Production of Heparin-Binding Epidermal Growth Factor–like Growth Factor (HB-EGF) at Sites of Thermal Injury in Pediatric Patients

Diane W. McCarthy,* Marc T. Downing,* David R. Brigstock,* Mark H. Luquette,† Kenneth D. Brown,‡ Mark S. Abad,§ and Gail E. Besner*

Departments of *Surgery and †Pathology, Ohio State University and Children's Hospital, Columbus, Ohio, and §Monsanto Company, Chesterfield, Missouri, U.S.A.; and ‡Department of Cellular Physiology, The Babraham Institute, Cambridge, United Kingdom

Fluids that accumulate at wound sites may be an important reservoir of growth factors that promote the normal wound healing response. The presence of heparin-binding growth factors was studied in burn wound fluid (BWF) from 45 pediatric patients who had sustained partial thickness burns. One of the growth factors present was similar to platelet-derived growth factor (PDGF) based on its heparin affinity, inhibition of bioactivity by a PDGF antiserum, and detection in a PDGF-AB enzyme-linked immunosorbent assay. A second growth factor was identified as heparin-binding epidermal growth factor–like growth factor (HB-EGF) based on its heparin affinity, competition with ^{125}I-labeled epidermal growth factor (EGF) for EGF receptor binding, and recognition in biological assays and Western blots by two HB-EGF antisera. Amino acid sequence analysis of one form of this second growth factor verified its identity as an *N*-terminally truncated form of HB-EGF. Immunohistochemical analysis of partial thickness burns demonstrated the presence of HB-EGF in the advancing epithelial margin, islands of regenerating epithelium within the burn wound, and in the duct and proximal tubules of eccrine sweat glands. HB-EGF in the surface epithelium of burn wounds was uniformally distributed, whereas it was restricted to the basal epithelium in nonburned skin. These data support a role for PDGF and HB-EGF in burn wound healing and suggest that the response to injury includes deposition of HB-EGF and PDGF into blister fluid and a redistribution of HB-EGF in the surface epithelium near the wound site. ***Key words: wound healing/partial thickness burn/epithelium/skin. J Invest Dermatol 106:49–56, 1996***

Heparin-binding epidermal growth factor–like growth factor (HB-EGF) was first described as a macrophage secretory protein that binds strongly to heparin and stimulates DNA synthesis in fibroblasts and vascular smooth muscle cells [1]. Purification of HB-EGF and cloning of its cDNA from the U937 macrophage-like cell line revealed that HB-EGF is a member of the epidermal growth factor (EGF) family [2,3]. Structural similarities of members in this group include three intra-chain disulfide bonds that result in an appropriate protein conformation for binding and activating EGF receptors (EGF-R) [4]. Between the first and sixth cysteine residues, human HB-EGF shares 40–53% homology with human EGF, transforming growth factor–α (TGF-α), and amphiregulin (AR) [2]. There is considerable cross-species conservation of the HB-EGF protein, with approximately 85–90% homology between the amino acid sequences of human, monkey, pig, rat, and mouse HB-EGF [2,5–7]. In common with several EGF family members, HB-EGF is predicted to be synthesized as a transmembrane precursor (208 amino acids) from which mature HB-EGF (approx. 86 amino acids) appears to be released by extracellular proteolytic cleavage [2,3]. The human HB-EGF precursor is a binding site for diphtheria toxin [8] that may also directly bind and activate EGF-R on neighboring cells in a juxtacrine fashion. HB-EGF and AR are distinguished from other EGF family members by their ability to bind strongly to heparin, a property which may result in their extracellular regulation by cell surface heparin-like molecules [9–11].

HB-EGF mRNA has been identified in multiple cell types including macrophages, vascular smooth muscle cells, endothelial cells, eosinophils, and endometrial epithelial cells [2,12–15]. Reverse transcriptase polymerase chain reactions (RT-PCR) and Northern blotting have shown wide tissue distribution of HB-EGF mRNA including skin, lung, heart, kidney, skeletal muscle, brain, male reproductive system, lymph node, thymus, and spleen [5,6]. Also, we have recently cloned a 380-bp HB-EGF cDNA from porcine uterus and purified HB-EGF from pig uterine tissues and secretions [7]. Target cells for HB-EGF include fibroblasts, smooth muscle cells, and keratinocytes [1,2].

HB-EGF may play a role in wound healing, since it is produced by and acts on cells that are involved in the wound healing process (i.e., macrophages, fibroblasts, and keratinocytes). From previous studies, a potential role for endogenously produced growth factors at the wound site has been inferred by their presence in wound

Manuscript received November 30, 1994; revised August 9, 1995; accepted for publication August 22, 1995.

Reprint requests to: Dr. Gail E. Besner, Division of Pediatric Surgery, Department of Surgery, Children's Hospital, 700 Children's Drive, Columbus, OH 43205.

Abbreviations: HB-EGF, heparin-binding EGF-like growth factor; AR, amphiregulin; EGF-R, EGF receptor; RRA, radioreceptor assay; BWF, burn wound fluid; FPLC, fast protein liquid chromatography.

0022-202X/96/$10.50 •

fluid. Platelet-derived growth factor (PDGF)–like molecules have been documented in wound fluid collected from surgical drains after mastectomies and orthopedic surgery [16–18], and from partial-thickness excisional porcine wounds [19]. The increased rates of collagen synthesis and re-epithelialization of superficial wounds covered with occlusive dressings have been attributed to the dressing maintaining high local concentrations of growth factors produced at the wound site [20]. Wound fluid obtained from beneath occlusive dressings stimulates proliferation of cultured endothelial cells and dermal fibroblasts [21] and contains EGF, PDGF, tumor necrosis factor–α, and basic fibroblast growth factor [22,23].

Since a detailed analysis of the growth factors that accumulate in blisters arising from partial thickness burns has not previously been reported, we characterized those that are present in burn wound fluid (BWF) up to 6 d after the initial burn injury. Here, we show that two of the principal fibroblast heparin-binding growth factors in BWF are PDGF and HB-EGF, and we describe the immunohistochemical localization of HB-EGF in the vicinity of the burn wound.

MATERIALS AND METHODS

Collection of Human BWF BWF was obtained from 45 pediatric patients (approximately 8 mo to 16 y of age) who had suffered accidental thermal injury. Intact blisters of partial thickness burns were aspirated up to 6 d after injury (day 0 = day of burn) and the fluid was collected, pooled, and frozen at −20°C. Informed consent was obtained for each patient. The study protocol conformed to the ethical guidelines of the Human Subjects Research Committee of the Children's Hospital Research Foundation.

Characterization of growth factor activities in BWF

Heparin-Affinity Chromatography: BWF was clarified by centrifugation (12,000*g* for 30 min) and passage through 0.45-μm membrane filters. It was then diluted 10-fold in 20 mM Tris HCl (pH 7.4) and applied at 1–3 ml/min to an EconoPac heparin affinity column (0.7 × 3.6 cm; BioRad Laboratories, Richmond, CA) or a TSK heparin 5PW column (0.75 × 8 cm; TosoHaas, Philadelphia, PA) that had been equilibrated in 20 mM Tris HCl (pH 7.4) containing 0.2 M NaCl. A fast protein liquid chromatography (FPLC) system (Pharmacia LKB Biotech Inc., Piscataway, NJ) was used to apply the sample, to wash the column with equilibration buffer until the absorbance (A_{280}) of the eluate was constant, and to generate a 40-ml gradient of 0.2–2.0 M NaCl for elution of bound proteins from the column. During protein elution, the flow rate was 1 ml/min and the eluate was collected into forty 1-ml fractions which were assayed for their ability to stimulate [^{3}H]thymidine incorporation in Balb/c 3T3 cells or to compete with ^{125}I-labeled EGF (^{125}I-EGF) for binding to the EGF-R.

