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Extracellular matrix molecular characterization in gingival fibroblasts from diabetics

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Extracellular Matrix Molecule Characterization in Gingival Fibroblasts from Diabetics

by

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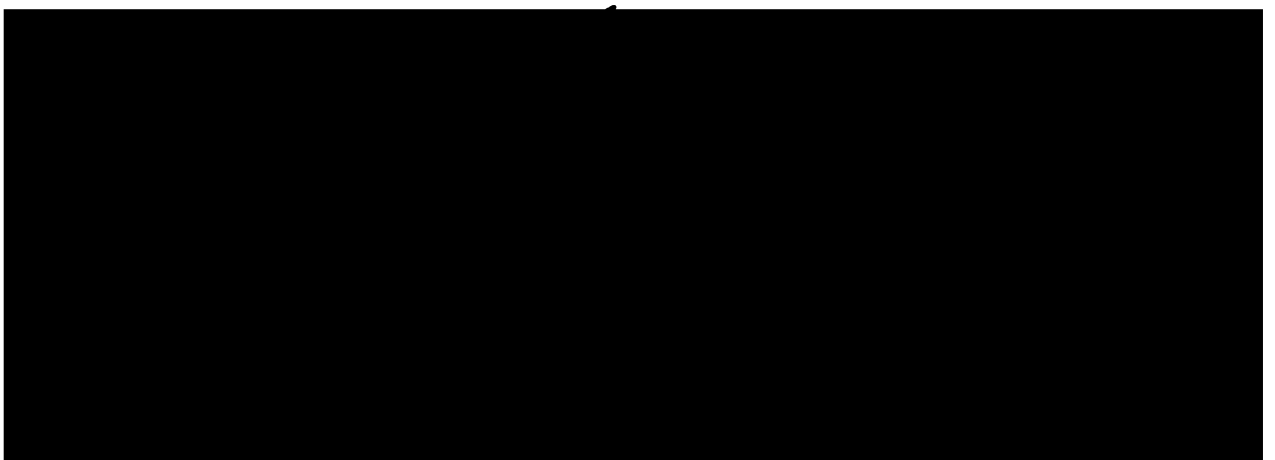

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INTRODUCTION

It is estimated that 12 to 14 million individuals in the United States have diabetes, with only half of the affected individuals diagnosed.[1] The classic signs and symptoms of diabetes mellitus include the triad of polyuria, polydypsia, and polyphagia. These signs and symptoms are the direct result of hyperglycemia. Systemic complications occur in association with prolonged hyperglycemia.[2] Uncontrolled or poorly-controlled diabetes is associated with increased susceptibility to oral infections, including periodontitis.[3] Periodontal disease may be more frequent and severe in diabetic individuals with more advanced systemic complications.[4] Evidence supports the theoretical relationship between periodontitis and diabetes especially in patients with poorly controlled diabetes mellitus or hyperglycemia.[5]

Several factors potentially contribute to the pathogenesis of periodontal disease in diabetic patients. Studies of polymorphonuclear leukocyte (PMNs) function suggest that dysfunction of PMNs may lead to impaired host resistance to infection in diabetic patients compared to non-diabetic controls.[6,7] Collagen synthesis appears to be affected by glucose levels. Gingival fibroblasts from diabetic patients synthesize less collagen compared to non-diabetic subjects.[8] It is probable that cumulative effects of altered cellular activities may contribute to impaired wound healing in diabetic patients. Decreased collagen synthesis by fibroblasts and increased collagenase production was found in diabetic patients which may also be responsible for defective wound healing.[9] The late

inflammatory response to wound healing is altered in diabetes.[10] The mechanisms responsible for compromised wound healing in diabetes are unknown.

A major problem in the diabetic population is delayed wound closure most likely due to a defect in cell matrix interactions. Therefore it was decided to evaluate the expression of extracellular matrix molecules and their corresponding integrin molecules in both diabetic and normal fibroblasts.

Cell migration is a required component of the complex process of wound healing. During this process cells must synthesize and migrate on specific constituents of the extracellular matrix. Cell surface receptor expression can modulate both the organization of and motility on the three-dimensional matrix. The integrin family of cell-adhesion receptors plays a significant role in this process.[11] Integrins are a large family composed of an α subunit noncovalently associated with a β subunit to form a fully functional receptor.[12] It has recently become evident that integrins can transduce a variety of signals to the inside of the cell using a cascade of signaling molecules.[13]

Wound healing in the context of extracellular matrix is a complex repair process influenced by extracellular matrix molecules such as ligands and their corresponding integrin molecules. The wound repair process begins as the blood clot first fills the wound site as a provisional matrix for cell migration from adjacent tissue and consists mainly of fibrin and plasma fibronectin secreted by fibroblasts. Cellular fibronectins are then secreted into cutaneous wounds by macrophages and fibroblasts during early granulation tissue formation. Anti-adhesive proteins such as tenascin, thrombospondin and secreted protein acidic

and rich in cysteine or SPARC, originally termed osteonectin, promote cell rounding and partial detachment and appear at a later time.[14] Cytokines are released initially during platelet degranulation, produced by macrophages and keratinocytes.

The attachment of cells to their surroundings is important in determining cell shape and in cell function and tissue integrity. The following families of adhesion molecules have been elucidated: 1) integrins, 2) selectins 3) cadherins, and 4) the immunoglobulin superfamily.[15] The interactions between the various molecules are mediated through a variety of structural domains dictating their different functions.

Hynes (1987) described a variety of molecules found on cell surfaces which linked the extracellular matrix to the cytoskeleton.[16] Integrins are cell surface receptors for extracellular matrix proteins. Integrins are found in several cells and are responsible for different functions. In fibroblasts (myofibroblasts) integrins are involved in wound contraction, re-epithelialization in keratinocytes, and epithelial cell integrins mediate angiogenesis. [17] Today, integrins include a large group of related heterodimeric glycoproteins composed of structurally unrelated α and β subunits found to span mammalian cell membranes. Eight β chains and eighteen α chains have been identified.

Diabetes and Wound Healing

It is the general belief that diabetic patients have impaired wound healing due to increased production of extracellular matrix proteins such as fibronectin,

laminin, and type IV collagen.[18] Production of these is triggered by hyperglycemia in vivo or high glucose concentration in vitro. Excess extracellular matrix proteins are considered to be key factors in thickening of basement membranes and modulation of cellular functions which may cause micro-angiopathy, a characteristic diabetic complication in tissues.[19] Periodontal disease is often more severe in diabetics than in patients who are systemically healthy. Long-duration diabetics have been found to have significantly more probing depths 6mm when compared to non-diabetics, and overall greater severity of periodontal disease prevalence.[20] Clinical studies have reported an increased tooth loss, more frequent attachment loss, and alveolar bone loss, as well as greater incidence of probing depths >5mm, and extensive gingival inflammation in poorly controlled diabetics.[21, 22]

Duration of diabetes appears to be related to the development of periodontitis as it is to retinopathy, neuropathy, nephropathy, and other complications.[21,22] Long-term diabetes alters the gingival composition in insulin-dependent diabetics as demonstrated by Seppälä et al. (1997), in which cellular, vascular, and connective tissue changes were indicative of increased catabolism rather than anabolism. Collagen synthesis is reduced in diabetic patients when compared to controls.[23]

