Changes in growth factor levels in human wound fluid

Victoria M. Dvonch, MD, Roberta J. Murphey, PhD, Junji Matsuoka, MD, PhD, and Gary R. Grotendorst, PhD, Tampa, Fla.

Background. It has been suggested that platelet-derived growth factor (PDGF) plays a central role in wound healing. Analysis of human wound fluid revealed the presence of PDGF AA (30 kd) and monocyte/macrophage-derived growth factor (MDGF) (12 to 14 kd) in the immediate postoperative period.

Methods. The amount of PDGF AA present was assayed by Western blot analysis. The chemotactic and mitogenic potential of purified wound fluid containing PDGF AA and MDGF was determined on a responsive cell line. The biologic activity of MDGF was assayed with a cell line that is unresponsive to the PDGF AA found in wound fluid.

Results. Both the concentration and the biologic activity were highest in the immediate postoperative period and declined to negligible levels by 24 hours after surgery. The chemotactic activity of MDGF was highest in the immediate postoperative period and declined during the first 24 hours in a manner similar to that of the combined PDGF AA and MDGF activity.

Conclusions. These data demonstrate the changing levels of PDGF and MDGF in human wound fluid over time, supporting the cascade model of wound repair. By demonstrating that MDGF acts on cell lines unresponsive to the PDGF AA found in wound fluid, these data suggest that MDGF may also play an important role in wound healing. (Surgery 1992;112:18-23.)

From the Departments of Ophthalmology, Biochemistry, and Molecular Biology, College of Medicine, University of South Florida, Tampa, Fla.

The ability of a tissue to repair itself depends on the mobilization, proliferation, and activation of specific cell populations. Many studies indicate that peptide growth factors are the choreographers of this complex interaction, comparing their mechanisms of action to the cascade model employed in blood clotting. A significant initiator of healing appears to be platelet-derived growth factor (PDGF), a basic protein (30 kd) stored in the α-granules of platelets and released when platelet aggregation occurs as a result of tissue trauma. PDGF exists in nature as a dimer with the subunits being designated as the A and B chains. All three forms of PDGF (AA, BB, and AB) have been identified in platelets; however, only PDGF AA is detectable in human wound fluid.

PDGF acts through at least two classes of receptors. The PDGF α-receptor has been shown to respond to all three isoforms of PDGF: PDGF AA, PDGF AB, and PDGF BB. The β-receptor binds predominantly the BB dimer and, to a lesser degree, the AB dimer but does not bind the AA dimer to any significant degree. The responsiveness of a specific cell line to a specific dimer of PDGF is a function of the cell's receptor population. PDGF BB is a chemoattractant and mitogen for both fibroblasts and smooth muscle cells that possess either or both receptors and has been shown to stimulate the formation of granulation tissue. PDGF AA has similar biologic activities but is limited to cell lines with α-receptors. Cell lines that do not display α-receptor include A10 smooth muscle cells and, more significantly, primary human dermal fibroblasts.

Monocyte/macrophage-derived growth factor (MDGF), a 12 to 17 kd protein that has biologic and immunologic properties similar to those of PDGF,
METHODS AND MATERIAL

Cells. NIH/3T3 cells were obtained in early passage from S. Aaronson (National Institutes of Health, Bethesda, Md.). A10 smooth muscle cells were obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and gentamicin (50 μg/ml) and maintained at 37°C in an atmosphere of 90% air/10% CO2. All mitotic and chemotactic assays were performed with density-inhibited cultured cells just after becoming confluent.

Human wound fluid. An experimental protocol was submitted, and written consent for collection of the wound fluid was obtained from the chief of staff of the Shriners Hospital for Crippled Children (Tampa). The fluid was collected by the Shriners' Hospital staff in strict compliance with the hospital's policies. Human wound fluids were collected 6, 12, 20, 28, 36, and 44 hours after surgery from six adolescent patients who had undergone orthopedic surgery. Wound fluid was collected at time points 1 through 4 in all patients and at all six time points in three patients. The wound fluid was obtained from the reservoir of the suction drainage system placed during surgery. Aside from their orthopedic condition, no other abnormality was noted in these patients. Specifically, there were no postoperative wound complications. Wound fluid was separated from tissue debris by centrifugation (10 minutes at 5000 g) and stored at −70°C.

Growth factors. Recombinant PDGF AA homodimer, BB homodimer, and AB heterodimer were supplied by Creative BioMolecules (Hopkinton, Mass.). The concentration and purity were determined by amino acid sequence analysis.

