with 4B4, a monoclonal antibody (Matsuyama *et al*, 1989) to the β1 integrin subunit. *Scale bar:* 20 μm. Modified from figures in Clark *et al*, 1996.)

microvessels in the periwound stroma (**Fig 2*A***). Previously we had found that the microvasculature in this area is permeable to large molecules and that it stains intensely for fibronectin (Clark *et al*, 1982a). Although the increased vascular permeability suggested that fibronectin within vessel walls might derive from the blood, this is not the case. Our early labeling studies revealed that most of the fibronectin, in fact, is produced *in situ* (Clark *et al*, 1982c). More recently we have shown that fibronectin-coated surfaces can induce endothelial cell expression of αv/β3 mRNA (Feng *et al*, 1999a) (vide infra) and that a 3-D ECM containing fibronectin can induce angiogenesis that is αvβ3 dependent (Feng, Clark, and Tonnesen, unpublished data).

On day 4 of wound repair, capillaries invade the fibrin- and fibronectin-rich provisional matrix in the wound. During this angiogenesis process, αvβ3 is highly expressed on capillary sprouts that are invading the fibrin clot (**Fig 2*B–D***; **Fig 3*A*, *B***). In fact, the expression of αvβ3 is most pronounced at the tips of the capillary sprouts (**Fig 3*A***). As newly forming blood vessels stain for laminin (Jerdan *et al*, 1991), double-label immunofluorescence technique

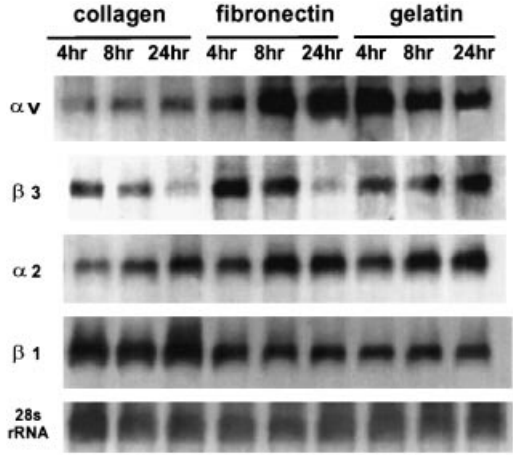

Figure 4. Expression of integrin subunit mRNA by HDMEC cultured on immobilized type I collagen, fibronectin, or gelatin. Total RNA was extracted from HDMEC cultured on immobilized type I collagen, fibronectin, or gelatin for 4 h, 8 h, and 24 h. Total RNA was sequentially probed with human integrin cDNA for αv, β3, α2, and β1. Uniformity of gel loading was monitored by UV light examination of the gel stained with ethidium bromide (data not shown). Uniformity of gel loading and uniformity of RNA transfer to the membrane were demonstrated by hybridization of the same blot with a ^{32}P-labeled probe for 28s ribosomal RNA. (From Feng *et al*, 1999a.)

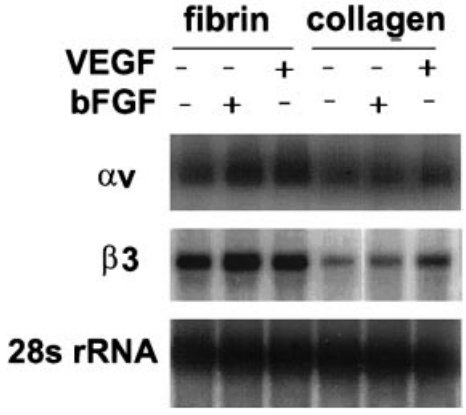

Figure 5. Fibrin gel, compared with collagen gel, enhances HDMEC αv and β3 integrin subunit mRNA levels in the presence or absence of the angiogenic growth factors bFGF or VEGF. HDMEC cultured on immobilized collagen were overlaid by fibrin gel or collagen gel, with or without bFGF (50 ng per ml) or VEGF (100 ng per ml) for 24 h. Total RNA was probed sequentially with human integrin αv and β3 cDNA. Uniformity of gel loading was monitored by ethidium bromide examined under UV light (data not shown). Uniformity of RNA transfer to the membrane was demonstrated by hybridization of the same blot with a ^{32}P-labeled probe for 28s ribosomal RNA. (Modified from figure in Feng *et al*, 1999a.)

for both laminin-1 and αvβ3 was used to confirm the localization of αvβ3 staining to the neovasculature in these wounds (**Fig 3*A*, *B***). The tips of capillary sprouts consistently stained weakly for laminin-1, probably secondary to blood vessel immaturity. Such weak staining for laminin in immature blood vessels has been observed by us before in the developing microvasculature of human fetal skin (Fig 4A in Tonnesen *et al*, 1985). In contrast to the focal expression of αvβ3 at the tips of capillary sprouts (**Fig 3*C***), β1 integrins are expressed along the full length of the wound neovasculature (**Fig 3*D***). αvβ3 appears to have a functional role