Nishimura et al. (1998) found periodontal ligament cells cultured in hyperglycemic conditions were impaired in adhesion and motility due to a possible impairment of cell-matrix interactions through advanced glycation end products or AGE response.[24] This process results in changes in cell-matrix

interactions induced by high glucose and diabetes that could cause compromised cell renewal and basement membrane thickening.[19] Prolonged hyperglycemia causes persistent inflammatory monocyte/ macrophages to release proinflammatory cytokines rather than differentiating into reparative or proliferative macrophages. These macrophages release growth factors necessary for wound repair such as platelet-derived growth factor, transforming growth factor-β, and basic fibroblast growth factor, all of which lead to delayed wound healing. These events may lead to decreased proliferation of resident cells and these conditions may cause altered cell-matrix ratio in the tissues.[25]

Uncontrolled diabetes adversely affects surgical wound healing and is often associated with abnormal proliferation of fibroblasts, excessive angiogenesis and poor bone regeneration. Human gingival fibroblasts from uncontrolled diabetic patients were found to significantly proliferate in vitro when exposed to high glucose concentrations.[26] Human periodontal ligament cells (PDL) cultured at high glucose concentration exhibit a higher expression of β_1 (VLA-5, fibronectin receptor) than cells cultured in physiologically normal glucose concentration. The levels of glucose concentration are correlated with varied VLA-5 expression.[27]

An increase in fibronectin receptors such as β_1 directly alters cellular functions including proliferation and migration by increased adhesiveness to its ligand or by non-specific binding.[28,29] Liu et al. (1998) found soluble fibronectin promotes migration of oral squamous-cell carcinoma cells, in particular results

suggest that fibronectin probably is the chemotactic factor in peritumor fibroblasts cultured from tissue surrounding oral squamous-cell carcinomas.[30]

Elevated glucose concentrations have a direct effect on many aspects of cell function, studies show that exposure to elevated levels of glucose altered synthesis of fibronectin (FN), laminin (LN), and type IV collagen.[31] Subsequently it was determined that the elevated glucose events are associated with an increase in cellular events such as an increase in protein kinase C, which can alter cellular functions.[32]

Spirin et al. (1999) found abnormal accumulation of extracellular matrix components in human retinas with diabetic retinopathy, in particular the proliferative nature of diabetic retinopathy was possibly associated with increased expression of vascular endothelial growth factor, placenta growth factor and transforming growth factor- β_1 which may trigger deposition of tenascin-C in blood vessel walls. Tenascin-C may promote neovascularization in diabetic retinas.[33]

Melcher (1976) described periodontal wound healing as a complex process requiring the deposition of at least four distinct connective tissues including gingival, periodontal ligament, bone and cementum.[34] Wound healing in periodontal tissues includes proliferation, migration and matrix synthesis. A crucial cellular event is cell recruitment which will determine healing by repair (new periodontal tissue restoration) or by regeneration (histologically determined regeneration of tooth's supporting structures).[17] The basic processes of healing in the oral cavity are similar to other systems, the difference being that periodontal

regeneration requires coordination and integration of each specific component of the periodontium in order to be successful.

Epithelial Integrins

Several cell types express integrins such as epithelial cells, fibroblasts, osteocytes, endothelial cells, leukocytes, lymphocytes and platelets.[35] Integrin expression in epithelium has been researched in great detail both in vivo and in vitro. Epithelial cells in culture have been shown to express eight integrin subunits and in combination form five integrins including $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_4$ and $\alpha_v\beta_5$[36] as well as $\alpha_v\beta_6$.[13] Immuno-histochemical studies have shown 5 integrin subunits are generally expressed by all epithelial cells in health, inflammation or in healing. However dispute exists over the expression of α_5 and α_v subunits in situ.[37] Pytela et al. (1985) have found the α_5 subunit complexed with the β_1 subunit to form the RGD-dependent receptor for fibronectin to be expressed by gingival connective tissue cells; in human skin this is only present during fetal development.[38] The α_v subunit has been found in healthy epidermis, gingival epithelium and junctional epithelium. Wounding skin has been associated with an upregulation of the α_v subunit and de novo expression of the fibronectin receptor $\alpha_5\beta_1$ during the stage of active migration (days 1-3 following wounding).[39] Integrins may appear in the healing wound at different times, as such the α_v subunit has been observed in migrating keratinocytes in wounded mucosa which were not present in healthy mucosa.[40] The associations of β subunits and α_v were expressed in oral cancer.[37] Ramos et al. (2001) found the

$\alpha_v\beta_6$ and $\alpha_5\beta_1$ fibronectin receptors appeared to mediate migration of squamous cell carcinoma in the presence of fibronectin (FN). The β_6 subunit modulated post-ligand binding events such as growth and cell motility rather than initial interactions with its ligand FN.[13]

A potential pathway to cell dysfunction has been demonstrated by Roth et al. (1993), as overexpression of integrins correlated with increased cell attachment to exogenous FN and LN as well as a complex matrix.[19] The adhesion molecules including integrins, and the interactions between the various molecules are mediated through a variety of structural domains dictating their different functions. The integrin/ligand interactions allow transmembrane signaling between the interstitial stroma and the cells to further express functions such as apoptosis, migration, and proliferation. Over 100 combinations of the integrin subunits are possible, but only a few combinations mediate certain functions, these will be discussed.

α_v Integrin Subfamily

The αv integrin subfamily mediates cell adhesion when combined with the β_1, β_3, and β_5 as well as β_6 integrins. Most epithelial derived tumor cells adhere to fibronectin by means of ligand/integrin interactions.[41] The distribution of $\alpha_v\beta_5$ is ubiquitous; it is the receptor for vitronectin, and is expressed on hepatoma cells, fibroblasts, and carcinoma cells.[42] This integrin has been found in epithelial and nonepithelial cell lines and tissues.[43]

$\alpha_v\beta_6$ appears to be epithelial-cell specific and functions as a receptor for fibronectin and tenascin-C (TN-C).[17] The $\alpha_v\beta_6$ integrin was isolated by homology PCR , with a distinct carboxy-terminus unlike that of any other integrins. Using Northern blotting, it was determined that it is only expressed in epithelial cells.[41] Huang et al. (1998) suggested $\alpha_v\beta_6$ integrin is involved in promoting cell migration.[44] Ramos et al. (2001) suggested that β_6 modulates post-ligand binding events such as growth and cell motility.[13]

Integrins may be substrate specific, for example $\alpha_v\beta_3$ integrin was found to mediate cell migration and p-3-kinase/AKT pathway activation. It was concluded that $\alpha_v\beta_3$ exists in multiple functional states that bind only VN or both VN and osteopontin and can activate cell migration and intercellular signaling pathways in a ligand-specific manner.[45]

A function-blocking antibody construct to the $\alpha_v\beta_1$ receptor has not been identified, however it is speculated that this may be involved in the post-ligand binding events similar to $\alpha_v\beta_6$ receptor found in oral cancer as well. (Personal communication, D. Ramos).

