Neutral Endopeptidase Expression and Distribution in Human Skin and Wounds

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Cutaneous sensory nerves mediate inflammation and wound healing by the release of neuropeptides such as substance P. Neutral endopeptidase is a cell surface enzyme that degrades substance P and thereby terminates its biologic actions. The distribution of neutral endopeptidase in normal skin and wounded human skin, however, has not been examined. The objectives of this study were to evaluate neutral endopeptidase expression in wounded and unwounded skin as well as in cells derived from human skin. Neutral endopeptidase was strikingly localized in normal skin by immunohistochemistry to keratinocytes of the epidermal basal layer, to hair follicles, eccrine and sebaceous glands as well as to endothelium of blood vessels and to large nerves. Standard incisional human wounds were studied at several time points between 1 h and 28 d after wounding. Staining for neutral endopeptidase was noted in the wound bed 6 h after wounding. In contrast to normal skin, staining of all the epidermal cell layers was noted in the migrating tongue of epithelium in l d wounds. Similar full-thickness staining was noted in 3 d and 7 d wounds in all layers of the new wound epithelium and in a "transition epithelium" near the wound edge. By 28 d post wounding neutral endopeptidase staining again was detected only in the basal layer of the epidermis. Neutral endopeptidase mRNA was detected in normal skin and wounds as well as cultured keratinocytes, fibroblasts and endothelial cells. Neutral endopeptidase enzymatic bioactivity was demonstrated in cultured keratinocytes. While it is known that several metalloproteinases important to tissue repair are produced by keratinocytes, this is the first evidence that keratinocytes produce neutral endopeptidase. Neutral endopeptidase may terminate the proinflammatory and mitogenic actions of neuropeptides in normal skin and wounds. ***Keywords: endothelial cells/epidermis/fibroblasts/hair follicles/keratinocytes/nerves/neutral endopeptidase/wound healing. J Invest Dermatol 112:873–881, 1999***

The cutaneous sensory nervous system comprises a network of fine C fibers within the skin that innervate multiple cell types and plays an important role in inflammation and wound healing (Hosoi *et al*, 1993; Ansel *et al*, 1996). These fibers convey information from the skin to the central nervous system, and thus have a sensory role. In addition, they have a local effector function by releasing neurotransmitters within the skin (Foreman and Jordan, 1984). Neuropeptides, notably substance p (SP) and calcitonin gene related peptide are released from afferent fibers both centrally and peripherally (Holzer, 1988). SP and calcitonin gene related peptide have multiple actions within the skin that are important in inflammation and healing (Otsuka and Yoshioka, 1993). These actions include increasing blood flow (Lembeck and Holzer, 1979), inducing plasma extravasation (Iwamoto *et al*, 1989) and neutrophil infiltration (Ohlen *et al*, 1989), stimulating adhesion molecule expression by endothelial cells (Matis *et al*, 1990; Quinlan *et al*, 1998) and macrophage chemotaxis (Ruff *et al*, 1985), stimulating DNA synthesis by keratinocytes (Tanaka *et al*, 1988) and fibroblasts (Nilson *et al*, 1985) as well as stimulating proliferation by endothelial cells and inducing neovascularization (Ziche *et al*, 1990). These effects require local secretion and are mediated by interaction with specific G-protein coupled receptors on target cells.

The biologic actions of neuropeptides are rapidly attenuated to prevent prolonged stimulation of cells in an uncontrolled manner (Bohm *et al*, 1997). One of the earliest mechanisms of attenuation is degradation of the neuropeptide within the extracellular fluid. Neutral endopeptidase (NEP, EC 3.4 24.11), also known as enkephalinase, common acute lymphoblastic antigen (CALLA) and CD10 is a cell surface enzyme that degrades several neuropeptides (Roques *et al*, 1993). From a kinetic standpoint, SP and bradykinin are favorable substrates (Matsas *et al*, 1984), although NEP also degrades calcitonin gene related peptide (Katayama *et al*, 1991). Thus, NEP inactivates several proinflammatory peptides and thereby terminates inflammation. Administration of NEP inhibitors magnifies the proinflammatory effects of SP and other tachykinin in several tissues (Nadel, 1990; Roques *et al*, 1993). Furthermore, genetic deletion of NEP results in elevated plasma extravasation in multiple tissues including the skin (Lu *et al*, 1997). These effects are reversed by administration of selective antagonists of SP and

Manuscript received January 26, 1999; revised February 23, 1999; accepted for publication February 25, 1999.

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Abbreviations: NEP, neutral endopeptidase; IHC, immunohistochemistry; SP, substance P; SPR, substance P receptor; HDF, human dermal fibroblasts; HDMEC, human dermal microvascular endothelial cells; MNA, methoxy-2 napthylamine.

bradykinin receptors and thus the observed effects are mediated by diminished degradation of these peptides.