Antibodies. Goat anti-human PDGF antibody was raised against purified PDGF from human platelets and synthetic peptides of PDGF A- and B-chain components. The goat anti-human PDGF antibody has been shown specifically to neutralize the biologic activity of PDGF and does not cross react with other growth factors including transforming growth factor β, human epidermal growth factor, or acidic fibroblastic growth factor. All three isoforms of PDGF are identified by this antibody.

Anti-A-chain- or anti-B-chain-specific antibodies were prepared with synthetic peptides containing the amino-terminal sequences of the PDGF A chain (amino acids 92 through 119) and the PDGF B chain (amino acids 79 through 107) as immunogens. Immune sera against PDGF A (92 through 119) and B (79 through 107) peptide has been shown previously to be sequence specific as determined by immunoblot, did not cross react with peptides containing the remaining amino acid sequence, and were shown to be chain specific.

Purification of PDGF-related peptide in human wound fluid. Because it is not possible to use wound fluid samples directly or acid-extracted samples in Western blot analysis because of the high concentration of proteins, PDGF-related peptides in human wound fluid were purified by the anti-PDGF immunoaffinity technique. Goat polyclonal anti-PDGF immunoglobulin (Ig) G was conjugated to Affigel-10 (Bio-Rad Laboratories, Richmond, Va.) as indicated by the manufacturer's protocol. PDGF-related peptides were isolated by incubation of 1 ml wound fluid, fresh or thawed from frozen stock, for 24 hours at 4°C with 100 μl Affigel-10 conjugated with anti-PDGF IgG. After incubation with the wound fluid, the matrix was washed five times with 1 ml 0.01 Hepes buffer, pH 7.4. The specifically bound peptides were released by treatment with 1.0N acetic acid for 24 hours. The supernatant was harvested by...
Western blot analysis of PDGF and MDGF in human wound fluid demonstrates declining levels of PDGF (upper arrow) over time, with persistence of low molecular weight species (lower arrow). Western blots on all six patients were essentially the same.

Centrifugation of the matrix and dialyzed against 1.0N acetic acid and stored at -70°C before assay. Identical amounts of wound fluid and affinity matrix were used to isolate the PDGF-related peptides from each sample. Preliminary studies demonstrated that this amount of affinity matrix is in greater than tenfold excess to recover all of the PDGF-related peptides in wound fluid samples.

Western blot analysis. Human wound fluid was electrophoresed under nonreducing and reducing conditions in a 12% polyacrylamide gel in the presence of sodium and dodecylsulfate and electroblotted to nitrocellulose as described previously. Immunoreactive PDGF peptides were detected with anti-human PDGF IgG and alkaline phosphate-conjugated rabbit antigoat IgG (Cappel, West Chester, Pa.).

Mitogenic and chemotactic assays. The mitogenic activity of purified wound fluid samples was determined as follows: NIH/3T3 fibroblasts were cultured in 48-well plates (Costar Corp., Norwood, N.J.) in DMEM with 10% FCS until confluent, and the medium was changed to DMEM with 2.5% FCS 24 hours before use. Samples in DMEM containing no serum were added to each well, and DNA synthesis was measured 17 hours later by tritiated thymidine incorporation into trichloroacetic acid–precipitable material.

The chemotactic assays were performed in modified Boyden chambers as described previously with collagen-coated polycarbonate filters 8 μm diameter pores; Nucleopore Corp., Pleasanton, Calif.). Assays were run for 4 hours in serum-free DMEM containing 2.0 mg/ml bovine serum albumin. The chemotactic response was quantitated by absorbance measurements (600 nm) of the stain extracted from the cells that migrated through the filter.