β_1 Integrin Subfamily

β_1 integrins are generally found in regions of intercellular contacts in epithelial sheets suggesting that they recognize a cell surface ligand.[46] Many integrins are expressed during wound healing in high proportions.[47] The role of integrins in matrix deposition and maturation is crucial; the initial step in matrix repair is binding of fibroblasts to fibronectin through the $\alpha_5\beta_1$ integrin. It is

concluded that integrin receptor involvement in active actin cable contraction may be involved in the process of wound contraction.[48]

Ligand Specificity

Extracellular matrix proteins and glycoproteins bind to epithelial integrins and mediate adhesion, migration and are particularly important in wound healing. In healthy gingival epithelium and connective tissue the following integrins and ligands are present: cell membrane integrins- $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_4$, $\alpha_5\beta_1$, and $\alpha_v\beta_5$; basement membrane ligands- collagen type IV($\alpha_2\beta_1$, $\alpha_3\beta_1$), entacin/nidogen ($\alpha_3\beta_1$), and epiligrin/kalinin ($\alpha_3\beta_1$, $\alpha_6\beta_4$), and laminin ($\alpha_6\beta_4$); connective tissue- collagen types I, III, IV, and V, and fibronectin and vitronectin.[49] A wound matrix consists of damaged basal lamina and proteins and glycoproteins of the subepithelial connective tissue. The gingival connective tissue extracellular matrix is a combination of collagenous fibrous tissue and associated glycoproteins as well as proteoglycans.[50] The ligands involved in wound healing include FN, LN, VN, and TN as well as collagen types I, III, IV, and V.[17] These ligands appear at different stages in development as well as during the wound healing process and as such can be divided into pre- and post-ligand binding events.[12]

Fibronectin

Fibronectin is a ubiquitous extracellular matrix protein that has been extensively studied and has been found to promote substrate attachment of cells grown in culture.[51] A large number of fibronectin isoforms exist due to alternative splicing of the fibronectin primary transcript. Two major forms exist: plasma

fibronectin (pFN), a soluble dimeric form found in plasma, and a dimeric or multimeric cross-linked form deposited as fibrils in the extracellular matrix of tissues, known as cellular fibronectin (cFN) that is produced by fibroblasts.[52] Fibronectins contain binding sites for major cell surface components such as collagen, heparan sulfate proteoglycans, fibrinogen and integrins. The multiple binding sites of FN are involved in the organization of the extracellular matrix and adhesive interaction of cells. FN also has specific domains to bind to several matrix components such as heparin and fibrin and as such FN plays a significant role in matrix assembly and stabilization.[53]

Proteolytic fragments of FN are present in wounds and are chemotactic to monocytes. Other roles of FN in wounds include 1) interaction with fibrin in clots and thickening of fibrin fibers, probably by cross-linking through transglutaminase, 2) acts as an opsonin and mediates clearance of fibrin from inflamed sites, 3) contains heparin- binding domains and collagen-binding sites.[17]

FN has been found in the interface of primate epithelium and subepithelial connective tissue[54] and cFN has also been detected in extracted human third molars.[55] These studies indicate that FN is widely distributed in the periodontium and support a role for FN as an extracellular matrix protein of importance in periodontium organization as well as repair during tissue inflammation.

Fibronectin Receptor ($\alpha_5\beta_1$)

The classical FN receptor is a dimer containing α_5 and β_1 subunits. While other integrins bind FN the $\alpha_5\beta_1$ displays specificity for FN alone.[56] The $\alpha_5\beta_1$

integrin binds to an RGD (Arg-Gly-Asp)-containing site in the cell-binding domain of FN. The cell- binding sequence RGD is important for biologic functions, for example the sequences located on the FN type III repeat participate in cell binding and appear necessary for affinity to the $\alpha_5\beta_1$ integrin receptor.[57] The RGD motif is a cell-attachment sequence shown to be critical for cell adhesion through α_v-containing integrins.

Tenascin-C

Tenascin-C (TN-C) is a large oligomeric glycoprotein located primarily in developing embryonic tissues. Three members of the tenascin family have been described: tenascin-C, tenascin-R (TN-R), and tenascin-X (TN-X). TN-C is expressed temporarily during embryogenesis at the sites of tissue interactions in organogenesis and is reexpressed in tumors. Tenascin-C is also expressed in the nervous system. TN-R is specific for the nervous system and is expressed in a more restricted fashion than TN-C. Preliminary evidence shows that mouse TN-X is primarily localized in heart and skeletal muscle as well as in blood vessels. TN-C expression has been documented in a variety of tumors, suggesting that this glycoprotein may play a role in tumor cell-stroma interdependence.[58] However tenascin is also present in tumorigenesis and during wound healing, where it may facilitate the migration of epithelial cells, but is not present in scar tissue.[17]

Tenascin, thrombospondin and SPARC act as soluble ligands and can provoke loss of focal adhesions in well spread endothelial cells [14] Tenascin structure has been determined and found to be similar to extracellular domains of human growth hormone receptor, the second domain of CD4, and PapD and RGD

(Arg-Gly-Asp) sequence functions in cell adhesion.[59] Tenascin and fibronectin have been tested for heparin binding and cell adhesion; results show heparin-binding activity was located on two different tenascin segments. Observations suggest cell attachment to a fibrinogen-like tenascin domain is mediated by cell surface proteoglycans.[60] Cell adhesion activity has been found greatest in the presence of endothelial cell receptors which may play a role in angiogenesis, blood vessel architecture, or in tenascin binding to cell surface in enhancing or eliciting a signaling function.[61] In vitro experiments have shown that tenascin production by human gingival fibroblasts can be modulated depending on serum factors and surface topography; results indicated that tenascin-positive cells exhibited greater migration on smooth titanium surfaces.[62]

In wound healing events tenascin has been demonstrated to accumulate in granulation tissue, in the deeper parts of wounds, and under advancing epithelium. Tenascin also appears in advance of re-epithelialization, possibly induced by cells such as macrophages and leukocytes.[63, 64]

Spirin et al. (1999) found abnormal accumulation of extracellular matrix components in human retinas with diabetic retinopathy, in particular the proliferative nature of diabetic retinopathy was possibly associated with increased expression of vascular endothelial growth factor, placenta growth factor and transforming growth factor- β_1 which may trigger deposition of tenascin-C in blood vessel walls. Tenascin-C may promote neovascularization in diabetic retinas.[33] An increase in tenascin-C expression was found in pathologic tissues in

angiogenesis, in particular tenascin-C was found as a marker for angiogenesis in human choroidal tissues.[65]

Vitronectin

Vitronectin (VN) is found in human serum and has been shown to promote attachment and spreading of cells grown in culture.[66] VN has been found associated with elastic fibers of loose connective tissues including myelofibrotic bone marrow and sclerotic glomeruli and unlike FN has not been detected in basement membranes.[67] These findings suggest a role for VN in the inflammatory and repair process. Specific bacterial binding sites for VN have been demonstrated for streptococci and staphylococci, thus this ligand may serve as a site for bacterial adherence and colonization on epithelial cells.[68]

VN has been found in several regions of primate gingivae including connective tissue fibers of the marginal gingival, the periodontal ligament, endosteum, and periosteum. The finding of VN in the periodontal ligament (PDL) may indicate that VN may be an important factor in repair processes and maintain integrity and function of the PDL.[54] Watt et al. (1993) found that VN did not occur in natural ECM of epithelial cells.[69]

Vitronectin Receptors ($\alpha_v\beta_3$ and $\alpha_v\beta_5$)

The $\alpha_v\beta_3$ integrin is the major receptor for VN. This integrin also binds other ligands such as osteopontin and fibrinogen through their RGD sequences. The localization of this receptor was investigated and found expressed by fibroblasts of the PDL.[54]

VN binds $\alpha_v\beta_5$ integrin or receptor during wound healing. The $\alpha_v\beta_5$ integrin is the receptor for VN which is highly important in the wound healing process and any alteration may hinder this process.[36]

Aims of study

It is generally believed that patients with diabetes are subject to an impaired wound healing ability. This may be partially substantiated by the increased production of extracellular matrix proteins such as FN which are triggered by hyperglycemia in vivo or by a high concentration of glucose in vitro.[18] These events lead to thickening of basement membrane and derangement of cellular function leading to microangiopathy, a characteristic feature in diabetic tissue complications. The interstitial stroma also has altered matrix expression in wound healing. The cell-matrix interactions are mediated by specific cell surface receptors or integrins for corresponding matrix molecules in a ligand specific manner. An excess production of matrix proteins will directly alter cellular functions via integrin molecules.

