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Appearance of heparin-binding EGF-like growth factor in wound fluid as a response to injury

(epidermal growth factor/platelet-derived growth factor/fibroblast growth factor/heparan sulfate/wound repair)

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ABSTRACT **Wound fluid was obtained from porcine partial-thickness excisional wounds and analyzed for heparin-binding growth factors. Two heparin-binding growth factor activities were detected, a relatively minor one that was eluted from a heparin affinity column with 0.65 M NaCl and a major one that was eluted with 1.1 M NaCl. These activities were not present in wound fluid 1 hr after injury but appeared 1 day after injury, were maximal 2–3 days after injury, and were not detectable by 8 days after injury. The heparin-binding growth factor eluted with 0.65 M NaCl was identified as a platelet-derived growth factor (PDGF)-like activity by the use of specific anti-PDGF neutralizing antibodies. The heparin-binding growth factor eluted with 1.1 M NaCl was shown to be structurally related to heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) by several criteria, including binding to heparin affinity columns and elution with 1.1 M NaCl, competition with the binding of ^{125}I-EGF to the EGF receptor, triggering phosphorylation of the EGF receptor, immunodetection on a Western blot, and stimulation of fibroblast and keratinocyte growth. It was concluded that HB-EGF is a major growth factor component of wound fluid and, since it is mitogenic for fibroblasts and keratinocytes, that it might play an important role in wound healing.**

The healing of a wound is characterized by fibroplasia, angiogenesis, and re-epithelialization. Inherent in this process is the migration and proliferation of cells such as fibroblasts, capillary endothelial cells, and epithelial cells. Growth factors produced by platelets, by monocytes/macrophages, and by other cells in the damaged tissue appear to mediate these migratory and proliferative events. These include platelet-derived growth factor (PDGF), a fibroblast and smooth muscle cell mitogen; transforming growth factor α (TGF-α), an epithelial cell mitogen; TGF-β, an angiogenesis factor and chemoattractant for monocytes; and basic fibroblast growth factor (bFGF), an endothelial cell mitogen and angiogenesis factor (1–4).

Recently, we described a member of the epidermal growth factor (EGF) family produced by monocytes and macrophages which, because of its affinity for heparin, was named heparin-binding EGF-like growth factor (HB-EGF) (5, 6). HB-EGF is a potent mitogen for fibroblasts, smooth muscle cells, and keratinocytes but not for endothelial cells. Given that the target cell specificities of HB-EGF and its production by monocytes and macrophages suggest a role in wound repair, we wished to know whether this growth factor could be found in wound fluid as a reflection of its physiological significance in the wound healing process. In addition, since other growth factors implicated in wound repair, such as PDGF and FGF, also bind heparin, we used heparin affinity chromatography to quantitate various heparin-binding growth factors in wound fluid.

Pig skin, because of its relative similarity to human skin, has been considered to be a good model for studying wound repair. We have recently developed a method in which liquid-tight vinyl chambers containing normal saline are placed over partial-thickness excisional wounds made on the back of a pig (7). By use of these chambers, it is possible to collect and sample wound fluid continuously for growth factor activity. Here we report that HB-EGF is the predominant heparin-binding growth factor mitogenic for BALB/c mouse 3T3 fibroblasts that is found in pig wound fluid in the first few days following injury. A PDGF-like mitogen is also found, but in much lesser amounts.

MATERIALS AND METHODS

Materials. Human EGF, PDGF BB, goat anti-human PDGF neutralizing antibody, and ^{125}I-labeled EGF were obtained from Collaborative Research. Phosphorylated EGF receptor was from Upstate Biotechnology (Lake Placid, NY). Heparin-Sepharose was from Pharmacia. Recombinant human TGF-α, rabbit antibodies to recombinant human TGF-α, and rabbit antibodies to human EGF were from Creative Biomolecules (Hopkinton, MA). Normal rabbit serum was from Pierce. Anti-phosphotyrosine antibodies (PY-20) were from ICN. Mouse monoclonal anti-human EGF receptor antibodies (Ab-1) were from Oncogene Sciences, (Union Dale, NY). Heparin (porcine intestinal mucosa, Lot PM 19486) was from Hepar (Franklin, OH). A 75-aa recombinant human HB-EGF corresponding to aa 73–147 of the HB-EGF precursor (6) was produced in an *Escherichia coli* expression system (S. Thompson, S. Pollitt, K. Lau, W. Ashton, and J.A., unpublished work).

