Basic Fibroblast Growth Factor in the Early Human Burn Wound

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The role of endogenous growth factors in normal wound healing is not clear. Most of the data on growth factors in healing wounds have been obtained from the application of recombinant exogenous growth factors to animal and human wounds. We describe the immunolocalization of basic fibroblast growth factor (bFGF) in the injured dermis of skin from patients with partial and full-thickness burns. Three antibodies demonstrate an extracellular staining pattern of bFGF corresponding to areas of tissue injury that was most intense in specimens collected between 4 and 11 days post-burn injury. In contrast, bFGF staining appeared markedly decreased by Postburn Day 17 and was more consistent with uninjured tissue in a 30-day-old burn that had virtually reepithelialized. Basic FGF staining in the nonburned skin from the same patients was restricted to the dermal capillary basement membranes and the sweat glands, which is consistent with other reports of immunoreactive bFGF localization in normal adult skin. The immunohistochemical results were confirmed with Western immunoblots of the same tissue. The major band at 16.5 kDa, which is within the recognized range of the bFGF molecule’s several forms, was detected in both burned and unburned tissue from the same patient. These findings support the hypothesis that bFGF is a presynthesized mediator that is stored in either the cells or extracellular matrix, is released locally from sites of direct injury, and may be important in early wound healing.

INTRODUCTION

The identification of growth factors has established new possibilities for interventions in wound healing [1–18]. The ability to augment impaired wound healing with exogenous growth factors is a potentially important option for the clinician. Most of the data on growth factors in healing wounds have been obtained from application of recombinant exogenous growth factors to animal and human wounds [5, 19–30]. Such studies help to identify potential functions of the individual growth factors in vivo, but they do not define the presence, time sequence, or activity of a particular endogenous growth factor in the normal healing wound. Although application of an exogenous growth factor might augment impaired healing, it does not determine whether there is an inherent deficit of that protein in the wound. Documentation of a normal temporal sequence is essential for the optimal application of appropriate regulatory proteins to wounds with insufficient endogenous growth factors. Because the healing of a burn wound is a dynamic process involving cell–cell and cell–mesenchymal interactions, it is an interesting model for wound repair.

In vitro and in vivo studies have identified basic fibroblast growth factor (bFGF) as a potent mitogen, chemotaxant, and angiogenic agent. It is one growth factor that affects all cell types involved in wound healing [31, 32]. Also, there is in vivo evidence that application of exogenous bFGF accelerates wound healing [5, 19–30].

Basic FGF is probably stored in a bound state in the extracellular matrix (ECM) and the cellular cytoplasm in normal tissue [31, 33–35] and may not be functionally available until tissue injury [15, 16, 35, 36] or enzymatic cleavage from heparan sulfate has occurred [3]. Immunohistochemical evaluation of normal skin has localized immunoreactive basic FGF in a restrictive pattern to the basement membranes of the dermal epidermal junction, capillaries, and dermal appendages [37, 38].

The possibility that tissue injury may result in growth factor release and mediation of early repair prompted us to investigate the burn wound for the presence of bFGF. We present evidence based on immunohistochemistry and Western immunoelectrophoresis that immunoreactive bFGF is diffusely present in the extracellular matrix of the early human burn wound and that with epithelialization and wound matrix remodeling, staining returns to the previously identified restrictive pattern.

METHODS

Human tissue. Samples of excised burn wounds were collected from eight patients undergoing excision and grafting for partial and full-thickness burn wounds at the University of Washington Burn Center (Table 1). Specimens of excess normal skin harvested as skin...
TABLE 1
Tissue Data

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Postburn day</th>
<th>Depth of injury</th>
<th>Burn etiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>4</td>
<td>Partial thickness</td>
<td>Scald</td>
</tr>
<tr>
<td>2*</td>
<td>5</td>
<td>Partial thickness</td>
<td>Grease</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>Full thickness</td>
<td>Flame</td>
</tr>
<tr>
<td>4*</td>
<td>6</td>
<td>Full thickness</td>
<td>Grease</td>
</tr>
<tr>
<td>5*</td>
<td>8</td>
<td>Partial thickness</td>
<td>Grease</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>Full thickness</td>
<td>Flame</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>Partial thickness</td>
<td>Scald</td>
</tr>
<tr>
<td>8</td>
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</tr>
<tr>
<td>10</td>
<td>30</td>
<td>Partial thickness</td>
<td>Scald</td>
</tr>
</tbody>
</table>

Note: Burn wound specimens were collected at the time of excision and grafting.
* Excess donor site skin also collected.
* Tissue also used for Western immunoblotting and Northern blotting.
* Specimens 4 and 9 represent two separate burns on Postburn Days 6 and 17 from the same patient.

grafts were collected from two of the patients. The tissue was collected in methyl Carnoy's fixative, paraffin embedded, and sectioned at 5 μm thickness. Burned and unburned skin specimens from two patients were snap-frozen for Western immunoblotting. All care and use of the tissue was in accord with the Human Subjects Review Committee’s Approval Guidelines and patient identifiable data was coded.