Growth Factor Assays: Aliquots of column fractions were tested for their ability to stimulate [^{3}H]thymidine incorporation into DNA of confluent quiescent Balb/c 3T3 cells [1]. Samples were also tested in an EGF radioreceptor assay (RRA) using human endometrial carcinoma cells (HEC-1-B) [10]. Some EGF RRAs were performed by incubating HEC-1-B cells in binding medium that contained either 10% (vol/vol) HB-EGF goat antiserum or 10% (vol/vol) goat pre-immune serum as described [7]. Measurement of PDGF-AB was performed using a human PDGF-AB Quantikine double sandwich enzyme-linked immunosorbent assay (ELISA) detection kit (R&D Systems, Minneapolis, MN).

SDS-PAGE and Western Blot Analysis: One milliliter of burn fluid (days 1–4) was centrifuged at 12,000*g* for 30 min and the supernatant filtered through 0.2-μm membranes. The filtrate was diluted 10-fold in 10 mM Tris HCl/0.5 M NaCl (pH 7.4) and passed through a 50-μl bed of heparin-Sepharose (Pharmacia LKB Biotech). The heparin-Sepharose beads were washed extensively with 10 mM Tris HCl/0.5 M NaCl (pH 7.4) prior to extraction of bound proteins by addition of 50 μl of 2X sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) sample buffer. Burn fluid samples were run in parallel on duplicate 18% SDS-PAGE under reducing conditions [1] and the electrophoresed proteins were Western blotted as described [7]. The nitrocellulose blots were blocked [24] and incubated overnight at 4°C with either a 1:5000 dilution of rabbit antiserum raised against the peptide KGLGKKRDPCLRKYKD corresponding to residues 26–41 of mature human HB-EGF (see [10] for numbering system) or with a 1:5000 dilution of pre-immune rabbit serum. Immunoreactive bands were developed by successive incubation of the blots with biotinylated anti-rabbit IgG (Amersham Corp., Arlington Heights, IL), streptavidin-alkaline phosphatase (Amersham) and nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Life Technologies Inc., Grand Island, NY) as described [7]. This protocol was repeated on 330 μl of a day 3 and day 6 pooled BWF sample obtained from three patients for which the Western blot was incubated with 10 μg/ml of affinity-purified HB-EGF antiserum or normal rabbit IgG. Sequence-grade recombinant human HB-EGF was used as a standard on Western blots and was produced in our laboratory essentially as described.[1]

Affinity Purification of Anti-HB-EGF(21–46) Antiserum The peptide HB-EGF(26–41) was produced on a Synergy 432A Peptide Synthesizer (Applied Biosystems, Norwalk, CT) and purified by C_{18} reverse-phase HPLC. Approximately 1 mg of the peptide was dissolved in 0.2 M $NaHCO_3$ containing 0.5M NaCl (pH 8.0) and coupled to a 1-ml NHS-activated HiTrap column (Pharmacia LKB Biotech) according to the manufacturer's specifications. HB-EGF antiserum was diluted 10-fold in PBS and cycled through the column three times, after which the column was washed with 20 ml of PBS followed by 20 ml of PBS/0.5 M NaCl. Bound proteins were eluted using 0.1 M glycine (pH 2.5) and collected into 0.5-ml fractions that were immediately neutralized with 50 μl of 1 M Tris. IgG concentrations of individual fractions were calculated from their absorbance at 280 nm.

Purification of HB-EGF from BWF

Heparin Affinity Chromatography: Two hundred milliliters of BWF was subjected to heparin affinity chromatography using an EconoPac heparin FPLC column as described above. Biologically active fractions that contained the 0.6–1.3 M NaCl eluate were diluted 5-fold in 20 mM Tris HCl (pH 7.4) and subjected to a second step of heparin affinity chromatography using a TSK heparin 5PW FPLC column as described above. Fractions containing the 1.0–1.2 M NaCl eluate were pooled (4.5 ml) and subjected to reverse-phase high-performance liquid chromatography (HPLC).

Reverse-Phase HPLC: Reverse-phase HPLC was performed on a Hitachi HPLC system using a Vydac C_4 column (0.46 × 25 cm, 5-μm particle size; The Separations Group, Hesperia, CA) as described [7]. Bound proteins were eluted from the column with a multilinear organic gradient comprising 5–15% acetonitrile over 5 min, 15–40% acetonitrile over 120 min, and 40–90% over 1 min. The column eluate was monitored at 214 nm using an in-line ultraviolet (UV) detector and the chromatogram was archived as described [25]. Individual fractions (1 ml) were collected in siliconized tubes containing NaOH to neutralize the TFA. Two-microliter aliquots of selected fractions were diluted 10-fold in PBS containing 1% bovine serum albumin and assayed for mitogenic activity on 3T3 cells. Three-microliter aliquots of selected fractions were assayed directly in the EGF RRA.

Amino Acid Sequencing: Five picomoles of a reverse-phase purified bioactive protein peak were subjected to *N*-terminal amino acid sequencing for 10 cycles using an Applied Biosystems Model 470A gas phase sequenator. Phenylthiohydantoin amino acid derivatives were identified by C_{18} reverse phase HPLC.

Immunohistochemical Detection of HB-EGF in Burn Wounds

Tissue Procurement: In accordance with the ethical guidelines of the Human Subjects Research Committee of the Children's Hospital Research Foundation, human burn wound tissues on day 2 and day 14 were procured from the leg of two patients at the time of excision and grafting of full thickness burns in the operating room. Burn specimens utilized in this study were from the burn wound margin and contained a rim of normal epidermis and underlying dermis. Nonburned normal chest skin was obtained at the time of autopsy of a different pediatric patient. Tissues were immediately placed in ice-cold Dulbecco's modified Eagle's medium prior to snap-freezing in Tissue-Tek OCT Compound (Miles Diagnostics, Kankakee, IL) and stored at −70°C. Frozen sections were mounted, wet-fixed in a 50% acetone/50% methanol solution for 5 min, and rinsed several times in PBS.

Immunohistochemistry: All immunohistochemical reactions were performed at room temperature in a humidity chamber. Tissue sections were blocked with 10% normal goat serum (Vector Laboratories Inc., Burlingame, CA) in PBS for 20 min and then incubated for 60 min in the same solution containing 1:100 dilutions of HB-EGF(26–41) antiserum or preimmune serum, or 19 μg/ml of affinity-purified anti-HB-EGF(26–41) or normal rabbit IgG. Sections were dipped 10 times in PBS/0.1% Tween 20 followed by three 3-min rinses with PBS. They were then incubated in a 1:100 dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories Inc.) in 2% normal goat serum for 30 min, washed as described above, and then incubated in a 1:100 dilution of streptavidin fluorescein (Vector Labs Inc. or Amersham Corp., Arlington Heights, IL) in 2% normal goat serum for 30

[1] Crissman-Combs MA, Brigstock DR, Besner GE: *Mol Biol Cell* 4(suppl): A109, 1993 (abst.)