Cell Proliferation. BALB/MK mouse epidermal keratinocytes were kindly provided by Stuart Aaronson (National Cancer Institute, Bethesda, MD). The proliferation of BALB/c 3T3 cells (5), bovine capillary endothelial cells (5), and BALB/MK cells (8) was assayed by measuring the incorporation of [^{3}H]thymidine into DNA as previously described. Some of the keratinocyte assays were kindly performed by J. Rubin (National Institutes of Health). Growth factor assays were performed in triplicate and background was subtracted. One unit of growth factor activity is defined as the amount required to stimulate half-maximal DNA synthesis in BALB/c 3T3 cells (5).

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Abbreviations: EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor.

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Wound Fluid Preparation. Wound fluid was collected as described (7). Briefly, medium partial-thickness excisional wounds (15 × 15 × 1.2 mm; 16–20 wounds per pig) were created on the backs of four female Yorkshire pigs. Each wound was covered with a separate liquid-tight vinyl chamber filled with 1.2 ml of normal saline containing penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml). The solution was exchanged every 24 hr, pooled daily, centrifuged, filtered through 0.45-μm filters, and frozen at −20°C. Wound fluid was collected from four different pigs with reproducible results.

Heparin Affinity FPLC. Wound fluid (20–25 ml) was applied to a heparin-TSK 5PW column (7.5 cm × 8 mm) (TosoHaas; distributed by The Nest Group, Southboro, MA) on a Pharmacia FPLC system (Pharmacia LKB) (5). Elution was with a linear gradient of 0.2–2.0 M NaCl in 10 mM Tris·HCl (pH 7.4). Fractions (1 ml) were collected and 10 μl was assayed for stimulation of DNA synthesis in BALB/c 3T3 cells.

EGF Assays. *Radioreceptor assay.* Competitive ^{125}I-EGF binding to A-431 cells was carried out as described (5). *Phosphorylation of the EGF receptor.* Triggering of EGF receptor phosphorylation in A-431 human epidermal carcinoma cells was analyzed by Western blotting with anti-phosphotyrosine antibodies (9). In some experiments EGF receptor was immunoprecipitated prior to Western blot analysis (10).

SDS/PAGE. Samples were concentrated on mini-heparin Sepharose columns (100 μl of heparin Sepharose beads in phosphate-buffered saline), eluted with SDS/PAGE loading buffer, and analyzed under reducing conditions by SDS/15% PAGE (5, 6). Prestained molecular weight markers (Bio-Rad) were used.

Preparation of ^{125}I-HB-EGF. Native HB-EGF was radiolabeled with Na^{125}I by using Iodo-Beads (Pierce) as described (6). The specific activity was 55,560 cpm/ng.

Western Blotting and Autoradiography. Transfer to nitrocellulose paper (Schleicher & Schuell) of HB-EGF and wound fluid material was done as follows. Polyacrylamide gels were washed for 5 min in water and for 5 min in transfer buffer containing 200 mM Caps (Sigma) (pH 11.0) and 20% methanol. Transfer was carried out at 4°C in transfer buffer for 3 hr at 200 mA. For Western blotting, a rabbit anti-HB-EGF antiserum (7541) was raised against the peptide YPN-KEEHGKRKKKGKGLGKKRD, which corresponds to amino acids 14–34 of the mature, 75-aa form of human HB-EGF (6), but with a tyrosine residue added at the amino terminus. The antiserum crossreacted with native and recombinant HB-EGF and in both cases could be blocked by excess (20 μg) peptide. The peptide amino acid sequence does not exist in human EGF or TGF-α, and the anti-HB-EGF antiserum did not crossreact with either one of these growth factors. The blot was incubated subsequently with alkaline phosphatase-conjugated anti-rabbit antibodies (Protoblot system; Promega). After SDS/PAGE of ^{125}I-HB-EGF, samples were transferred to nitrocellulose paper and autoradiography was carried out with Kodak XAR-5 film.