Immunohistochemistry. An antibody to cytokeratin, 34βE12 (courtesy of Allen Gown, Seattle, WA) was used at a concentration of 1:2000 to identify epidermal squamous cells [39]. For monocytes/macrophages, the CD-68 antibody (DakoPatts, Denmark) was used at a concentration of 1:400; the CD-45 antibody (DakoPatts) was used at a concentration of 1:20 to identify T lymphocytes. A polyclonal antibody to basic FGF (courtesy of Andrew Baird, Whittier Institute, CA) was used at a concentration of 1:1000 [34]. A monoclonal anti-bFGF type II antibody (U.B.I., NY) was used at a concentration of 1:200 [40]. A monoclonal anti-bFGF antibody, DE6 (DuPont–Merck, DE) was used at a concentration of 1:1000 [41]. All three of these antibodies had been demonstrated to have no cross-reactivity with acidic FGF.

Sections were deparaffinized and rehydrated and endogenous peroxidase activity was blocked with 3% H2O2 for 5 min. Using 1% bovine serum albumin as diluent, the primary antibody was applied for 60 min. Biotinylated anti-mouse IgG (Vector Laboratories, CA) was used at a concentration of 1:200 for 30 min followed by avidin–biotin peroxidase amplification (Vector Laboratories, CA) for 30 min according to the manufacturer’s instructions. Incubation with 0.1% 3',3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and nickel chloride at 37°C for 10 min produced a black reaction product. Nuclear counterstain with 1% methyl green was followed by section dehydration. Control specimens without primary antibody and with rabbit serum for the polyclonal antibody were included in each immunohistochemical run.

Competition assay. As an additional control, each of the antibodies to bFGF was incubated for 1 hr with an excess of human recombinant bFGF (U.B.I.) and applied to the tissue slides prior to the usual immunoperoxidase method.

Western immunoblotting. Equal-weight specimens of burned and normal skin from the same patient were homogenized (TissueMizer; Tekmar Industries, OH) in sample buffer (50 mM Tris–HCl, 1% sodium dodecyl sulfate, 10% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol) and were heat reduced. Electrophoresis was performed using 12% SDS–polyacrylamide gels [42] followed by transfer to a nitrocellulose membrane (BA-85; Schleicher & Schuell, Inc., Keene, NH). The membrane was blocked for 2 hr with either horse serum (for the monoclonal antibody) or goat serum (for the polyclonal) followed by incubation with the primary antibody, either polyclonal or monoclonal anti-bFGF, for 2 hr. The biotinylated secondary antibody was applied for 1 hr, followed by avidin–biotin peroxidase amplification (Vector Laboratories, CA) for 1 hr, followed by 4-chloro-1-naphthol (Sigma Chemical Co.) as the chromagen.

RESULTS

The two samples of normal skin show intact epidermis and normal glandular structures as evidenced by staining with the cytokeratin antibody 34βE12. Immunostaining with cell type-specific antibodies identifies no monocytes/macrophages or lymphocytes in the uninjured tissue and increased numbers of inflammatory cells in the injured dermis. The antibodies to bFGF demonstrate staining in the dermal capillary basement membranes and in the sweat glands without other extracellular staining (Fig. 1).

The eight burn wounds excised between 4 and 11 days postinjury demonstrates diffuse extracellular staining for immunoreactive bFGF (Figs. 2a and 3a). These positive-staining areas correspond on serial sections to regions lacking viable epidermis, as evidenced by absence of cytokeratin antibody 34βE12 staining (Fig. 3b). In the adjacent regions of viable epidermis staining positive with 34βE12, there is no extracellular staining for bFGF. This relationship persists for all specimens examined. Evaluation with cell-specific antibodies reveals dense infiltration of monocytes/macrophages and leukocytes in the areas of tissue injury (results not shown).

One patient underwent excision and grafting of one wound on Postburn Day 6 and another on Postburn Day 17. The intensity of the bFGF staining in the 17-day specimen is diminished compared to that in the 6-day-old burn (Figs. 2a and 2b). A 30-day-old burn specimen
FIG. 1. Indirect immunoperoxidase staining of normal noninjured skin with monoclonal anti-bFGF type II antibody (U.B.I., NY) demonstrates a staining pattern restrictive to the basement membranes. (a) bFGF distribution at 93× magnification. (b) bFGF localization in a capillary basement membrane at 372× magnification.
FIG. 2. (a) Postburn Day 6 (Specimen No. 4). Indirect immunoperoxidase staining with monoclonal anti-bFGF type II antibody (U.B.I.) shows intense extracellular immunoreactive bFGF. (b) Postburn Day 17 (Specimen No. 9). The 17-day-old burn from the patient in (a) shows less extracellular immunoreactive bFGF than the wound excised on PBD 6.
FIG. 3. (a) Postburn Day 9 (Specimen No. 6). The monoclonal anti-bFGF type II antibody (U.B.I.) intensely localizes bFGF in the extracellular matrix. (b) Indirect immunoperoxidase staining of the same specimen in (a) with the monoclonal antibody to cytokeratin, 34βE12, demonstrates the boundary between intact and disrupted epithelium.
from a different patient shows virtually no extracellular staining for bFGF (Fig. 4). While this is a qualitative comparison, it does represent a trend.