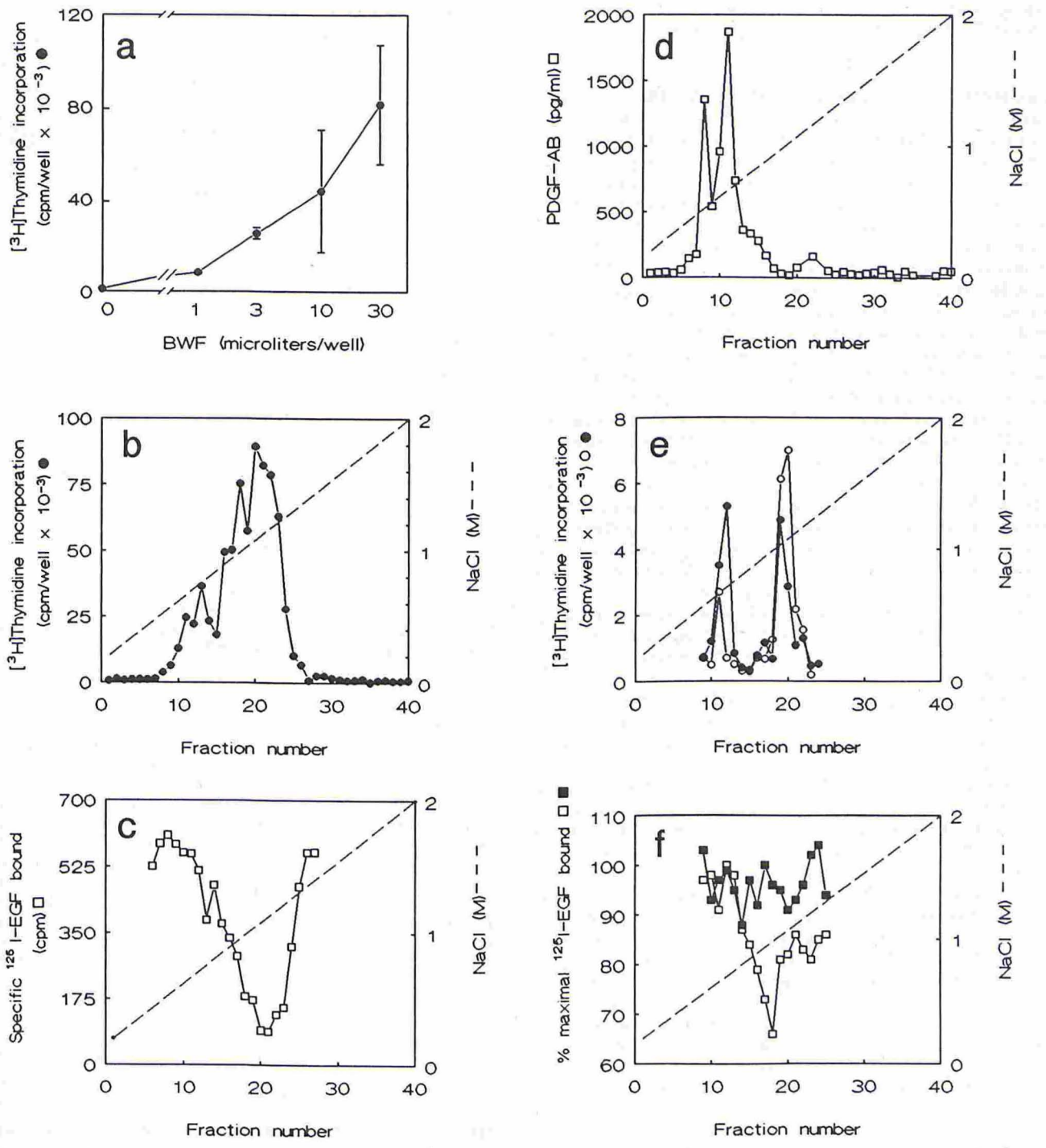


Figure 1. Biologically active PDGF and HB-EGF are present in human BWF. *a*) BWF aspirated 6 d after injury from the blister of a partial thickness burn was clarified by centrifugation at 12,000*g* for 10 min and the supernatant assayed for its ability to stimulate DNA synthesis in Balb/c 3T3 cells. *Error bars*, SD; triplicate determinations of [^{3}H]thymidine incorporation (●). *b*) Twelve milliliters of BWF aspirated from intact blisters of partial thickness burns were clarified by centrifugation and filtration. The supernatant was diluted 10-fold in 20 mM Tris HCl (pH 7.4) and applied to an EconoPac heparin column which was then developed with a 0.2–2 M NaCl gradient. Individual fractions were tested at 5 μl for their stimulation of [^{3}H]thymidine incorporation in 3T3 cells (●). The same fractions were tested at (*c*) 25 μl for their ability to compete with ^{125}I-labeled EGF for binding to the EGF-R (□) and (*d*) 200 μl for the presence of immunoreactive PDGF-AB using a double sandwich ELISA (□). *e*) Seven milliliters of BWF prepared as described above were diluted 10-fold in 20 mM Tris HCl (pH 7.4) and applied to a TSK heparin FPLC column. The figure shows the stimulation of 3T3 cell DNA synthesis by 25 μl of individual fractions in the presence (○) or absence (●) of 10 μg/ml neutralizing antiserum to PDGF. *f*) Twenty-five milliliters of BWF from a day 1 patient was subjected to EconoPac heparin affinity chromatography as described above. An EGF RRA was performed in binding medium containing 10% (vol/vol) of either goat pre-immune serum (□) or goat anti-HB-EGF antiserum (■) plus 25 μl of fractions 9–25 which contained the 0.6–1.3 M NaCl eluate. Results are expressed as percentage of maximal specific ^{125}I-EGF binding.

min. The specimens were then washed, mounted using Aquamount (Lerner Laboratories, Pittsburgh, PA), and examined by fluorescence microscopy.

RESULTS

Human BWF Contains PDGF and HB-EGF BWF stimulated [^{3}H]thymidine incorporation in Balb/c 3T3 cells in a dose-dependent manner **(Fig 1*a*)**. When a 12-ml pool of BWF from several patients was subjected to heparin affinity chromatography, two main peaks of [^{3}H]thymidine incorporation–stimulating activity were detected that were eluted from the column by 0.5–0.7 M NaCl (fractions 9–14) and by 0.8–1.2 M NaCl (fractions 16–24) **(Fig 1*b*)**. When these fractions were tested in an EGF RRA, a competitor of ^{125}I-EGF binding was present in the 0.8–1.2 M NaCl eluate **(Fig 1*c*)** that co-eluted with the second mitogenic peak, suggesting that they might be shared properties of the same molecule. In contrast, the fractions containing the 0.5–0.7 M NaCl eluate were inactive in the EGF RRA, suggesting that they did not contain an EGF-R ligand. Since the heparin-binding property of the first mitogenic peak was comparable to that of PDGF [26], fractions were tested in a PDGF ELISA with the result that immunoreactive human PDGF-AB was detected in the 0.5–0.7 M NaCl eluate **(Fig 1*d*)**. Fractions from heparin-affinity chromatography of a different 7-ml pool of BWF were also assayed for 3T3 cell-stimulatory activity in the presence and absence of a neutralizing PDGF antiserum. As a result the mitogenic activity of fractions containing the 0.5–0.7 M NaCl eluate was reduced suggesting that at least one biologically active isoform of human PDGF (AA,BB or AB) was present **(Fig 1*e*)**. The mitogenic activity of the 0.8–1.2 M NaCl eluate was somewhat enhanced by the PDGF antiserum, suggesting the second mitogenic peak was not PDGF-like and that it was able to interact synergistically or additively with non-IgG components of the antiserum.