Platelet Lysate. Human platelet lysate was kindly prepared by J. Erban (Tufts University, Boston, MA) from 20 ml of platelet-rich plasma (4–6 × 10^9 platelets).

RESULTS

Appearance of Heparin-Binding Growth Factor Activity in Wound Fluid After Injury. Pig wound fluid samples were collected daily for 8 days and analyzed for growth factor activity on BALB/c 3T3 cells. It was estimated that there were 7000 units of total 3T3-stimulating growth factor activity 1 day after injury and that the activity diminished to 150 units by day 8. The wound fluid samples were fractionated by heparin affinity chromatography (Fig. 1). No heparin-binding mitogenic activity was found 1 hr after injury (Fig. 1*B*), nor in 3 ml of control serum prepared from a wounded pig, the protein content of which was similar to that of wound fluid obtained 3 days after injury (Fig. 1*A*). However, from 1 to 5 days after injury, two peaks of growth factor activity were observed, a relatively minor peak eluted with 0.65 M and a major peak eluted with 1.1 M NaCl. The two peaks of mitogenic activity were maximal at 2–3 days after injury. The heparin column elution at 2 days demonstrated an ≈4:1 ratio of 1.1 M NaCl- to 0.65 M NaCl-eluted growth factor activity (Fig. 1*C*). By day 7 the only heparin-binding growth factor activity present was a relatively small amount eluted with 1.1 M NaCl (Fig. 1*D*), and by day 8 no heparin-binding growth factor activity was detectable at all (data not shown).

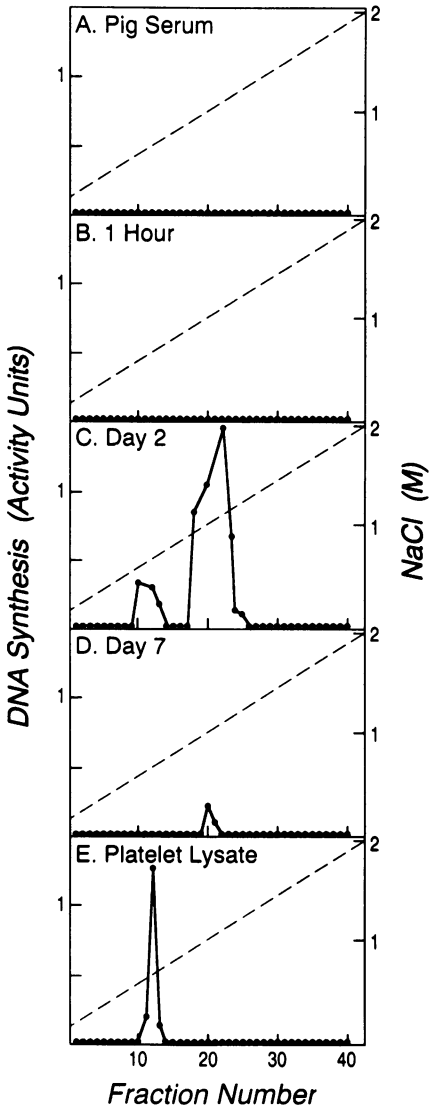

FIG. 1. Heparin affinity column analysis of pig wound fluid. Pig wound fluid was collected 1 hr (*B*), 2 days (*C*), and 7 days (*D*) after injury and samples were applied to heparin-TSK columns. Pig serum (*A*) and platelet lysate (*E*) were also analyzed. Fractions were eluted with a 0.2–2 M NaCl gradient and examined for the ability to stimulate DNA synthesis in BALB/c 3T3 cells. These results were reproducible in wound fluid samples obtained from four separate pigs.