Tissue responsiveness to neuropeptides depends on the presence of specific receptors and on the distribution of neuropeptide degrading enzymes such as NEP. NEP is highly expressed in the brush border of the kidney and small intestine, and in lymph nodes, although substantial NEP levels are also found in the brain, salivary glands, adrenal glands, pancreas, gut wall, nasal mucosa, and lungs (Roques *et al*, 1993; Ansel *et al*, 1996). The skin also contains neuropeptide degrading proteases, although they have not been fully characterized and localized. Frog skin secretes a 100 kDa prototypical metallopeptidase that degrades neuropeptides but which is distinct from NEP (Carvalho *et al*, 1992). Homogenized mouse skin (Paus *et al*, 1994) and guinea pig skin (Iwamoto *et al*, 1989) have peptidase activity that can be blocked with specific inhibitors of NEP. A previous study of human skin has failed to demonstrate NEP by immunohistochemistry (IHC) in a patient with scleroderma. Cultured skin fibroblasts from the same patient, however, did express NEP measured by flow cytometry (Bou-Gharios *et al*, 1995).

In view of the lack of information about localization of NEP in the skin, and the established role of neuropeptides in cutaneous inflammation and in wound healing, we decided to study in detail the distribution of NEP in normal and wounded human skin. Our aims were to: (i) localize NEP in normal and wounded human skin by IHC using a specific antibody; (ii) examine expression of NEP mRNA in normal human skin, in wounds and in cells derived from the human skin; and (iii) confirm expression of enzymatically active NEP in keratinocytes.

MATERIALS AND METHODS

Generation of an antibody to NEP An antibody was raised in rabbits to recombinant human NEP (Khepri Pharmaceutical, San Francisco, CA) conjugated to keyhole limpet hemocyanin (KLH) (Calbiochem, La Jolla, CA). The recombinant human NEP was produced in Chinese hamster ovary (CHO) cells. This human NEP was shown to be enzymatically active using the fluorometric assay described in *Materials and Methods* below. The NEP (5 mg) was dissolved in 3 ml 50 mM phosphate-buffered saline (PBS), pH 7.4, containing 5 mg KLH, 30 mg carbodiimide (ICN, CA). The mixture was agitated at room temperature for 3 h and overnight at 4°C. The efficacy of conjugation, estimated by incorporation of iodinated NEP (labeled using chloramine T) was ~30%. Three 10 wk old New Zealand rabbits were immunized at intervals of 6–8 wk by multiple intradermal injections. Emulsions were prepared of equal parts of conjugated antigen and complete Freund's adjuvant (Difco Labs, Detroit, MI). Each rabbit received 2 ml of emulsion, containing 500 μg conjugate, divided into 12–15 sites. At the first immunization, 0.5 ml Tri-immunol vaccine (Lederle Labs, Pearl River, NY) was injected intramuscularly. Useful antibodies were obtained after the second boost. All rabbits generated antibodies with titers of ~1:10,000, as determined by ELISA. Affinity column purification was performed with recombinant NEP.

Western blots: [Enhanced chemiluminescence (ECL)] Total protein from homogenized adult human skin, cultured adult human keratinocytes, and cultured fetal human keratinocytes (human keratinocytes) was extracted in urea–Tris (Fleckman *et al*, 1997) and centrifuged. The protein concentration of the supernatants was determined for each sample, then 15 μg of protein was loaded and separated by discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970). Purified recombinant NEP (1 μg total protein) was also fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to 0.2 μm nitrocellulose membranes (Bio-Rad, Hercules, CA) (Towbin *et al*, 1979). The membranes were incubated with antibodies to NEP at 1:500 dilution or pan keratin AE1/AE3 at 1:200/1:1000 dilution (gift T.T. Sun) (Cooper and Sun, 1986) for 1 h at room temperature. Membranes were washed and incubated with secondary antibodies. Secondary antibody for NEP was biotinylated goat anti-rabbit–horseradish peroxidase (Bio-Rad) and secondary antibody for AE1/AE3 was biotinylated goat anti-mouse–horseradish peroxidase (BioRad), each at 1:2000 dilution with a 1 h incubation. All washes used 2% dried milk, 0.1% Tween in PBS. Blots were exposed on autoradiography film then visualized using ECL detection reagents (Amersham, Arlington Heights, IL).