To evaluate more specifically whether HB-EGF was present in BWF, fractions obtained by heparin affinity chromatography of 25 ml of day 1 BWF were tested in an EGF RRA in the presence of a HB-EGF goat antiserum that blocks EGF receptor binding by HB-EGF but not by EGF, TGF-α, or AR [7]. When tested in this manner, the 0.8–1.2 M NaCl eluate retained its ^{125}I-EGF–competing activity in the presence of preimmune serum but was rendered ineffective in the presence of the HB-EGF antiserum **(Fig 1*f*)**.

The presence of EGF-R ligands in BWF taken on day 0, 1, 2, 3, 4, and 6 was shown by the ability of crude (i.e., unpurified) samples to inhibit ^{125}I-EGF binding by 18–42% when tested in the EGF RRA at 0.066% (vol/vol) (data not shown). To test whether this effect was due, at least in part, to HB-EGF, 1-ml BWF samples were passed through mini-heparin columns and bound proteins were subjected to SDS-PAGE followed by Western blotting using a rabbit antiserum raised against the peptide HB-EGF(26–41). This antiserum, which recognizes human HB-EGF but not EGF, TGF-α, or AR (data not shown), detected multiple immunoreactive bands of M_r 10,000–15,000 in a day 1 BWF sample that were not detected by pre-immune rabbit serum **(Fig 2*a*)**. To confirm and extend this data, Western blotting was repeated using BWF collected between day 1 and 4 from four other patients. As shown in **Fig 2*b***, similar immunoreactive bands were detected in burn fluid on each of the days tested. The apparent sizes of the proteins were clearly distinct from those of 20- to 22-kDa native HB-EGF [2] and more closely resembled that of nonglycosylated HB-EGF such as the recombinant form of the protein produced in *Escherichia coli*.[1] In view of the concern that these immunoreactive proteins might not be authentic forms of HB-EGF, the HB-EGF antiserum was affinity purified and the immune (bound) fraction incubated on a Western blot of day 3 and 6 BWF that was pooled from three different patients. As shown in **Fig 2*c***, the purified antiserum reacted strongly with 12-kDa recombinant human HB-EGF as well as with multiple proteins in BWF of approximately 10–16 kDa; normal rabbit IgG tested at the same concentration failed to react with either the human HB-EGF standard or the proteins

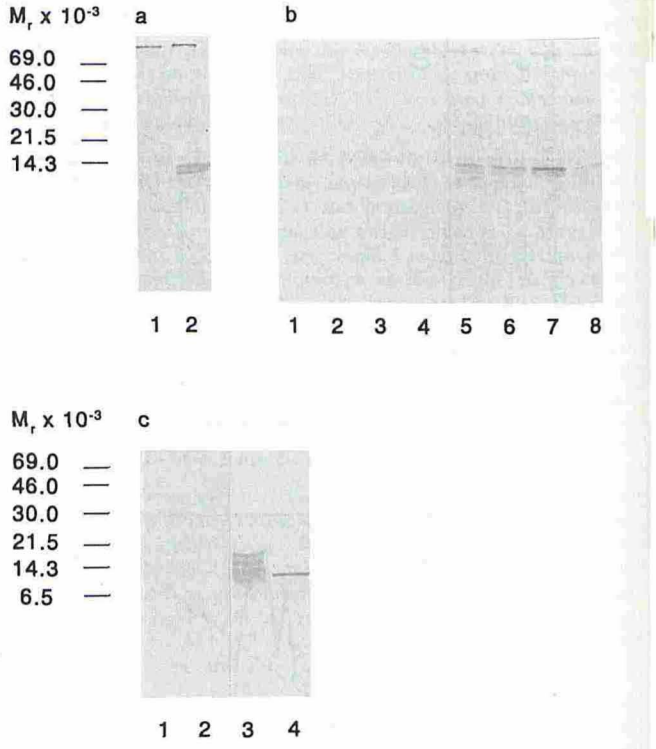


Figure 2. Heparin-purified BWF contains immunoreactive HB-EGF proteins. Aliquots of day 1–4 BWF was prepared and passed through mini-heparin columns as described in *Material and Methods*. Bound proteins were extracted using 2X SDS-PAGE sample buffer and were electrophoresed on duplicate 18% SDS-polyacrylamide mini-gels. Proteins were transferred to nitrocellulose which was then blocked and incubated with a 1:5000 dilution of either rabbit anti-HB-EGF(26–41) or rabbit pre-immune serum. Immunoreactive bands were visualized by successive incubations of the blots with biotinylated goat anti-rabbit IgG, streptavidin-alkaline phosphatase and chromogenic substrates. The figure shows (*a*) BWF from day 1 analyzed with pre-immune serum (*lane 1*) or anti-HB-EGF(26–41) (*lane 2*) and (*b*) BWF from day 1 (*lanes 1 and 5*; different patient to that in [*a*]), day 2 (*lanes 2 and 6*), day 3 (*lanes 3 and 7*), and day 4 (*lanes 4 and 8*) treated with pre-immune serum (*lanes 1–4*) or HB-EGF antiserum (*lanes 5–8*). Each sample lane received heparin-binding proteins derived from 400 μl of BWF. The figure also shows (*c*) the equivalent of 165 μl of heparin-purified day 3 and day 6 pooled BWF (*lanes 1 and 3*) and 100 ng recombinant human HB-EGF (*lanes 2 and 4*) incubated with 10 μg/ml of normal rabbit IgG (*lanes 1 and 2*) or affinity-purified anti-HB-EGF(26–41) (*lanes 3 and 4*).

detected in BWF **(Fig 2*c*)**. These data thus provided further evidence that the immunoreactivity in BWF represented genuine forms of HB-EGF, despite their lower mass.