The aims of the study are: 1.) to compare the expression of extracellular matrix molecules and the corresponding integrin molecules in diabetic and normal gingival fibroblasts and gingival tissues, and 2.) to compare two subfamilies of integrins, the α_v and β_1 and corresponding ligand interactions important in cell motility in normal and diabetic gingival fibroblasts.These comparisons may further characterize fibroblasts from diabetic patients and elucidate the complexity of oral wound healing in diabetes.

Materials and Methods

I. Gingival Fibroblast Cell Cultures

Normal human gingival fibroblasts (NHF) were isolated from tissue removed during periodontal surgery from non-diabetics at the University of California, San Francisco. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS).[58] Diabetic human gingival fibroblasts (DHF) were similarly obtained at the time of periodontal surgery from type II well-controlled diabetic patients and cultivated. Fibroblasts were grown according to protocol as follows: 5ml of DME with10% FBS (supplemented with 0.5X fungizone, 1X penicillin-streptomycin, 1X sodium pyruvate, and 1X non-essential amino acids) added to 100mm cell culture plate. Serial passaging was subsequently carried out and cells of the 5^{th} to the 9^{th} passage derived for the experiments.[30]

II. Flow Cytometry Analysis Scan (FACS)

The cells were washed once using 1X phosphate buffered solution (PBS), and detached using EDTA, spun at 500-600 rpm for 4-5 minutes. The cells were then washed once with 1X PBS, and blocked using 2% bovine serum albumin (BSA) for 20-30 minutes at room temperature. The cells were again spun at 600 rpm for 5minutes. The primary antibodies were added for 45 minutes at room temperature. The cells were washed once with 1X PBS twice. The second antibody was then added (fluorescein isothiocyanate-labelled goat anti-mouse IgG, FITC) (Jackson Immunoresearch, West Grove, PA) for 30 minutes at room

temperature. The cells were washed 1X using PBS. Then 0.5ml PBS was added. The tubes were then prepared using 1-2 μl propidium iodide into each tube. The FACS scan was then completed using the flow cytometry machine (FACScan at UCSF). NHF and DHF cells were used to perform functional assays including migration and adhesion with and without antibodies to α_v, β_1, β_3, and β_5 integrins.

III. Functional Assays.

Adhesion Assay

The adhesion assay as described by Ramos et al. [30,58] was used in this project. A 96-well plate was coated with TN, FN, and VN (each at 10 μg/ml) at 37°C for 1 hour and then blocked with 0.1% BSA for 20 minutes. Cells were removed from tissue culture plates using 2mM EDTA: 0.05% BSA in PBS for 10 minutes at 37°C, washed and resuspended in serum-free DMEM (containing 200 μg/ml $CaCl_2$, and 200 μg/ml $MgCl_2$) with 0.1% BSA. The cells were seeded at 2 x10^5 cells/well using 50 μl of medium (with or without antibodies) and then incubated at 37° C in a humidified 5% CO_2 incubator for 3hours. Function-blocking assays were done in the presence of anti-integrin antibodies (10 μg/ml) to α_v, β_1, β_3, and β_5. The plate was then placed in the shaker for 45 seconds six times. The medium was then aspirated from the wells, and 50 μml/well of 0.1% BSA was added and the plate shaken twice for 45 seconds. The media was aspirated and the crystal violet stain added for 20 minutes and subsequently

washed using tap water. Then 80 µl 2% sodium dodecyl sulfate (SDS) added per well, and the plate was then read using the microplate reader.

Migration Assay

The Ramos lab protocol for migration assay was used in this project .[30,58] Cell migration on surfaces coated with respective proteins (TN, FN, VN, at 5 µg/ml, see Appendix B) using the Transwell filter system (8-µm pore size) (Fisher). Briefly, the lower surfaces of the filters were coated with the respective proteins overnight at 4° C. Cells were removed with 2mM EDTA from tissue culture dishes as described above. The wells are washed briefly using 0.1% BSA in DME, and function blocking antibodies to the following integrins were added (at10 µg/ml) to the transwells: α_v, β_1, β_3, and β_5. The transwells were then placed in a 37° C humidified incubator at 5% CO_2 for 2 hours. The cells were then fixed using 2% paraformaldehyde for 5 minutes. The cells were stained for 20 minutes using crystal violet. The wells were then washed using PBS several times and 1-2 times with distilled H_2O. The tops of the wells were then dried carefully using cotton swabs. The cells were counted at 40X in triplicate under the light microscope.

IV. Matrix Organization

To detect extracellular matrix production, DHF and NHF cells (2 x 10^4 cells/ml) were seeded on uncoated glass coverslips for 24 hours at 37 C and then fixed and permeabilized with 2% paraformaldehyde, 0.1% Triton X-100 (Sigma,

St. Louis, MO) for 10 minutes. The coverslips were rinsed 1X with PBS, then incubated with the primary antibody (Appendix B), (1:100) Mab (monoclonal antibody) to tenascin-C (TN-C) or polyclonal antibodies to FN for 1 hour at room temperature, followed by a PBS wash. Coverslips were then incubated with FITC-labelled goat-anti-rabbit second antibody (for FN, at1:100) and FITC-labelled goat-anti-mouse second antibody (for TN-C, at 1:100) (Amersham) for 30 minutes at room temperature, washed with PBS and mounted with Vectashield (Vector).[30]

V. Immunohistochemical Analysis

Gingival Tissue Sections

Specimens from normal oral mucosa and diabetic human gingival tissue were obtained from a tissue bank maintained in the Department of Stomatology, University of Californi, San Francisco. All tissue specimens were directly embedded in OCT (optimal cutting temperature compound; Miles, Elkhart, IN) embedding medium and snap-frozen, or first immersed in 10% sucrose at 4 C for 4 hours and then embedded and frozen. 5 μm frozen sections were cut in ultracryomicrotome (Reichert-Jung 1800) and stored at -70° C until ready for staining.

Immunohistochemistry Staining

Frozen tissue sections were retrieved from the –70° C freezer, were air-dried for 30 minutes, then dipped in cold acetone for 10 minutes and dried again.

Sections were rinsed in PBS and endogenous peroxidase activity was blocked with 0.1% hydrogen peroxide. After rinsing again with PBS, sections were incubated in 10% normal goat serum for 30 minutes at room temperature. The slides were incubated with primary antibodies to FN and TN-C for 60 minutes at room temperature in a humid chamber for 1 hour. After washing with PBS, sections were incubated with polymeric HRP-linked goat anti-rabbit secondary antibody (PowerVision TM Two-Step Histostaining Kit, ImmunoVision Technologies, Daly City, CA.). The slides were then incubated using biotin-conjugated second antibody for 45 minutes at 1:100 concentration at room temperature. Tissue sections were washed and incubated with diaminobenzidine (DAB) (PowerVision TM Two-Step Histostaining Kit, ImmunoVision Technologies, Daly City, CA.) for 5-10 minutes. Slides were counterstained with hematoxylin and then mounted in DAKO® Faramount (DAKO, Carpinteria, CA). The slides were then air dried and mounted using polymer and viewed using the light microscope.