Tissue collection for IHC Simplate-II (General Diagnostics Company, Organon Teknika, Durham, NC) bleeding time devices were used to create pairs of wounds on both legs of 11 normal males and three normal female volunteers 66 ± 6 y of age (mean ± SD). The Simplate-II is a spring-loaded instrument which, when activated, projects a pair of blades 5 mm in length, 1 mm in depth, and 3 mm apart. This human wound model has been described previously in detail (Olerud *et al*, 1995). Volunteers were recruited using methods approved by the University of Washington Institutional Review Board for Human Subjects and with informed consent. All subjects were screened and shown to be free of neuropathy and diabetes mellitus as previously described (Olerud *et al*, 1995).

Pairs of wounds were collected with a 4 mm biopsy punch at 1 h, 6 h, 1 d, 3 d, 7 d, 14 d, and 28 d after injury. Wounds were created and collected such that each time point was represented in four or five subjects. All 14 subjects contributed unwounded control samples from both legs and one sample from thigh, sacrum, or medial arm to test for regional variation in innervation and NEP expression. Half of each biopsy was frozen in OCT (Tissue Tek Miles, Torrance, CA) for IHC studies.

To examine more thoroughly NEP immunohistochemistry in hair follicles a single 6 mm biopsy was taken from the vertex scalp of a 53 y old volunteer with mild androgenetic alopecia. The cylindrical biopsy was bisected and mounted in OCT in order to obtain sections perpendicular to the epidermis (vertical sections) as well as sections parallel to the epidermis (horizontal sections). Parallel sections were taken below the dermal–epidermal junction at depths between 1 and 2 mm.

A sample of human jejunum was taken after informed consent from redundant tissue excised during cancer surgery. The specimen was immediately frozen in OCT and used as a positive control for IHC studies of NEP because of the high level of NEP found in the intestinal brush boarders.

Immunohistochemistry Six micron frozen tissue sections of wounds and control skin washed in 0.05 M Tris-buffered saline were postfixed with 2% paraformaldehyde/Sorensons buffer for 5 min. The slides were then washed with 20 mM glycine in Tris-buffered saline to saturate any free aldehydes and incubated in H_2O_2/Tris-buffered saline for 30 min. Slides were incubated in 1.3% goat serum/Tris-buffered saline for 30 min and then incubated with NEP anti-serum at 1:200 dilution for 1 h at room temperature. Control slides were either incubated without primary antibody or with NEP antibody at 1:200 adsorbed with recombinant NEP at a concentration of 10 μg per ml. Biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) at 1:200 dilution for 30 min was used as secondary antibody, followed by streptavidin–biotin complex (SABC Universal Kit, Zymed Laboratories, San Francisco, CA) at 1:200 dilution for 30 min. All antibodies were diluted with 0.1% bovine serum albumin in Tris-buffered saline and all incubations were done at room temperature. Sections were then visualized for immunoreactivity using 3,3′ diaminobenzidine (Sigma, St. Louis, MO) as a chromogen (0.12% in H_2O with 0.1% H_2O_2 and 20% 0.5 M Tris buffer for 20 min), coverslipped with glycergel (Dako, Carpenteria, CA) and photographed using a Nikon-Microphot-SA microscope using either standard image capture or differential interference contrast image capture.

Tissue collection for RNA isolation For studies of mRNA in normal skin and wounds a 53 y old subject allowed pairs of wounds 1, 3, and 7 d of age to be created on the leg with a Simplate-II bleeding time device. These wounds were removed with a 6 mm biopsy punch and immediately frozen in liquid nitrogen. Two biopsies of adjacent unwounded skin were likewise taken and immediately frozen in liquid nitrogen. Total RNA was extracted with Trizol (Gibco, Gaithersburg, MD) for mRNA studies. The subject met the same entry criteria as the other volunteers with regard to the absence of diabetes and neuropathy.

Detection of NEP mRNA in normal skin, wounds, and cultured newborn human keratinocytes by reverse transcriptase–polymerase chain reaction (PCR) Skin and wound extracts were studied using oligonucleotide primers synthesized (Gibco) corresponding to bp 903–921 (5′ primer, sense-ATG ACA TTG GCC CAG ATC C) and the complementary reverse sequence against base pairs 1330–1311 (3′ GCA ATC AAA TCC TCG ACC AC). The melting temperature of the primers is 46°C. RNA was extracted with Trizol (Gibco) from a pair of 6 mm punch biopsies of normal skin from the lateral leg (day 0) as well as the day 1, 3, and 7 wound specimens. Reverse transcriptase–PCR was performed using Super Script One-Step reverse transcriptase–PCR System (Gibco). Synthesis of cDNA was performed at 50°C for 30 min followed by PCR using a denaturing temperature of 94°C and an annealing temperature of 58°C and an extension temperature of 72°C for 30 cycles. Included were samples treated with RNase to control for the possibility

of a PCR product generated from genomic DNA and samples were tested for consistent levels of glyceraldehyde-3-phosphate dehydrogenase expression.