HB-EGF Isolated from BWF Is Highly Truncated at Its *N* Terminus Although use of two different HB-EGF antisera provided strong evidence for the presence in BWF of HB-EGF rather than AR, to obtain definitive proof we elected to isolate and molecularly characterize the growth factor in question. Preparative heparin affinity chromatography was performed on a 200-ml pool of BWF from two patients on day 2 and 3 using an EconoPac heparin column followed by a TSK heparin 5PW column **(Fig 3*a*)**. The active fractions containing the 1.0–1.2 M NaCl eluate were pooled and applied to a C_4 reverse-phase HPLC column that was developed with a multilinear acetonitrile gradient. The column eluate contained three activity peaks with retention times of 38 min, 42–46 min, and 48 min that were detected in both biological assays **(Fig 3*b*)**. Although multiple protein peaks were eluted from the column during this time course, only the protein with a retention time of 42–43 min was present in sufficient quantity for structural analysis. When approximately 5 pmol of this protein were subjected to 10 cycles

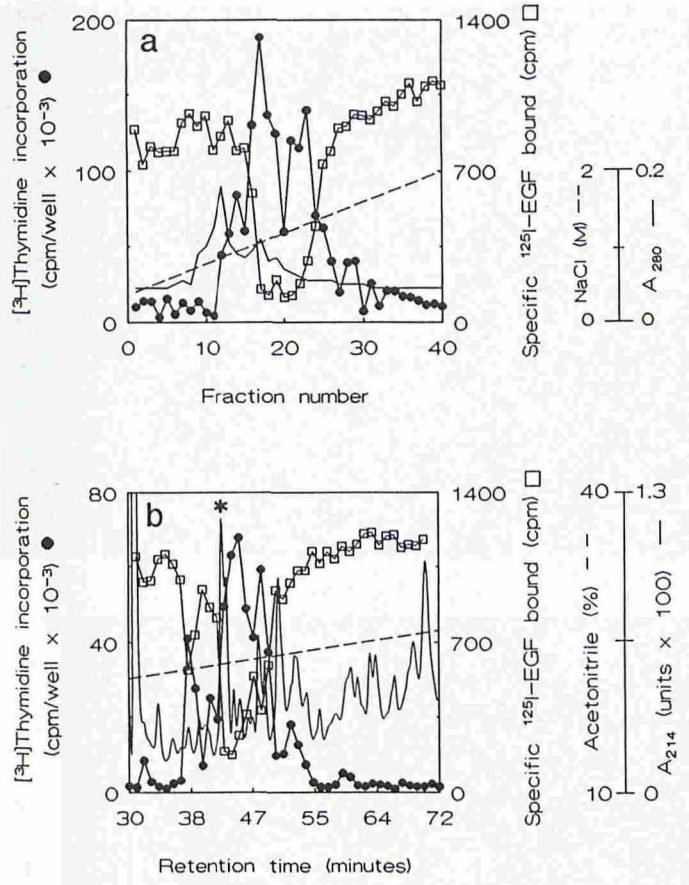


Figure 3. Isolation of HB-EGF from BWF. *a*) A pool of 200 ml of BWF from obtained from two patients on day 2 and 3 was subjected to two cycles of affinity chromatography using EconoPac- and TSK-heparin columns. The figure shows the absorbance (280 nm) of the eluate (solid line) when the TSK heparin column was developed with the indicated NaCl gradient (– – –), and the stimulation of 3T3 cell DNA synthesis by 3 μl (●) and inhibition of ^{125}I-EGF binding by 5 μl (□) of 1-ml fractions collected during protein elution. Fractions containing the 1.0–1.2 M NaCl eluate were pooled (approx. 4.5 ml) and subjected to C_4 reverse-phase HPLC. *b*) Elution from the C_4 column of protein (A_{214}) (——) between 30 and 72 min after sample injection (approximately 20–27% acetonitrile [– – –]) and the stimulation of 3T3 cell DNA synthesis by 2 μl (●) and inhibition of ^{125}I-EGF binding by 1 μl (□) of the fractions collected over this time course. *The protein peak selected for amino acid sequencing.

of *N*-terminal degradation, no amino acids were identified in the first cycle, three were identified in the second cycle, two were identified in the third cycle, and one was identified in each of the remaining seven cycles **(Table I)**. The last seven amino acids were identical to residues 28–34 of human HB-EGF **(Table I)**. With this alignment, the Lys residue identified in the second cycle matched that in position 26 of human HB-EGF. Although this alignment resulted in a mismatch between residues identified in the third cycle (Leu or Tyr) and position 27 of human HB-EGF (Gly), this was probably due to a sequencing error. It is of note that the Leu, Lys, Asp, and Pro residues definitively identified in the 4th, 7th, 9th, and 10th cycles, respectively, clearly distinguish this peptide sequence as originating from HB-EGF rather than AR **(Table I)**.

The Distribution of HB-EGF in Burn Wounds Is Different from That in Normal Skin Excision of a day 14 burn wound and its margin demonstrated positive staining of the entire epithelium at the unburned margin and of keratinocytes within a regenerating island from a hair follicle when incubated with

Table I. *N*-Terminal Amino Acid Sequence of BWF Growth Factor Demonstrates Its Identity as a Truncated Form of HB-EGF[a]

BWF[1–10]	HB-EGF[25–34]	AR[30–39]
Xaa	Gly	Gly
Lys,Gly,Ser	Lys	Gly
Leu,Tyr	Gly	Lys
Leu	Leu	Asn
Gly	Gly	Gly
Lys	Lys	Lys
Lys	Lys	Asn
Arg	Arg	Arg
Asp	Asp	Arg
Pro	Pro	Asn

[a] Fractions containing the 43- to 46-min HPLC-purified bioactive peak (see **Fig 3*b***) were pooled and reduced in volume to approximately 100 μl. The sample (approx. 5 pmol) was then subjected to automated *N*-terminal amino acid sequence analysis. The table shows the amino acids that were identified in each of the first 10 cycles of Edman degradation. Also shown is the alignment of the sequence with that of an internal domain of human HB-EGF (residues 25–34) and the corresponding domain of human AR (residues 30–39).

HB-EGF(26–41) antiserum. This pattern of staining was absent when an adjacent tissue section was treated with pre-immune serum (data not shown). To verify these findings, immunohistochemistry was repeated on the same burn specimen using affinity-purified HB-EGF(26–41) with which immunoreactivity was localized throughout the epithelium of the skin at the burn margin and to epithelial cells of the hair follicle **(Fig 4*a*)**. This pattern of staining was very similar to that seen with the crude antiserum and was absent in adjacent tissue sections treated with purified rabbit IgG **(Fig 4*b*)**, pre-absorbed antiserum, or blocking solution alone (data not shown). Whereas the HB-EGF was spread throughout the regenerating surface epithelium at the burn wound margin **(Fig 4*a*)**, in normal skin it was highly localized to the basal keratinocytes of the surface epithelium **(Fig 4*c*)**. In contrast, dermal appendages, identified as hair follicles and eccrine sweat gland ducts, stained positively for HB-EGF but did not demonstrate obvious differences in distribution of the growth factor between burned and nonburned tissues **(Fig 4** and data not shown). While the entire duct of the sweat gland stained positively for HB-EGF in both normal and burned skin, only a portion of the coiled sweat gland tubules stained positively. Using either unpurified antiserum **(Fig 4*d,e*)** or affinity-purified antiserum (data not shown), the proximal portion of the eccrine gland consistently showed intense staining whereas the distal region demonstrated only slight or no HB-EGF immunoreactivity **(Fig 4*d,e*)**. High power magnification of eccrine sweat glands demonstrated that the immunoreactivity was localized to epithelial cells lining the tubule, with apparently higher concentrations in the lumen and luminal surfaces **(Fig 4*f*)**. Epithelial cells of the epidermis and eccrine sweat glands also stained positively when burn wound tissue excised on day 2 was incubated with an antiserum raised against residues 9–26 of HB-EGF [7] (data not shown). Dermal connective tissue cells reproducibly did not stain for HB-EGF and while blood vessels appeared to stain slightly with unpurified anti-HB-EGF(26–41) **(Fig 4*d*)**, this finding was not substantiated using affinity-purified antiserum (data not shown).