RESULTS

Integrin expression in normal and diabetic gingival fibroblasts

Flow cytometry was used to evaluate expression of specific integrins in normal gingival fibroblasts (NHF) and in diabetic gingival fibroblasts (DHF). Using α_v-specific antibodies (L230) it was found that NHF expressed higher levels of α_v when compared with DHF (figure 1A). When further evaluated using anti-$\alpha_v\beta_5$ antibodies (P1F6), an increase in expression of $\alpha_v\beta_5$ in NHF compared

with DHF was found (figure 1B). No difference was seen in the expression of β_1 (P5D2) integrins (figure 1C). These results suggest that shifts in β_5 (P1F6) integrin may be important in the behavior of NHF cells.

Anti-$\alpha_v\beta_5$ suppresses NHF, not DHF adhesion to FN and VN

NHF and DHF cells were seeded onto FN or VN substrates for 1 hr to evaluate initial adhesion. Cyclohexamide ($5x10^{-5}$ M) was used in the media to rule out the effects of protein synthesis on adhesion.[70] On both substrates NHF adhesion was between 30 and 60% greater when compared with DHF (figure 2A and 2B). This increased adhesion could be inhibited with anti-$\alpha_v\beta_5$ function-blocking antibodies (P1F6). Incubation with P1F6 had no effect on DHF attachment to either substrate. These results suggest that $\alpha_v\beta_5$ is important for initial adhesion of NHF to both FN and VN substrates. When adhesion was evaluated in the presence of anti-β_1 antibodies (P5D2), attachment to FN and VN was completely suppressed in DHF not NHF (figure 2A and 2B); further indicating that it plays an important role in attachment of NHF to FN and VN.

Anti-$\alpha_v\beta_5$ suppresses NHF, not DHF migration on FN and VN

To investigate the role that $\alpha_v\beta_5$ plays on motility on both FN and VN, migration assays were performed in the presence and absence of anti-$\alpha_v\beta_5$ function-blocking antibodies (P1F6). 2×10^4 cells/well were applied to the top of 24-well transwell chambers (containing 8 μm pore size polycarbonate membranes), on which the undersurface had previously been coated with 10 μg/ml of either FN or VN. Cells were allowed to migrate for 2hrs. The upper

surfaces were wiped clean, filters were fixed and stained. The number of cells migrating under the surface was determined (see Materials and Methods). It was found that incubation with P1F6 completely suppressed NHF, but not DHF migration on FN and VN (figure 3A and 3B). These results suggest that $\alpha_v\beta_5$ is important for post-ligand binding events such as cell motitlity in NHF cells. Although anti-β_1 partially suppressed motility of DHF and NHF on both substrates, DHF were more profoundly affected on FN by anti-β_1 (figure 3A) than were NHF suggesting a greater role for β_1 in migration of DHF on FN compared with NHF. These results suggest a differential role for both β_5 and β_1 in migration of DHF and NHF on FN and VN.

Differential organization of FN matrix in DHF and NHF

2×10^4 cells were plated on glass coverslips under serum-free conditions and allowed to grow for 24 hrs at 37°C. Coverslips were then prepared for immunofluorescence microscopy as described in the Materials and Methods. Cultures were stained with anti-FN and anti-TN-C antibodies. It was found that cultures of NHF cells organized a dense, robust FN matrix with delicate, long fibrils (figure 4A). In contrast, DHF cells organized a less-dense and truncated FN matrix suggesting that matrix organization had been interrupted (figure 4B). These results suggest an inherent difference in the ability of DHF and NHF to organize a 3-dimensional FN matrix. Neither NHF nor DHF were able to organize TN-C into a matrix. This suggests that TN-C organization may be dependent upon additional factors supplied by keratinocytes in vivo. This immunoflourescence

stain for FN showing matrix organization at the cellular level, demonstrates the delicate dynamic of matrix deposition, any subtle change in this could produce dramatic alterations in wound healing.

Differential deposition of FN in diabetic tissue and normal tissue

Tissue sections were prepared for immunohistochemical analysis for the presence of TN-C and FN as described in the Materials and Methods section. The results of the immunostaining reveal similar deposition in both tissue types when treated with antibodies to TN-C; the antibody reacted more strongly at the basement membrane-interstitial stroma interface of the vasculature in diabetic tissue, with a similar distribution found in normal tissue (figure 5A and 5C). In contrast, the deposition of FN in diabetic tissue (figure 5B) appears greatly different than that organized by the normal tissue (figure 5D). The localization of FN in normal mucosa was restricted to the epithelial invaginations with increased localization to the basement membrane. In contrast, expression of FN in diabetic tissue was expressed throughout the stroma with an apparent loss of spatial regulation. These results indicate that there is a dramatic difference in the organization and distribution of FN, a major ECM component, between normal and diabetic gingiva. Although only an observation, this could represent one aspect of altered wound repair seen in the diabetic population.

DISCUSSION

Extracellular matrix proteins and glycoproteins bind to epithelial integrins and mediate adhesion and migration and are particularly important in wound

healing.The expression of extracellular matrix molecules FN, VN and TN-C and the corresponding integrin receptors in both diabetic and normal fibroblasts and gingival tissues were evaluated to better understand wound healing in diabetic tissues. In particular the focus was on the integrins α_v, β_1, β_3, and β_5 and corresponding ligand interactions important in cell motility in normal and diabetic stromal cells and tissue. The mechanisms responsible for compromised wound healing in diabetes are unknown. However it is known that fibroblasts from the oral connective tissues are the preferred cells to repopulate the wound after traditional periodontal treatment although epithelial cells intervene to prevent reformation of normal periodontal attachment. Identifying the ECM receptors and their ligands present in diabetic gingival tissue may allow a greater understanding of molecular complexity of healing in diabetic gingiva as compared to normal gingiva as is relevant to periodontal wound healing.

The α_v integrin subfamily mediates cell adhesion when combined with the β_1, β_3, β_5, and β_6 integrins. β_1 integrins are generally found in regions of intercellular contacts in epithelial sheets suggesting that they recognize a cell surface ligand.[46] Many integrins are expressed during wound healing in high proportions.[47] The role of integrins in matrix deposition and maturation is crucial; the initial step in matrix repair is binding of fibroblasts to fibronectin through the $\alpha_5\beta_1$ integrin. Integrin receptor involvement is involved in the process of wound contraction.[48]

To compare expression of integrins in the two cell lines flow cytometry was utilized. Using α_v-specific antibodies (L230) it was found that NHF

expressed higher levels when compared with DHF. Additionally, anti-$\alpha_v\beta_5$ antibodies (P1F6) were used and a higher expression in NHF compared with DHF was found. Shifts in β_5 integrin may be important in the behavior of NHF cells. $\alpha_v\beta_5$ is one VN receptor important in wound healing in non-differentiating keratinocytes[36] and this altered expression could be related to a difference in wound healing between NHF and DHF cells. Data from this study suggest that initial adhesion of both NHF and DHF cells is mediated by $\alpha_v\beta_5$ receptor interactions with VN or FN or in combination with other integrin receptors. There was no detectable difference between NHF and DHF cells with respect to β_1 and β_3 receptors.