PDGF-Like Activity in Wound Fluid. The wound fluid-derived heparin-binding growth factor eluted with 0.65 M NaCl (Fig. 1*C*) was eluted at the same NaCl concentration as the major heparin-binding growth factor found in human

platelet lysate, PDGF (Fig. 1*E*). Anti-human PDGF antibodies, which neutralized 100% of the human platelet lysate activity, neutralized about 70% of the pig wound fluid activity eluted at 0.65 M NaCl but had no inhibitory effect on the peak of mitogenic activity eluted at 1.1 M NaCl (Fig. 2). These results suggested that at least 70% of the wound fluid growth factor activity eluted with 0.65 M NaCl is PDGF-like, but the percentage could be higher, since anti-human PDGF antibodies might not be totally efficient in detecting pig PDGF.

HB-EGF-Like Activity in Wound Fluid. Human macrophages secrete HB-EGF, a heparin-binding growth factor that is eluted from a heparin column with 1–1.2 M NaCl (5, 11). Since macrophage products are found in wound fluid, it was plausible that the mitogenic activity eluted with 1.1 M NaCl might be HB-EGF-like. The EGF-like activity of HB-EGF has been previously demonstrated by its ability (*i*) to compete with ^{125}I-EGF for binding to the EGF receptor and (*ii*) to trigger phosphorylation of the EGF receptor (5, 6). The wound fluid-derived peak of growth factor activity eluted with 1.1 M NaCl had these properties as well. A pooled sample of 1.1 M NaCl peak material obtained after heparin affinity chromatography of wound fluid collected 1–4 days after injury competed in a concentration-dependent manner with the ability of ^{125}I-EGF to bind to A-431 cells (Fig. 3*A*), as did recombinant human EGF (Fig. 3*B*). In addition, the growth factor activity eluted with 1.1 M NaCl also induced phosphorylation of the EGF receptor as measured by immunoblotting with anti-phosphotyrosine antibodies (Fig. 4, lanes 3 and 8). The receptor phosphorylated by the wound fluid-derived heparin-binding mitogenic activity comigrated with a 170-kDa phosphorylated EGF receptor standard (Fig. 4, lane 1) and with EGF receptor phosphorylated by EGF (lanes 2 and 6) and by HB-EGF (lane 7). It was concluded that the wound fluid-derived growth factor activity eluted at 1.1 M NaCl was heparin-binding and EGF-like, two characteristic properties of HB-EGF.

For more direct evidence that the wound fluid-derived 1.1 M NaCl peak contained HB-EGF, immunoblotting was carried out with anti-human HB-EGF antibodies prepared against a peptide corresponding to an amino-terminal region of HB-EGF (Fig. 5). These antibodies cross-reacted with human 20-kDa native U-937 cell-derived HB-EGF (5, 6) (Fig. 5*A*, lane 4) and with 14-kDa recombinant human HB-EGF (lane 3). When the wound fluid-derived mitogenic peak eluted with 1.1 M NaCl was analyzed, the anti-HB-EGF antibodies crossreacted with a 14-kDa species (Fig. 5*A*, lane 1) that

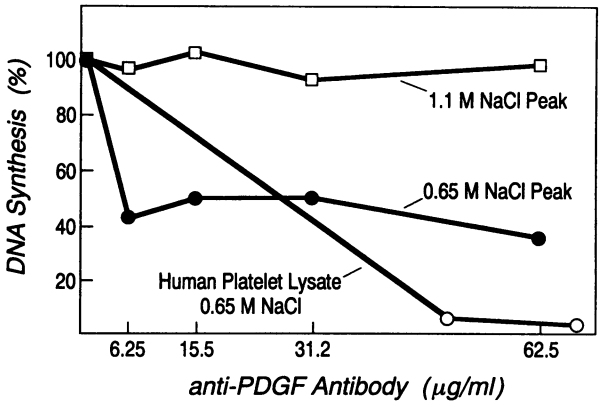

FIG. 2. Effect of anti-PDGF neutralizing antibodies on heparin affinity-purified mitogenic activity in wound fluid. Various concentrations of neutralizing antibodies were incubated at room temperature for 3 hr with the peak of activity eluted with 0.65 M NaCl obtained from human platelet lysate as shown in Fig. 1*E* (○), with the pig wound fluid-derived peak of activity eluted with 0.65 M NaCl shown in Fig. 1*C* (●), and with the wound fluid-derived peak of activity eluted with 1.1 M NaCl shown in Fig. 1*C* (□).