DISCUSSION

Although numerous growth factors and cytokines have been implicated in the wound healing response [27,28], the roles played by each factor and their synergistic interactions have yet to be fully understood. Here, we show that HB-EGF and PDGF may also be involved in healing of human burn wounds since they can be recovered from BWF up to at least the fourth day after injury. Evidence for the presence of HB-EGF included its heparin-binding and EGF-R-binding properties, the antagonism of its EGF-R binding by a HB-EGF-specific goat antiserum, the detection of 10-

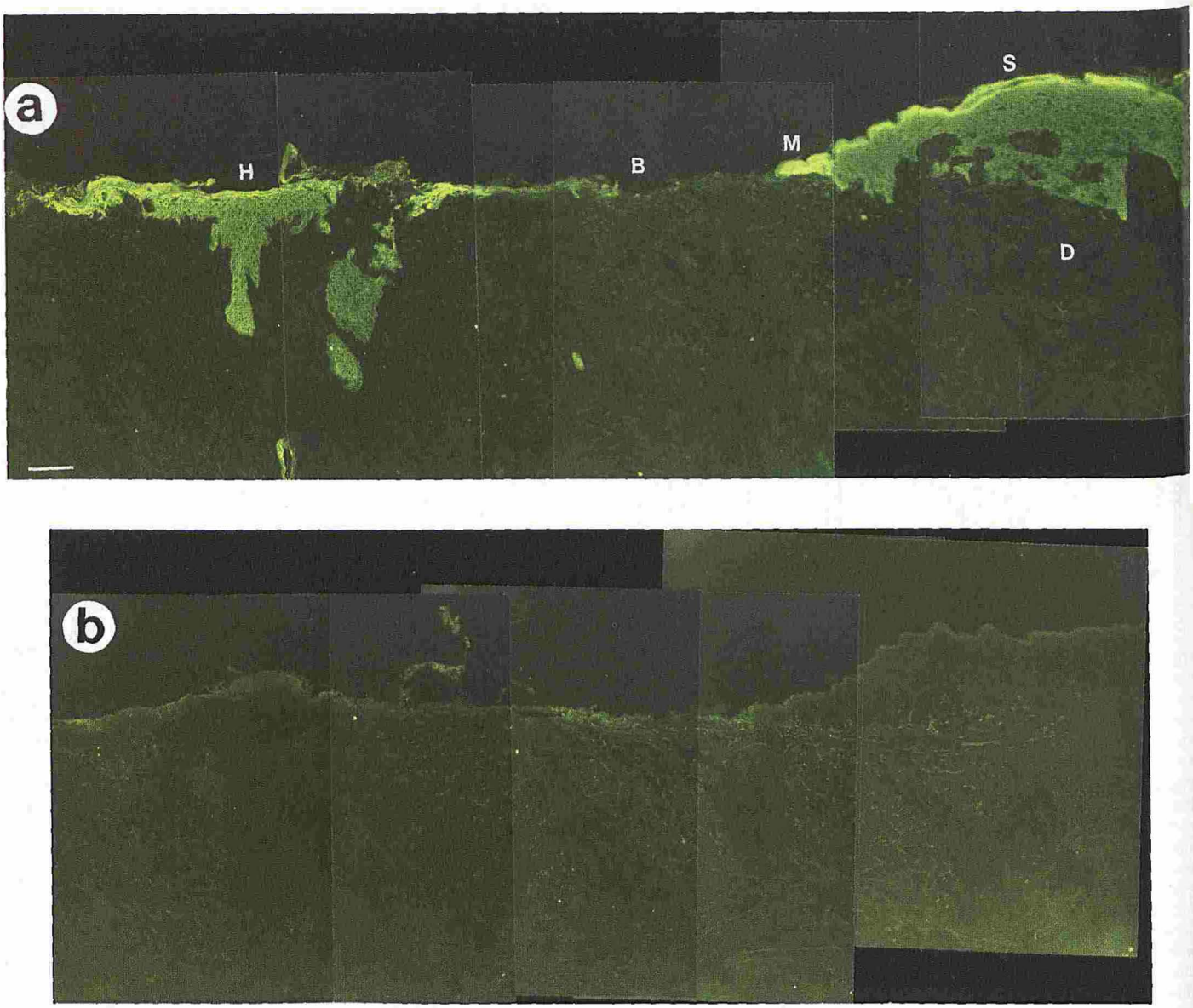
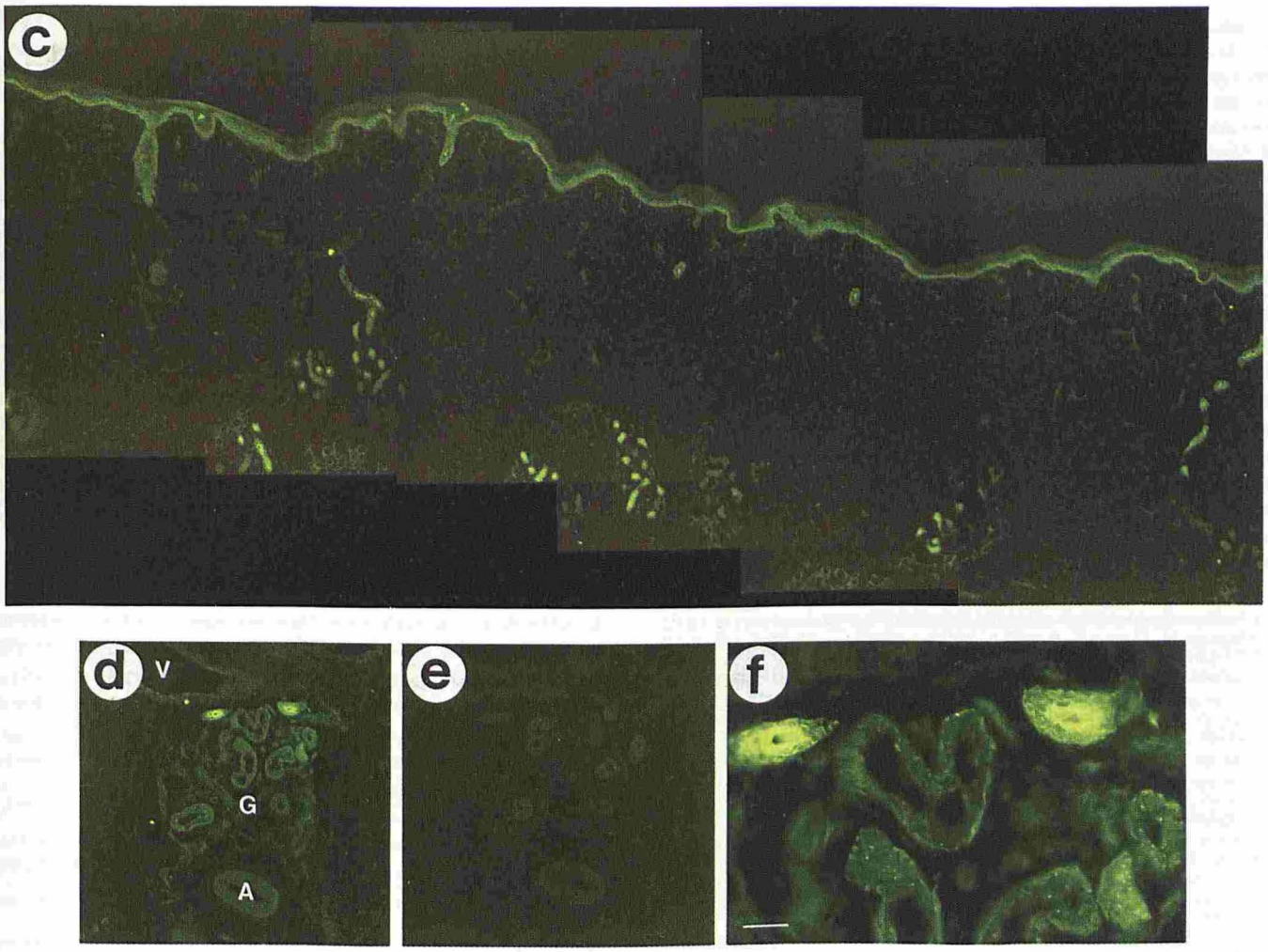