Wound closure is mediated by a combination of matrix remodeling, cell migration and eventual scar formation. Therefore, functional motility assays were performed to determine putative differences in cell lines in presence or absence of function blocking anti-integrin antibodies. Function-blocking antibodies to $\alpha_v\beta_5$ (P1F6) blocked NHF adhesion between 30 and 60% on FN and VN, respectively. In contrast DHF was not affected by P1F6. These results support the work of others in that β_5 mediates initial adhesion to VN and FN by NHF along with normal keratinocytes.[36,54]

FN has been found at the interface of primate epithelium and subepithelial connective tissue and the cFN has also been described in extracted human third molars.[55] FN is also widely distributed in subepithelial connective tissue found generously throughout human oral mucosa.[58] These studies indicate that FN is widely distributed in the periodontium and support a role of FN as an extracellular

matrix protein of importance in periodontium organization as well as repair during tissue inflammation.[39]

Evaluation of adhesion in the presence of anti-β_1 antibodies (P5D2), and attachment to FN and VN resulted in complete suppression in DHF cell adhesion but not NHF. DHF adhesion was totally mediated by the β_1 receptor compared to other integrins evaluated. This indicates that β_1 plays an important role in adhesion of DHF to FN and VN. This corresponds to integrin/ligand interaction between β_1 and FN.

Migration assays were performed in the presence and absence of anti-$\alpha_v\beta_5$ function-blocking antibodies (P1F6). It was found that incubation with $\alpha_v\beta_5$ (P1F6) completely suppressed NHF migration on FN and VN and did not affect DHF cells. These results suggest that it is important for post-ligand binding events such as cell motility in NHF cells. Although anti-β_1 partially suppressed motility of DHF and NHF on both substrates, DHF were more profoundly affected on FN by anti-β_1 than were NHF suggesting a greater role for β_1 integrin in migration of DHF on FN compared with NHF. These results suggest a differential role for both β_1 and β_5 in migration of DHF and NHF on both FN and VN substrates.

Results from the functional assay indicate an altered response by $\alpha_v\beta_5$ and β_1 receptors in DHF cells when compared to normal. This may be due to a difference in expression of the receptors and the interactions with their respective ligands in this cell line. Human gingival fibroblasts and periodontal ligament fibroblasts have been found to express α_v, β_1, β_3, and β_5 integrin subunits in similar amounts.[71] α_v is important for motility of both cell migration on both VN

and FN. Both cell lines are significantly suppressed on both substrates by antibodies to β_3 or β_5. In addition β_1 antibodies also suppressed migration. The results suggest both α_v, and β_1 subfamilies modulate cell migration on FN and VN. It is possible that other integrins may be present, but inactive, and may alter cell adhesion due to hyperglycemic effects which may alter gingival cell phenotype. This would require further investigation at the molecular level (PCR, Northern blots, Southern Blots, In Situ hybridization).

As part of the newly forming wound there is a dynamic deposition, degradation and motility on extracellular matrices. Therefore, the possible differences in the ability of the two cell types to organize a matrix was examined by immunoflourescence microscopy. The results suggest an inherent difference in the ability of DHF and NHF to organize a 3-dimensional FN matrix. Neither NHF nor DHF were able to organize TN-C into a matrix. One explanation may be due to a lack of keratinocytes present in the matrix organization experiment on the coverslip to supply growth factors responsible for organization of the matrix. In vivo gingival keratinocytes supply growth factors necessary for organization of TN-C matrix. The FN matrix appeared robust in the NHF cell line, and the DHF matrix appeared weak and lacked robustness which may be due to phenotypic differences in the gingival cell lines.

Other authors have demonstrated deposition of FN and TN deposition in periodontal tissues.[54] Immunohistochemical analysis of diabetic and normal diabetic tissues revealed greater FN deposition in diabetic tissue with respect to spatial organization. The normal tissue appeared to have a more regulated

deposition along the epithelial-stromal invaginations (rete pegs) and not at all present in the connective tissue stroma. This may indicate a less regulated expression of FN in diabetic tissue, which may be due to an alteration of expression in the FN receptor β_1 or β_5.

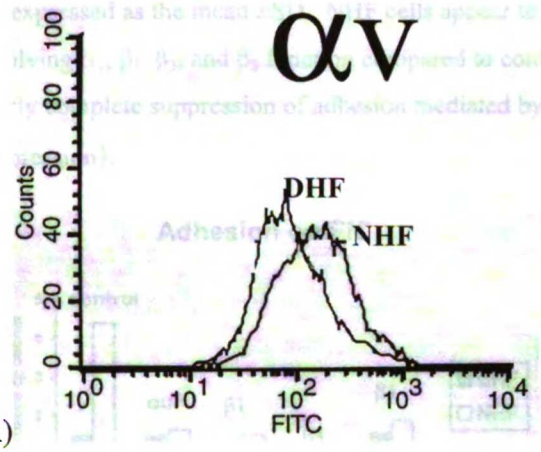
Conclusions

Elucidating integrin expression in vivo is an exceedingly complex process. Characterization of the integrin expression in vivo would allow directions of therapy as are occuring in cancer treatments, using integrins to block angiogenesis in tumors. In periodontal wound healing the use of integrin enhancing or blocking may allow directed regeneration. By looking at diseased tissue which may be present in uncontrolled or poorly controlled diabetic patients it may be possible to better understand wound healing processes. By characterizing the differences present in DHF cells compared to NHF cells it may be possible to better understand the altered expression between these two cell lines. An understanding at the molecular level would allow further insight into prevention of early granulation tissue into periodontal sites or possibly enhanced motility by other cell types into the periodontal wound site.

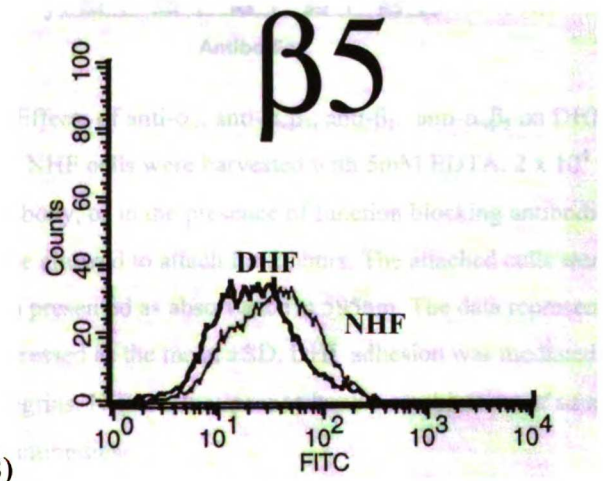
The finding that DHF cells had altered expression of α_v, β_1, β_3, and β_5 may be the beginning of characterization of this cell line with respect to presence of other receptors and the alterations in function as is found in other tissues of diabetic patients such as eye, kidney and heart tissues. Further directions might

include in vivo characterization of these integrins and their ligand interactions in healing wounds.

Figure 1. Flow cytometry was used to characterize integrin expression in normal and diabetic gingival fibroblasts. Using α_v-specific antibodies (L230), NHF expressed higher levels of α_v when compared with DHF (A). Using anti-$\alpha v\beta 5$ antibodies (P1F6) the expression of $\alpha_v\beta_5$ was higher in NHF compared with DHF (B). No difference was seen in the expression of β_1 integrins (C).