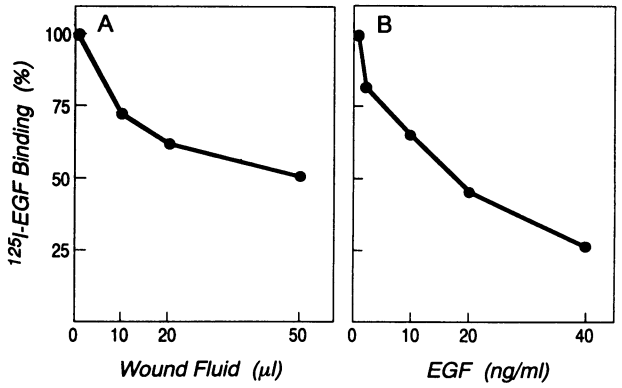

FIG. 3. Competition for ^{125}I-EGF binding to the EGF receptor. Samples of growth factor and ^{125}I-EGF (0.6 ng/ml) were added to A-431 cells for 2 hr at 4°C, the cells were washed, and the amount of ^{125}I-EGF bound to cells was measured. (*A*) Increasing concentrations of a pool of the pig wound fluid-derived peaks of growth factor activity eluted with 1.1 M NaCl 1–6 days after injury. (*B*) Increasing concentrations of human EGF.

comigrated with 14-kDa recombinant HB-EGF (lane 3). Crossreactivity with the anti-HB-EGF antibody was blocked totally by preincubation with excess (20 μg) peptide (Fig. 5*A*, lane 2). A plausible explanation for the lowered molecular mass of wound fluid-derived HB-EGF, 14 kDa, compared to the 20-kDa U-937 cell-derived native HB-EGF, was that it might have been degraded in the wound fluid. Accordingly, ^{125}I-labeled native human HB-EGF (18–20 kDa), a mixture of multiple forms (6) (Fig. 5*B*, lane 1), was incubated for 4 hr with pig wound fluid at 37°C with the result that its molecular mass was lowered to 12–14 kDa (lane 2). These results suggest that the pig HB-EGF could be enzymatically degraded in wound fluid.

Mitogenic Activity for BALB/MK Keratinocytes. The HB-EGF-like activity eluted from heparin affinity columns with 1.1 M NaCl was mitogenic not only for BALB/c 3T3 fibroblasts but also for BALB/MK keratinocytes in a dose-dependent manner, just as was purified native human HB-EGF (Table 1). Amphiregulin is an EGF-like growth factor (12) with about 40% sequence identity to HB-EGF (5). Like