Figure 4. HB-EGF is present in the burn wound surface epithelium and shows a differential distribution as compared to nonburned skin. A day 14 burn from a 17-y caucasian male was excised, fixed, sectioned, and mounted as described in *Materials and Methods* and then incubated in with 19 μg/ml of either (*a*) affinity-purified HB-EGF(26–41) antiserum or (*b*) normal rabbit IgG. The tissue shown in (*c*) is normal nonburned skin that was incubated with a 1:100 dilution of HB-EGF(26–41) antiserum. Additional tissue sections from different regions of the burn specimen shown in (*a*) and (*b*) were also incubated with 1:100 dilutions of either (*d*) HB-EGF(26–41) antiserum or (*e*) preimmune serum. A higher magnification of the immunoreactive regions in (*d*) are shown in (*f*). *H*, hair follicle epithelium; *B*, burn wound; *M*, margin of wound; *S*, surface epithelium; *D*, dermis; *G*, eccrine sweat gland; *V*, vein; *A*, artery. *Scale bars*, 150 μm (*a–e*) and 30 μm (*f*).

to 15-kDa immunoreactive proteins on Western blots by HB-EGF(26–41) antiserum and by amino acid sequence analysis of the purified protein. Evidence for the presence of PDGF included its relative heparin affinity, the neutralization of its biological activity by PDGF antiserum, and the detection of PDGF-AB using a specific ELISA.

While PDGF and PDGF-like factors have previously been detected in a variety of wound fluids, the presence of HB-EGF in wound fluids has not been widely reported. A recently characterized growth factor in puncture wound fluid of pigs appeared to be HB-EGF-like based on its heparin-affinity, target cell specificity, binding to and autophosphorylation of the EGF-R, and immunodetection on Western blots [19]. However, given the inherent difficulty of distinguishing between HB-EGF and AR due to their structural and biological similarities, our study was greatly facilitated by the availability of two highly specific HB-EGF antisera and by amino acid sequence analysis of the bioactive protein in BWF.

An obvious point of interest is that of the time course of HB-EGF production or accumulation in BWF. Although we have been unable as yet to perform a quantitative analysis in view of the lack of highly specific and sensitive assays for HB-EGF (e.g., ELISA), we found from the 45 patients who donated BWF for these studies that, at least qualitatively, HB-EGF is detectable in BWF as early as day 0 and appears to be present until at least day 4. Unfortunately, we have not yet been able to determine the rate of production of HB-EGF on each of the days following injury. However, the sustained presence of HB-EGF in BWF and burn wound tissues suggests that the biological effects of HB-EGF, either alone or in combination with other factors such as PDGF, could be maintained for at least 2 wk at the burn site.



Whereas glycosylated HB-EGF purified from U937 cells migrates on SDS-polyacrylamide gels with a M_r of 20,000–22,000 [2], the HB-EGF in BWF appeared to have a M_r of 10,000–16,000 and was more similar in size to nonglycosylated HB-EGF. The presence of multiple HB-EGF bands on Western blots of heparin-purified BWF is suggestive of the presence of a variety of the proteins that were variably truncated and/or glycosylated. Amino acid analysis of the HB-EGF protein in BWF that was isolated and sequenced clearly show that it had an *N*-terminal truncation of 24 residues. Of interest is that some of the truncated domain (i.e., residues 20–24) have been suggested to contribute to the heparin-binding domain(s) of HB-EGF [10,29]. Since the purified protein was isolated by two cycles of heparin affinity chromatography, our interpretation of this data is that either (i) the truncation occurred after the heparin-affinity steps; the positive staining by anti-HB-EGF(9–26) of burn wound tissues (data not shown) suggests that HB-EGF *in vivo* exists, at least partially, in a form that is much less truncated than that which was purified and sequenced, or (ii) additional heparin-binding regions are present downstream of the lysine residue at position 24 of HB-EGF. Support for the latter possibility has come from peptide mapping and site-directed mutagenesis, which have suggested that additional sites between residues 30 and 41 also contribute to the heparin-binding domain(s) of HB-EGF [10,29].

Since HB-EGF levels in serum and plasma are undetectable ([1] and data not shown), its presence in BWF is suggestive of local production at the burn wound site rather than accumulation from the circulation. This is consistent with our immunohistochemical data showing that HB-EGF is present in epithelial cells lining the margins of the wound and dermal appendages. Moreover, compared with normal skin which exhibits HB-EGF staining in the basal epithelium, there is a striking difference at the burn wound site in that HB-EGF is uniformally distributed throughout the entire epithelium. Thus thermal injury appears to be associated with a distinct distribution of HB-EGF, as well as HB-EGF accumulation in blister fluid.

Although the basis for the differential staining for HB-EGF in eccrine sweat glands was not investigated further, it may be relevant that the proximal portions of these glands are epithelial in nature whereas the distal portions are composed of secretory or myoepithelial cells. Since the transition between secretory and ductal epithelial cells is histologically abrupt, our data may be suggestive of differences in HB-EGF production by these distinct cell populations. While we cannot eliminate the possibility that other cell types produce lower nondetectable amounts of HB-EGF in the burn wound, it is of interest that high levels of EGF-R expression have been demonstrated in the early post-burn period in humans in marginal keratinocytes, in adjacent hypertrophic epithelium and in marginal and non-marginal hair follicles, and in sweat ducts and sebaceous glands [30]. Therefore, HB-EGF produced by surface and glandular epithelial cells may act at the burn site through autocrine, paracrine, or juxtacrine mechanisms to stimulate epithelial regeneration.

The finding that HB-EGF is produced *in vivo* in the partial-thickness burn wound adds this molecule to the growing list of cytokines and growth factors that may regulate the wound healing process. Since HB-EGF is present in BWF and undergoes epithelial redistribution in the vicinity of the burn wound, it may play an integral role in the normal healing response to thermal injury.