A)



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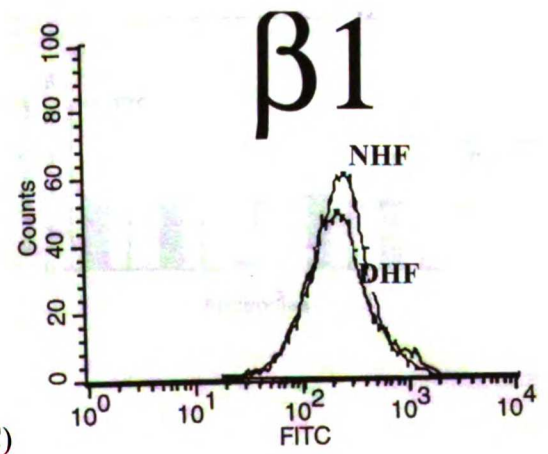
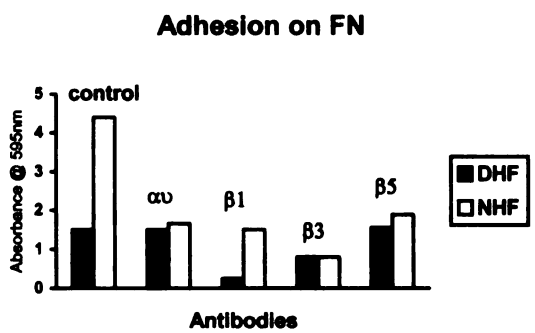


C)

Figure 2:

A) Effects of anti-α_v, anti-$\alpha_v\beta_3$, anti-β_1, anti-$\alpha_v\beta_5$ on DHF and NHF cell adhesion to FN. DHF and NHF cells were harvested with 5mM EDTA. 2 x 10^4 cells/well were plated in the absence of antibody, or in the presence of function blocking antibodies (10ug/ml), α_v, $\alpha_v\beta_5$, β_1, and $\alpha_v\beta_5$. Cells were allowed to attach for 2 hours. The attached cells were fixed, stained and counted. Adhesion was presented as absorbance at 595nm. The data represent triplicate determinations and are expressed as the mean ±SD. NHF cells appear to have a combination of suppressed adhesion involving α_v, β_1, β_3, and β_5 function compared to control and to DHF cells. The DHF cells had nearly complete suppression of adhesion mediated by the β_1 blocking antibodies (>60% suppression).



B) Effects of anti-α_v, anti-$\alpha_v\beta_3$, anti-β_1, anti-$\alpha_v\beta_5$ on DHF and NHF cell adhesion to VN. DHF and NHF cells were harvested with 5mM EDTA. 2 x 10^4 cells/well were plated in the absence of antibody, or in the presence of function blocking antibodies (10ug/ml) α_v ,$\alpha_v\beta_5$, β_1, and $\alpha_v\beta_5$. Cells were allowed to attach for 2 hours. The attached cells were fixed, stained and counted. Adhesion was presented as absorbance at 595nm. The data represent triplicate determinations and are expressed as the mean ±SD. DHF adhesion was mediated by the β_1 receptor compared to the other integrins. NHF cells appear to have a combination of suppression of adhesion by the α_v, β_1, β_3, and β_5 antibodies.

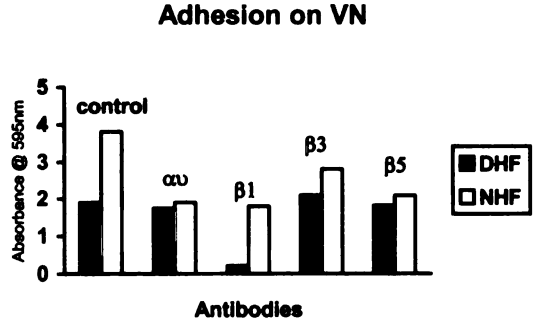
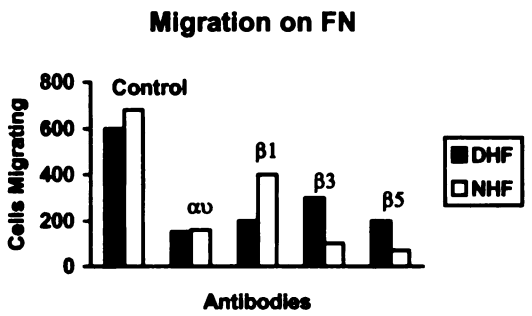


Figure 3:

A) Effects of anti-integrin antibodies on DHF and NHF cell migration across semiporous membranes and FN. 8 x 10^4 cells/well were applied to the top of 24-well transwell chambers containing 8 um pore size polycarbonate membranes, in the presence of function blocking antibodies to α_v, β_1, $\alpha_v\beta_3$, and $\alpha_v\beta_5$. Both cell lines are significantly suppressed on FN substrates by β_1, β_3, and β_5 antibodies. The results suggest both α_v and β_1 subfamilies modulate cell migration on FN.



B) Effects of anti-integrin antibodies on DHF and NHF cell migration across semiporous membranes and VN. 8 x 10^4 cells/well were applied to the top of 24-well transwell chambers containing 8 um pore size polycarbonate membranes, in the presence of function blocking antibodies to α_v, β_1, $\alpha_v\beta_3$, and $\alpha_v\beta_5$. α_v is important for motility of both cell migration on both VN and FN. Both cell lines are significantly suppressed on both substrates by β_1, β_3, and β_5 antibodies. The results suggest both α_v, and β_1 subfamilies modulate cell migration on FN and VN.

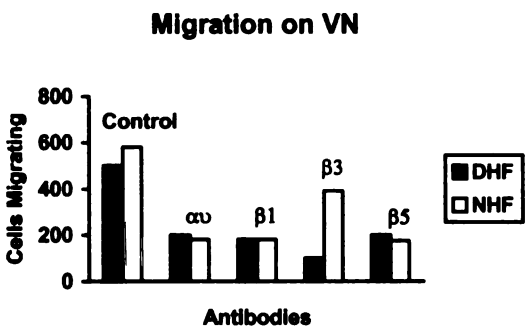


Figure 4. Matrix organization:To detect extracellular matrix production, DHF and NHF cells (2×10^4 cells/ml) were seeded on uncoated glass coverslips for 24 hours at 37 C and then fixed and permeabilized with 2% paraformaldehyde, 0.1% Triton X-100 (Sigma, St. Louis, MO) for 10 minutes. The coverslips were rinsed with PBS 1X, then incubated with the primary antibody (1:100) Mab to TN-C (BC-7) or polyclonal antibodies to FN for 1 hour at room temperature, followed by a PBS wash. Coverslips were then incubated with FITC-labelled goat-anti-rabbit second antibody (for FN at 1:100) and goat-anti-mouse second antibody (for TN-C at 1:100) (Amersham) for 30 minutes at room temperature, washed with PBS and mounted with Vectashield (Vector). (A) Demonstrates FN matrix organization by NHF, with robust and thick fibrils formed. (B) Represents DHF FN matrix formation which is less robust and thinner, shorter fibrils formed.