RESULTS

PDGF AA and MDGF in human wound fluid. Western blot analysis (Fig. 1) of the human wound fluid from each patient studied revealed two major immunoreactive peptides of approximately 30 to 32 kD and 12 to 14 kD. The peptide at 30 kD (upper arrow) comigrated with the PDGF AA standard. The highest concentration of the peptide was in fluid collected during the immediate postoperative period. The levels of the peptide then decreased over time, so by 30 hours after surgery only trace amounts of peptide were present. Analysis of the reduced samples with the polyclonal anti-PDGF A chain antibody revealed the presence of an immunoreactive species at 15 kD comigrating with the reduced PDGF AA standard. A similar Western blot probed with the anti-B-chain antibody failed to identify an immunoreactive band comigrating with the reduced PDGF BB standard (data not shown). A second species of immunoreactive peptides migrated at 12 to 14 kD (lower arrow), which is the known weight of MDGF. This species was present at the earliest time point and persisted through all the time points examined. These findings were essentially the same for the six patients whose wound fluid we examined.
Fig. 2. Measurement of biologic activity of PDGF and MDGF. A, Chemotactic activity in NIH/3T3 cells is highest in earliest sample and declines over time. B, Mitogenic activity in NIH/3T3 cells correlates with mitogenic response. C, Chemotactic activity in A10 cells is highest at earliest time point but is less than activity seen at earliest time point in NIH/3T3 cells and declines over time.
Proper handling and storage of the wound fluid is necessary for an accurate determination of the level of PDGF present. The time interval between collection and processing of the wound fluid markedly affected the amount of PDGF AA detected in the sample. The decrease in PDGF AA caused by degradation was most noticeable when the wound fluid was allowed to stand at room temperature for more than 24 hours. Storage of the fluid for more than 48 hours at 4°C also resulted in a marked decrease in the detectable level of PDGF AA. Repeated freeze-thaw cycles decreased the level of PDGF AA detected in matched samples.

Biologic activity of PDGF AA and MDGF in human wound fluid. Biologic activity of PDGF AA and MDGF in three patients in whom fluid was collected at all six time points was measured by chemotactic and mitogenic activity of the immunoabsorbed fractions in NIH/3T3 fibroblast cells and by chemotactic assay in A10 smooth muscle cells. In the assays employing the NIH/3T3 cells, the response to both PDGF AA and MDGF, the wound fluid collected in the immediate postoperative period demonstrated the highest level of chemotactic activity (Fig. 2, A). This biologic response declined with increasing time from surgery (p = 0.05). The results of the mitogenic assay (DNA synthesis asayed) showed a similar pattern in biologic activity with time (Fig. 2, B). Mitogenic activity was highest in immunopurified samples obtained immediately after surgery, and the activity declined with increasing time after surgery (p < 0.05).

In assays employing the A10 cell line that is responsive only to MDGF, the wound fluid collected immediately after surgery again contained the highest level of chemotactic activity, with the amount of activity declining with increasing time after surgery (p < 0.05) (Fig. 2, C).

DISCUSSION

Platelet aggregation resulting from tissue trauma releases a wide array of factors into the wound environment. Some are associated with the coagulation cascade; others such as PDGF are presumed to play a role in wound healing. Of particular interest to both basic scientists and clinicians are the factors that initiate wound repair. The recruitment of cells into the wound site followed by their proliferation are crucial steps in initiating the healing process. The identification of only PDGF AA with its limited ability to attract cell lines that do not possess the α-receptor, such as primary human dermal fibroblasts, raises interesting questions about its role in wound healing. Although it is possible that the body could synthesize, store, and release a large amount of a useless protein at a wound site, it is not, in our opinion, probable. Rather, we hypothesize that in the unique environment of the wound, PDGF AA interacts by a presently unknown mechanisms with the recruited cell populations active in repair.

Our inability to identify PDGF AB and BB in the wound fluid supports our observation that the significant proportion of PDGF in human platelets is A chain with a small amount of B chain. Based on a ratio of approximately 7:1 of A chain/B chain, only 1 to 3 mg would be expected to be present in the earliest sample. Any binding to cell receptors or matrix interaction would reduce the amount of BB to levels undetectable by Western blot analysis.

The presence of MDGF at the earliest time point suggests that, like PDGF, it is a significant connective tissue growth factor. Matsuoka and Grotendorst demonstrated that, unlike PDGF, the chemotactic activity of MDGF persisted until at least 7 days after surgery. Their Western blot analysis of human wound fluid showed a gradual decrease in the immunoreactive band consistent with MDGF until, at day 7, only trace amounts were present. The persistence of the low molecular weight immunopeptide that includes MDGF despite the decrease in biologic activity can be explained by the presence of inactive isoforms in the wound fluid. An alternative explanation would be the presence of MDGF or PDGF degradation products accumulating in the wound fluid over time. Having cloned the gene and expressed MDGF, we are currently preparing an anti-MDGF antibody that would allow us to study the protein more efficiently.

Although limitations of the use of wound fluid to predict the wound environment are obvious, this sampling method provides meaningful data about how repair occurs and when certain growth factors are active in the healing process. What this technique fails to find cannot be ruled out, but the proteins identified by this method are truly present.

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