We thank the Pediatric Surgical Faculty of The Ohio State University, the nursing staff of the Burn Unit at Columbus Children's Hospital, Diane Cooney, R.N., and Lizette Levieux-Anglin, R.N., for their assistance with collection of BWF. We also thank Jane Tolley for help with protein sequencing and Christy Steffen for helping prepare the figures. D.R.B. and G.E.B. were supported by Ohio State University Seed Grants and by Children's Hospital Research Foundation (020-871 and 020-872). G.E.B. was also supported by an American College of Surgeons Faculty Fellowship and by the Bremer Foundation, the Department of Surgery Medical Research and Development Fund, and Surgical Research, Inc. D.W.M. and M.T.D were both recipients of E. Thomas Boles Scholarships in Pediatric Surgery and contributed equally to this work.

REFERENCES

1. Besner GE, Higashiyama S, Klagsbrun M: Isolation and characterization of a macrophage-derived heparin-binding growth factor. *Cell Regul* 1:811–819, 1990
2. Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M: A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* 251:936–939, 1991
3. Higashiyama S, Lau K, Besner GE, Abraham JA, Klagsbrun M: Structure of heparin-binding EGF-like growth factor multiple forms, primary structure, and glycosylation of the mature protein. *J Biol Chem* 9:6205–6212, 1992
4. Prigent SA, Lemoine NR: The Type 1 (EGFR-related) family of growth factor receptors and their ligands. *Prog Growth Factor Res* 4:1–24, 1992
5. Vaughan TJ, Pascall JC, Brown KD: Tissue distribution for heparin-binding epidermal growth factor. *Biochem J* 287:681–684, 1992
6. Abraham JA, Damm D, Bajardi A, Miller J, Klagsbrun M, Ezekowitz RAB: Heparin-binding EGF-like growth factor: characterization of rat and mouse cDNA clones, protein domain conservation across species, and transcript expression in tissues. *Biochem Biophys Res Commun* 190:125–133, 1993
7. Kim GY, Besner GE, Steffen CL, McCarthy DW, Downing MT, Luquette MH, Abad MS, Brigstock DR: Purification of heparin-binding EGF-like growth factor from pig uterine luminal flushings and its production by endometrial tissues. *Biol Reprod* 52:561–571, 1995
8. Naglich JG, Metherall JE, Russell DW, Eldels L: Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* 69:1051–1061, 1992
9. Cook PW, Mattox PA, Keeble WW, Pittlekow MR, Plowman GD, Shoyab M, Edelman JP, Shipley GD: A heparin sulfate-regulated human keratinocyte autocrine factor is similar or identical to amphiregulin. *Mol Cell Biol* 11:2547–2557, 1991
10. Besner GE, Whelton D, Crissman-Combs MA, Steffen CL, Kim GY, Brigstock DR: Interaction of heparin-binding EGF-like growth factor (HB-EGF) with epidermal growth factor receptor: modulation by heparin, heparinase, or synthetic heparin-binding HB-EGF fragments. *Growth Factors* 7:289–296, 1992
11. Higashiyama S, Abraham JA, Klagsbrun M: Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. *J Cell Biol* 122:933–940, 1993
12. Temizer DH, Yoshiumi M, Perrella MA, Susanni EE, Quertermous T, Lee M-E: Induction of heparin-binding epidermal growth factor-like growth factor mRNA by phorbol ester and angiotensin II in rat aortic smooth muscle cells. *J Biol Chem* 267:24892–24896, 1992
13. Yoshizumi M, Kourembanas S, Temizer DH, Cambria RP, Quertermous T, Lee M-E: Tumor necrosis factor increases the transcription of the heparin-binding epidermal growth factor-like factor in vascular endothelial cells. *J Biol Chem* 267:9467–9469, 1992
14. Powell PP, Klagsbrun M, Abraham JA, Jones RC: Eosinophils expressing heparin-binding EGF-like growth factor mRNA localize around lung microvessels in pulmonary hypertension. *Am J Pathol* 143:784–793, 1993
15. Zhang Z, Funk C, Roy D, Glasser S, Mulholland J: Heparin-binding epidermal growth factor–like factor is differentially regulated by progesterone and estradiol in rat uterine epithelial cells. *Endocrinology* 134:1089–1094, 1994
16. Matsuoka J, Grotendorst GR: Two peptides related to platelet-derived growth factor are present in human wound fluid. *Proc Natl Acad Sci USA* 86:4416–4420, 1989
17. Dvonch VM, Murphey RJ, Matsuoka J, Grotendorst GR: Changes in growth factor levels in human wound fluid. *Surgery* 112:18–23, 1992
18. Soma Y, Dvonch V, Grotendorst GR, Platelet-derived growth factor AA homodimer is the predominant isoform in human platelets and acute human wound fluid. *FASEB J* 6:2996–3001, 1992
19. Marikovsky M, Breuing K, Liu PY, Eriksson E, Higashiyama S, Farber P, Abraham J, Klagsbrun M: Appearance of heparin-binding EGF-like growth factor in wound fluid as a response to injury. *Proc Natl Acad Sci USA* 90:3889–3893, 1993
20. Alvarez OM, Mertz PM, Eaglstein WH: The effect of occlusive dressings on collagen synthsis and re-epithelialization in superficial wounds. *J Surg Res* 35:142–148, 1983
21. Katz MH, Alvarez AF, Kirsner RS, Eaglstein WH, Falanga V: Human wound fluid from acute wounds stimulates fibroblast and endothelial cell growth. *J Am Acad Dermatol* 25:1054–1058, 1991
22. Chen WYJ, Rogers AA, Lydon MJ: Characterization of biologic properties of wound fluid collected during early stages of wound healing. *J Invest Dermatol* 99:559–564, 1992
23. Grayson LS, Hansbrough JF, Zapata-Sirvent RL, Dore CA, Morgan JL, Nicolson MA: Quantitation of cytokine levels in skin graft donor site wound fluid. *Burns* 19:401–405, 1993
24. Brigstock DR, Klagsbrun M, Sasse J, Farber PA, Iberg N: Species-specific high molecular weight forms of basic fibroblast growth factor. *Growth Factors* 4:45–52, 1990
25. Bray W, Brigstock DR: Raw integrator data storage and retrieval using data acquisition software. *Am Laboratory* 26(16):38, 1994
26. Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M: Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science* 223:1269–1299, 1984
27. Deuel TF, Kawahara RS: Growth factors and wound healing: platelet-derived growth factor as a model cytokine. *Annu Rev Med* 42:567–584, 1991
28. McKay IA, Leigh IM: Epidermal cytokines and their roles in cutaneous wound healing. *Br J Dermatol* 124:513–518, 1991
29. Thompson SA, Higashiyama S, Wood K, Pollitt NS, Damm D, McEnroe G, Garrick B, Ashton N, Lau K, Hancock N, Klagsbrun M, Abraham JA: Characterization of sequences within heparin-binding EGF-like growth factor that mediate interation with heparin. *J Biol Chem* 269:2541–2549, 1994.
30. Wenczak BA, Lynch JB, Nanney LB: Epidermal growth factor receptor distribution in burn wounds. Implications for growth factor-mediated repair. *J Clin Invest* 90:2392–2401, 1992