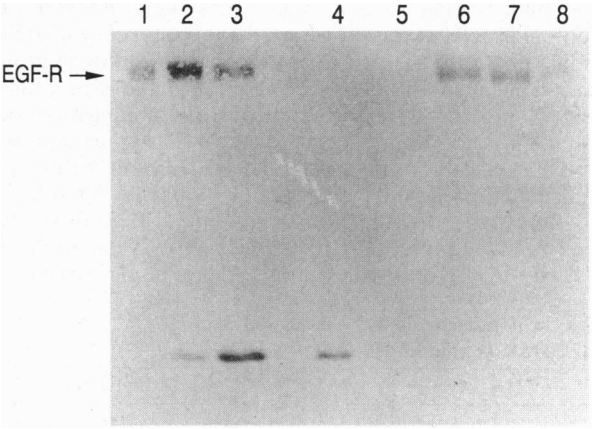

FIG. 4. EGF receptor phosphorylation. A-431 cells were incubated for 6 min at 37°C with human EGF (25 ng/ml) (lanes 2 and 6), with purified human HB-EGF (3 ng/ml) (lane 7), with the peaks of wound fluid-derived growth factor activity eluted at 1.1 M NaCl collected on days 1–5 post-injury and pooled (lanes 3 and 8), and with no addition of growth factor (lanes 4 and 5). A-431 lysates were either immunoprecipitated with anti-EGF receptor antibodies prior to SDS/7.5% PAGE and Western blot analysis with anti-phosphotyrosine antibodies (lanes 2–4) or directly analyzed by Western blotting with anti-phosphotyrosine antibodies (lanes 5–8). Purified phosphorylated EGF receptor (EGF-R) standard is shown in lane 1.

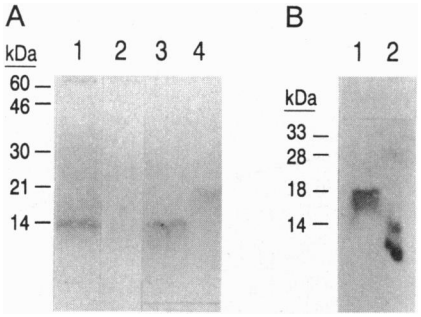

FIG. 5. Western blot and autoradiography. Heparin affinity-purified wound fluid-derived mitogenic activity eluted with 1.1 M NaCl was concentrated on heparin-Sepharose minicolumns and analyzed by SDS/15% PAGE in a minigel. Proteins were transferred to nitrocellulose paper and incubated with anti-HB-EGF antiserum 7541 as described in *Materials and Methods*. (*A*) Western blot. A pool of the peaks of mitogenic activity eluted with 1.1 M NaCl, collected 1–5 days post-injury, were incubated with antibody (lane 1) or with antibody preincubated with 20 μg of peptide used to prepare the antibody (lane 2). Recombinant HB-EGF (10 ng) (lane 3) and native HB-EGF (25 ng) (lane 4) were also analyzed. (*B*) Autoradiography after transfer to nitrocellulose. Lane 1, ^{125}I-labeled native HB-EGF (a mixture of multiple 18- to 20-kDa forms); lane 2, ^{125}I-labeled native HB-EGF incubated with wound fluid for 4 hr at 37°C.

HB-EGF, amphiregulin binds to heparin (data not shown) and stimulates keratinocyte proliferation (13). The mitogenic activity of amphiregulin is totally inhibited by heparin (13). Similar levels of heparin, however, potentiated the growth factor activities of both native HB-EGF and the wound fluid-derived 1.1 M NaCl heparin-binding peak (Table 2). These results are consistent with the presence of HB-EGF in the 1.1 M NaCl heparin-binding peak and the probable lack of substantial amphiregulin in this material.

DISCUSSION

Heparin-binding growth factors such as HB-EGF (5, 6), PDGF (1), bFGF (4), keratinocyte growth factor (8), and vascular endothelial cell growth factor (14) stimulate the migration and proliferation of cell types known to be involved in wound healing such as fibroblasts, keratinocytes, and endothelial cells. Accordingly, we have analyzed wound fluid for its content of biologically active heparin-binding growth factors because this analysis might provide clues to the identity and quantity of the active heparin-binding migration- and proliferation-stimulatory factors that participate in wound repair. We have found that HB-EGF is the predominant heparin-binding growth factor mitogenic for BALB/c 3T3 fibroblasts that appears in pig wound fluid in the first week following injury. A PDGF-like activity, neutralizable with anti-PDGF antibodies, also appears in pig wound fluid

Table 1. Mitogenic activity of HB-EGF for BALB/MK keratinocytes

HB-EGF		1.1 M NaCl peak	
ng/ml	DNA synthesis, cpm × 10^{-3}	μl per well	DNA synthesis, cpm × 10^{-3}
0.3	4 ± 0.6	0.2	42 ± 2
0.5	10 ± 0.8	0.5	108 ± 26
1	29 ± 3	1	270 ± 23
5	103 ± 3	2	305 ± 10

HB-EGF (peaks 1 and 2 from U-937 cell conditioned medium; ref. 6) and the wound fluid-derived peak eluted with 1.1 M NaCl (collected on days 1–4 post-injury, pooled, and concentrated) were analyzed for the ability to stimulate DNA synthesis in BALB/MK keratinocytes in the presence of insulin (5 μg/ml).