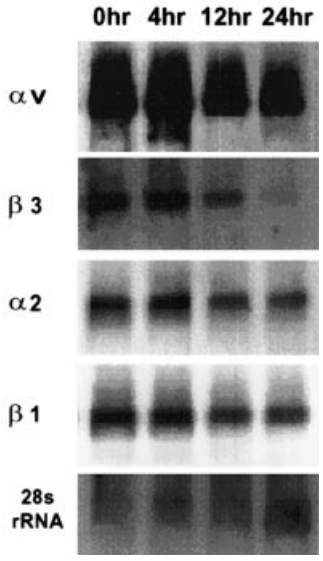

Figure 6. Integrin β3 subunit mRNA is unstable compared with mRNA of subunits αv, α2, and β1. After 24 h of culture on immobilized collagen, HDMEC were treated with 60μM 5,6-dichloro-1b-D-ribofuranosyl-benzimidazole (DRB), a RNA transcription initiation inhibitor. Total RNA was isolated at 0 h, 4 h, 12 h, and 24 h and probed with αv, β3, α2, and β1 cDNA. Uniformity of gel loading was monitored by UV light examination of the gel stained with ethidium bromide (data not shown). Uniformity of gel loading and uniformity of RNA transfer to the membrane were demonstrated by hybridization of the same blot with a ^{32}P-labeled probe for 28s ribosomal RNA. (Modified from figure in Feng *et al*, 1999a.)

in wound angiogenesis because monoclonal antibodies and cyclic peptides specific for αvβ3 transiently inhibit granulation tissue formation and alter αvβ3 distribution on the surface of capillary sprout endothelial cells (Clark *et al*, 1996).

Recently we have accumulated experimental evidence that the spatial restriction of αvβ3 to the fibronectin-rich, hypertrophied blood vessels in the periwound stroma and the tips of capillary sprouts invading the fibrin clot appears to be secondary to ECM control of integrin subunit expression. mRNA levels of αv/β3 were higher in HDMEC plated on the immobilized provisional matrix protein fibronectin compared with levels in HDMEC plated on collagen (**Fig 4**) (Feng *et al*, 1999a). Denatured type I collagen (gelatin) had an even stronger inductive effect than fibronectin **Fig 4**). In fact denatured type I collagen, which expresses 5 arg-gly-asp (RGD) sites that are cryptic in the native collagen molecule, may also be inductive to angiogenesis. At least one of these RGD sites can bind cells through the αvβ3 integrin (Davis, 1992). Interestingly, monoclonal antibodies raised to denatured type IV collagen through subtractive hybridization can inhibit angiogenesis (Brooks, unpublished data presented at this conference). Denatured type IV collagen, like denatured type I collagen, also expresses RGD sites that are cryptic in the native collagen molecule.

To delineate ECM regulation of integrin expression further, HDMEC were overlaid with fibrin or collagen gels. This construct better simulates the *in vivo* environment. αv/β3 mRNA levels at 24 h were higher in HDMEC under a fibrin gel compared with a collagen gel, whether angiogenic factors were present or absent (**Fig 5**). In fact with collagen gel overlay, the effect of bFGF on αv/β3 mRNA levels was negligible and the effect of VEGF only modest. Furthermore, with a fibrin gel overlay neither angiogenic factor had a substantial stimulatory effect on αv/β3 mRNA levels. Interestingly, in monolayer HDMEC cultures, β3 mRNA decayed much faster than αv, α2, and β1 mRNA (**Fig 6**). Whereas fibrin gel overlay enhanced αv/β3 mRNA stability, collagen gel overlay did not (Feng *et al*, 1999a). These data support the contention that provisional matrix molecules, particularly fibrin and fibronectin, in

the wound clot positively regulate wound angiogenesis through their modulation of αvβ3 integrin receptor expression.

SUMMARY AND SPECULATIONS ON WOUND ANGIOGENESIS

Given the information outlined above, a series of events leading to wound angiogenesis can be hypothesized. Substantial injury causes tissue-cell destruction and hypoxia. Potent angiogenesis factors such as FGF-1 and FGF-2 are released secondary to cell disruption, whereas VEGF is induced by hypoxia. Proteolytic enzymes released into the connective tissue degrade ECM proteins. Specific fragments from collagen, fibronectin, and elastin, as well as many phylogistic agents, recruit peripheral blood monocytes to the injured site where these cells become activated macrophages that release more angiogenesis factors. Certain angiogenesis factors, such as FGF-2, stimulate endothelial cells to release plasminogen activator and procollagenase (Magnatti *et al*, 1989). Plasminogen activator converts plasminogen to plasmin and procollagenase to active collagenase and in concert these two proteases digest basement membrane constituents.

The fragmentation of the basement membrane allows capillary sprouts to form and migrate into the injured site in response to FGF, VEGF, and other angiogenesis factors. In fact chemoattraction and mitogenesis are two major attributes of angiogenesis factors. To migrate through a fibronectin infiltrated basement membrane zone into the fibrin/fibronectin-rich wound clot, endothelial capillary sprouts express αvβ3 integrin. The highly regulated temporal and spatial expression of αvβ3 on the tips of capillary sprouts appears to be controlled by the fibrin/fibronectin-rich provisional ECM of the wound.

The newly forming blood vessels first deposit a provisional matrix containing fibronectin and proteoglycans but ultimately form mature vascular basement membrane. TGF-β may induce endothelial cells to produce the fibronectin and proteoglycan provisional matrix as well as assume the correct phenotype for capillary tube formation. FGF, and other mitogens such as VEGF, stimulate endothelial cell proliferation, resulting in a continual supply of endothelial cells for capillary extension. Capillary sprouts eventually branch and join to form capillary arcades through which blood flow begins. New sprouts then extend from these loops to form capillary networks, apparently under the influence of VEGF, FGF, mast cell tryptase, and other factors. As the provisional matrix clears from the wound and is replaced by collagen-rich scar tissue, most of the new blood vessels degenerate through apoptosis (unpublished data). Those mature blood vessels that remain no longer express αvβ3.

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