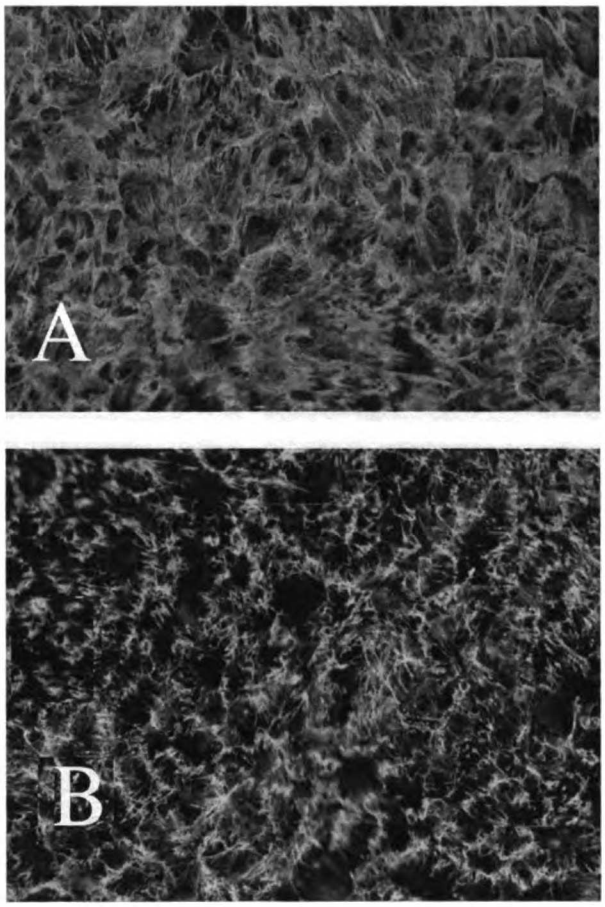


Figure 5. Immunohistochemical analysis. The immunostaining reveal similar deposition in both tissue types when treated with antibodies to TN-C (A and C), (A) a concentration of expression at the basement membrane-interstitial stroma interface of the vasculature in diabetic tissue, with a similar distribution found in normal tissue (C). The anti-FN deposition in normal tissue (D) appears largely different than that organized by the diabetic tissue (B). Expression of FN in diabetic tissue was expressed throughout the stroma with an apparent loss of spatial regulation (B). In contrast, the localization of FN in normal mucosa was restricted to the rete pegs with increased localization to the basement membrane (D).

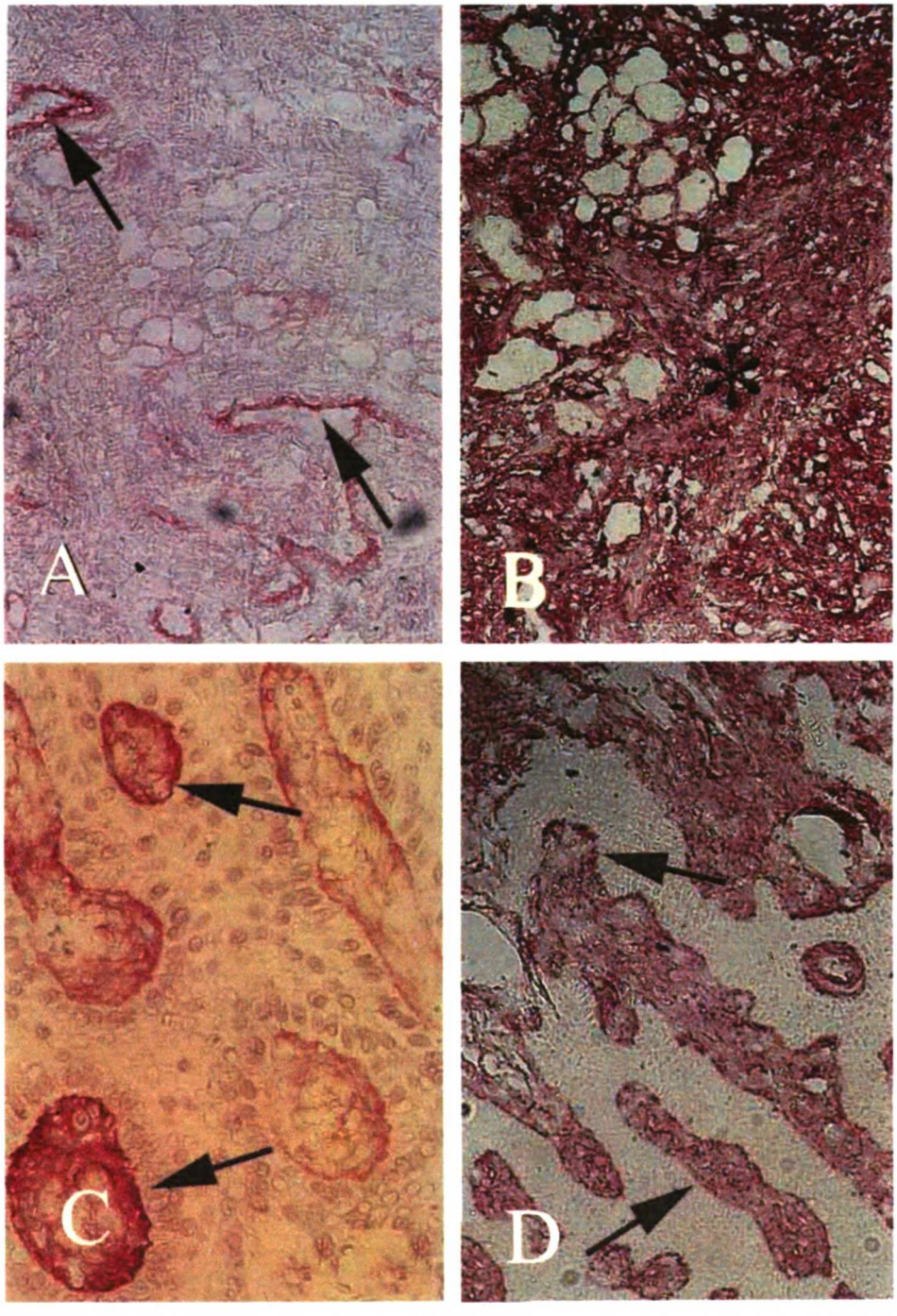


Table 1: Interactions between α and β subunits of integrins[17,49]

	β_1	β_3	β_5	β_6
α_1	X			
α_2	X			
α_3	X			
α_4	X			
α_5	X			
α_6	X			
α_7	X			
α_8	X			
α_v		X	X	X

Table 2: Integrin and Ligand Interactions[17,49]

Integrin	Ligand
β_1 integrins	
$\alpha_1\beta_1$	Collagen, Laminin
$\alpha_2\beta_1$	Collagen, Laminin
$\alpha_3\beta_1$	Collagen, Laminin, Entactin, Fibronectin
$\alpha_4\beta_1$	Fibronectin
$\alpha_5\beta_1$	Fibronectin
$\alpha_6\beta_1$	Laminin
$\alpha_7\beta_1$	Laminin
$\alpha_v\beta_1$	Fibronectin, Vitronectin
β_3 integrins: $\alpha_v\beta_3$	Bone sialoprotein, fibrinogen, fibronectin, thrombospondin, vitronectin, von Willebrand's factor
β_5 integrins: $\alpha_v\beta_5$	Fibronectin
β_6 integrins: $\alpha_v\beta_6$	Fibronectin

APPENDIX A: Media

FBS: 1 10% Fetal Bovine Serum 0.5Xfungizone, 1X Penicillin-Streptomycin, 1X Sodium Pyruvate, and 1X non-essential amino acids

DME: 10% FBS with 1X Penicillin-Streptomycin, 0.5X Fungizone, and 1X non-essential amino acids.

BSA: 0.1%Bovine Serum Albumin, Sigma

PBS: Phosphate Buffered Solution

SDS: 2% SDS= 1% paraformaldehyde, 0.5% Crystal Violet and 20% methanol in PBS

APPENDIX B: Antibodies Used

Primary Antibodies	Source	Second Antibody
α_v (L230)	Nishimura	Goat anti mouse
β_1 (P5D2)	Carter, Wayner	Goat anti mouse
$\alpha_v\beta_3$ (Lm 609)	Cheresh	Goat anti mouse
β_5 (P1F6)	Carter, Wayner	Goat anti mouse
Ligands		
Tenascin (BC-7)	Zardi	Goat anti-mouse
Fibronectin	Sigma	Goat anti-rabbit
Vitronectin	Chemicon	Goat anti-rabbit

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