Table 2. Effect of heparin on mitogenic activity

	DNA synthesis, cpm × 10^{-3}	
Heparin, μg/ml	HB-EGF	1.1 M NaCl peak
0	13.3 ± 4.3	21 ± 0.7
1	29.5 ± 3	37.7 ± 1.7
5	35.7 ± 1	36.3 ± 7
30	29 ± 0.1	30 ± 1.3

HB-EGF (0.5 ng/ml) and the wound fluid-derived 1.1 M NaCl peak collected on day 2 after injury (5 μl) were analyzed in the presence of various amounts of heparin for the ability to stimulate DNA synthesis in BALB/MK keratinocytes.

but is much less significant, about 5–10% of the HB-EGF levels when measured in terms of mitogenic activity for BALB/c 3T3 cells. Interestingly, there is little evidence in pig wound fluid in the first week following injury for the presence of any readily detectable bFGF, a potent BALB/c 3T3 mitogen that is eluted from heparin columns with 1.7 M NaCl.

The major heparin-binding growth factor in pig wound fluid is HB-EGF-like: (*i*) it is eluted from heparin-TSK columns with 1.1 M NaCl, (*ii*) it is mitogenic for BALB/c 3T3 cells and keratinocytes but not for endothelial cells, (*iii*) heparin potentiates its mitogenic activity for BALB/MK keratinocytes, (*iv*) it competes with ^{125}I-EGF for binding to the EGF receptor, (*v*) it induces phosphorylation of the EGF receptor, and (*vi*) it crossreacts specifically on a Western blot with antibodies that recognize an amino-terminal region of HB-EGF.

The pig wound fluid-derived HB-EGF-like activity, 14 kDa, is considerably smaller than HB-EGF purified from U-937 cell conditioned medium, ≈20 kDa (5, 6). The most plausible explanation for the lowered molecular mass is deglycosylation. Native HB-EGF is heavily glycosylated, unlike recombinant HB-EGF produced in *E. coli*, which is not glycosylated (5, 6). Lack of glycosylation does not appear to affect mitogenic activity or heparin binding. On an immunoblot, pig wound fluid HB-EGF, at 14 kDa, comigrates with recombinant HB-EGF. Incubation of native 18- to 20-kDa ^{125}I-HB-EGF with pig wound fluid lowers its molecular mass to 14 kDa. Thus, it is plausible that the molecular mass of pig wound fluid-derived HB-EGF is lowered by the action of endoglycosidases that are found at the site of a wound. HB-EGF might also be degraded by wound-derived proteases. A possible source of degradative enzymes could be macrophages, since HB-EGF secreted by macrophage-like U-937 cells exists in multiple forms, all biologically active, some of which appear to be of lower molecular mass (6).

It cannot be ruled out that other EGF-like heparin-binding growth factors are also present in wound fluid. In particular, amphiregulin is a member of the EGF family (12, 13) with 40% sequence identity to HB-EGF (5). It binds to heparin-TSK columns and is eluted with 1 M NaCl, slightly less than HB-EGF (S.H. and M.K., unpublished results). One potential difference between amphiregulin and HB-EGF is the effect of heparin on their mitogenic activity for keratinocytes. The mitogenic activity of amphiregulin for BALB/MK keratinocytes is completely inhibited by heparin at 30 μg/ml (13). On the other hand, the mitogenic activity of native human HB-EGF and the wound fluid-derived HB-EGF-like activity for keratinocytes are potentiated by heparin, consistent with the wound fluid-derived material being preponderantly HB-EGF rather than amphiregulin. More definitive studies using specific anti-amphiregulin antibodies would be needed to ascertain the presence of amphiregulin in wound fluid. Unfortunately, neither amphiregulin protein nor antibody is readily available for analysis of wound fluid at present. Another member of the EGF family, recently de-