Evidence that this was actual bFGF-specific staining and not random detection of nonspecific epitopes in the burned tissue was confirmed in three ways. The controls show no staining for bFGF. The competition assays with human recombinant bFGF eradicate all extracellular staining (results not shown). Western immunoblotting (Fig. 5) confirms that all three anti-bFGF antibodies recognize a 16.5-kDa polypeptide as the major band in preparations of equal weights of normal and burned skin. These two respective bands appear to have the same density.

**DISCUSSION**

*In vitro* and *in vivo* studies have documented basic fibroblast growth factor’s effects on cell morphology, proliferation, and differentiation of the cells normally involved in the healing wound [31, 32]. Synthesis by the various cell types involved in wound healing, including macrophages, fibroblasts, endothelial cells, and smooth muscle cells, has also been well described [43–46].

The bFGF molecule is present in different tissues in more than one form, ranging from 16.5 to 27 kDa [38, 46, 47], while recombinant human bFGF appears as a 17.9-kDa band. The bFGF mRNA lacks a consensus signal sequence [48, 49], suggesting that bFGF is not secreted via the classic exocytotic route, but is manufactured and
is stored in the cytoplasm and in the ECM bound to heparan sulfate [33, 50]. Normal regulation of cell growth in skin and gut epithelium may rely on low-level release of bFGF from the cytoplasm of injured cells [15, 16, 51].

Our results suggest that bFGF in uninjured ECM and cellular cytoplasm is not immunoreactive. The distribution in our specimens of normal skin is consistent with previous descriptions of immunoreactive bFGF confined to the sweat glands and basement membranes of the dermal capillaries, but absent in the remaining ECM [37, 38]. This bound state may protect the bFGF from degradation and may also serve as a ready pool, unable to interact with the cell surface receptors until it is released with tissue injury [15, 16, 35, 52, 53].

Injury, such as a burn, may result in release of bound bFGF from injured cells, basement membrane, or extracellular proteins. This would explain the heavy extracellular staining pattern obtained in the burned areas and the absence of staining in adjacent, viable regions within the same excised specimen. Furthermore, the presence of 16.5-kDa electrophoretic bands of comparable density in the burned and unburned skin suggests that they have similar amounts of bFGF which is not recognized by immunostaining until tissue injury occurs. However, our immunoblots were performed using matching weights of injured and noninjured skin; since the injured dermis may be edematous, the protein contents of the two tissues may be different. This may result in a bFGF band that is smaller in the burned skin than it would be if the specimens were matched for total protein content rather than weight.

The areas of intense bFGF extracellular immunostaining correlate with increased inflammatory cell concentrations. The heavy infiltration of monocyte/macrophages and T-cells in the burned tissue could certainly lead to de novo synthesis of bFGF in the early stages of the response to injury. Preliminary data using in situ hybridization and Northern blot analysis on specimens excised on Postburn Days 5 and 8 have not demonstrated the presence of bFGF mRNA (unpublished data) despite an abundance of immunoreactive bFGF in the extracellular matrix of these tissues. Therefore, there is unlikely to be significant bFGF synthesis in the wound during this period of repair. Because we have not evaluated wounds earlier than Postburn Day 4, we are unable to say whether the bFGF in the extracellular matrix precedes the inflammatory response or whether the early inflammatory cells exhibit the message for bFGF mRNA during the initial 48 hr. Our data do not correlate with another report of immunolocalization of bFGF in punch wounds in mice. Those authors described bFGF localization in the advancing keratinocytes and adjacent hair bulbs, but specifically absent from the extracellular dermis [54]. This may be due to recognition of different epitopes with our respective antibodies. However, the different mechanisms of injury provide a more likely explanation. Punch biopsies result in well-defined precise wounds while thermal injuries create more diffuse mesenchymal destruction.

One explanation for the early presence of immunoreactive bFGF and the subsequent loss of staining is that "available" bFGF is utilized by endothelial cells or fibroblasts involved in angiogenesis and fibroplasia during the early stages of wound healing and is then either enzymatically degraded or reincorporated into the ECM during remodeling. A high concentration of this mediator may be necessary to initiate the cascade of events leading to the initial cellular proliferation, migration, and differentiation. The hypothesis that bFGF in the early wound is necessary for initiation of normal repair is supported by observations that antibodies to bFGF retard the formation of granulation tissue in mechanically induced injury [55].

CONCLUSIONS

Our data suggest that immunoreactive bFGF is present in the extracellular matrix of early human burn wounds with gradual disappearance of bFGF staining in older wounds. These findings support the role of bFGF as a presynthesized mediator released locally from cells in the site of direct injury. The implications of this study include the potential recognition of a specific time during which application of exogenous growth factor to wounds deficient in basic fibroblast growth factor may be beneficial.

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REFERENCES


46. Gospodarowicz, D., Neufeld, G., and Schweigerer, L. Molecular and biological characterization of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. *Cell Differ.* **19**: 1, 1986.