scribed, is the neuroblastoma differentiation factor (NDF) also known as heregulin (15, 16). NDF/heregulin is a 44-kDa protein that binds to heparin columns. However, this protein does not compete with ^{125}I-EGF for binding to the EGF receptor (15) and could therefore not account for the heparin-binding, EGF receptor-binding activity found in wound fluid.

The appearance of HB-EGF in wound fluid may be a relatively early response to injury. HB-EGF is not found in wound fluid 1 hr after injury, appears by 24 hr, is maximal at 2–3 days after injury, and is undetectable 8 days after injury, at which time wounds are healed macroscopically. HB-EGF is produced by human peripheral monocytes and macrophages in culture (11). The kinetics of HB-EGF appearance in wound fluid parallels that of macrophages. Macrophages accumulate at the wound site within 24 hr after injury, reach maximal levels 3 days after injury, and decrease immediately afterwards (17). Keratinocytes also migrate into the wound and proliferate soon after injury. Pig skin keratinocytes harvested from a wound and grown in culture express HB-EGF mRNA (M.M. and M.K., unpublished results). Thus, it is possible that the sources of HB-EGF in wound fluid include macrophages, keratinocytes, and possibly other cell types.

What is the significance of finding HB-EGF in wound fluid? First, it is plausible that HB-EGF is involved in wound healing. It is a mitogen for fibroblasts and keratinocytes, cell types that migrate and proliferate in response to wounding. It is produced by macrophages, cells that play a central role in wound healing (17). There is also considerable evidence that members of the EGF family promote wound healing *in vivo*. For example, EGF applied topically increases the rate of wound healing in skin (18), shortens the length of time for healing of partial-thickness wounds on human skin grafts (19), and accelerates the rate of epidermal repair in split-thickness wounds in pigs (20, 21) and in full-thickness ulcers in the rabbit ear (22) and in mice (23). TGF-α applied topically to partial-thickness burns in pigs accelerates epidermal regeneration (24).

Second, HB-EGF by virtue of its heparin-binding properties might have advantages *in vivo* when compared with EGF and TGF-α, which are not heparin-binding proteins (11). The importance of growth factor–heparin interactions has been best illustrated for bFGF (25). bFGF binds to heparan sulfate proteoglycan (HSPG) on cell surfaces (26) and in extracellular matrix (27). The binding of bFGF to cell surface HSPG, which act as low affinity receptors, is a prerequisite for its binding to the high affinity FGF receptor and for its mitogenic activity (28–30). The association of bFGF with HSPG of extracellular matrix has been suggested to be a mechanism for sequestering growth factors as part of a highly stable complex (27). Other heparin-binding growth factors, such as interleukin 3 and granulocyte/macrophage-colony-stimulating factor, also bind to HSPG in extracellular matrix and in tissue stroma *in vivo* (31). HB-EGF binds to cell surface HSPG (S.H. and M.K., unpublished results), and its mitogenic activity for keratinocytes is potentiated by heparin. Thus, it is plausible that heparin–HB-EGF interactions might be biologically significant. We speculate that after wounding, HB-EGF is released into the wound site by monocytes, macrophages, keratinocytes, or other cell types and binds to HSPG. Binding to cell surface HSPG might enhance HB-EGF mitogenicity, as is the case with bFGF. HB-EGF could also bind to HSPG of extracellular matrix and subsequently be released in a sustained manner by heparitinases or proteases found in the damaged tissue, possibly of macrophage origin (32–34). Thus, while HB-EGF might be similar to EGF and TGF-α in target cell specificity, its availability and mitogenic activity could be markedly greater than those of EGF or TGF-α because of its heparin-binding properties.

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