RNA was isolated from cultured newborn human keratinocytes using Ultraspec RNA Isolation Reagent (Biotex Labs, Alberta, Canada). Oligonucleotide primers were synthesized (Bio-Synthesis, Lewisville, TX) corresponding to base pairs 461–481 (5′ primer, sense-CCT CTA CTC AAA CTG TTA CC) and the complementary reverse sequence against base pairs 754–774 (3′ primer, anti-sense-GT CTA ACT AAG CAG TCC TTC) of NEP cDNA (Malfroy *et al*, 1988). Reverse transcription was performed on keratinocyte RNA to make a complementary cDNA strand to NEP mRNA by combining 3′ primer, Maloney murine leukemia virus reverse transcriptase (MMLV RT, Gibco), 1 OX d4NTP (Pharmacia LKB Biotechnology, Piscataway, NJ), 5 × first strand buffer (Gibco), RNasin (Promega, Madison, WI), and dithiothreitol (Gibco). Reverse transcriptase mixture was incubated at 42°C for 1 h followed by heat inactivation of the MMLV RT at 65°C. PCR was performed on 0, 1, 2, and 5 μl of the preconfluent and confluent reverse transcriptase reaction products by adding equal amounts of the 3′ and 5′ primers, 1.25 × d4NTP mixture (Pharmacia LKB Biotechnology) and 1 OX PCR buffer + Mg (Boehringer Mannheim, Indianapolis, IN). *Taq* polymerase (Boehringer Mannheim) was added during the hot start at 85°C. The PCR reaction was carried out for 35 cycles using a denaturing temperature of 94°C, an annealing temperature of 53°C, and extension temperature of 72°C.

The reverse transcriptase–PCR products from extracts of normal skin and wound as well as from keratinocytes were gel-purified (Qiagen, Valencia, CA). The products were then sequenced in both directions using a PCR sequencing reaction containing the 5′ and 3′ NEP oligonucleotide primer and Dye Terminator with AmpliTaq DNA Polymerase FS (ABI Prism, Perkin-Elmer, CT) followed by sequencing using a model 377 Fluorescence Sequencer (ABI Prism, Perkin-Elmer).

Northern blot analysis Northern blot analysis was performed using total RNA extracted from 3 and 7 d wounds with Trizol (Gibco). RNA was hybridized to a NEP specific ((32P) UTP-labeled anti-sense RNA probe (Plowman *et al*, 1993) transcribed from base pairs 945–1346 in the NEP cDNA sequence. Ten and 20 μg samples of total RNA were studied. Normalization of the data for variability in gel loading and transfer efficiency was accomplished by staining the RNA with 0.5 M NaOAc/0.04% methylene blue.

Newborn human keratinocytes (Dale *et al*, 1990), human dermal fibroblasts (HDF) (Dale *et al*, 1990), or human dermal microvascular endothelial cells (HDMEC) (Swerlick *et al*, 1991) were cultured in appropriate media to 50%, 70%, or 90% confluence, respectively, then subcultured in serum-free media overnight before mRNA isolation. Cells were harvested with or without 100 nM SP for 3 h and mRNA was isolated using a commercially available mRNA Isolation Kit (Boehringer Mannheim). Northern blot analysis was performed as previously described (Sambrook *et al*, 1989). An 860 bp NEP cDNA fragment probe isolated by reverse transcriptase–PCR was radiolabeled according to the random priming method of Feinberg and Vogelstein (1983, 1984). The cDNA fragment used for northern blotting spanned base pairs 101–961. Identity with the published sequence for NEP cDNA (Malfroy *et al*, 1988) was established by DNA sequencing. The positively charged nylon membrane blot was probed at 65°C for 3–4 h in Rapid-hyb buffer (Amersham) and washed with low stringency solution (2 × sodium citrate/chloride buffer, 0.1% sodium dodecyl sulfate) at room temperature then high stringency solution (0.1 × sodium citrate/chloride buffer, 0.1% sodium dodecyl sulfate) at 65°C for 20 min prior to exposure to X-ray film at 70°C for 2–3 d. Normalization of the data for variability in gel loading and transfer efficiency was accomplished by determining the level of β-actin using the appropriate cDNA probe (Alonso *et al*, 1986).

NEP enzymatic assay Frozen keratinocytes were weighed and homogenized in 50 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris, pH 7.5) containing 1 μM pepstatin A and 1 mM phenylmethylsulfonylfluoride, and assayed for protein using Coomassie Blue. Cell membranes containing a variety of enzymes (presumably including NEP) were incubated with Glu-Ala-Ala-Phe-MNA. Enzymatic degradation of Glu-Ala-Ala-Phe-MNA results in a fluorescent product (MNA) that can be quantitated. Incubations with keratinocyte membranes were carried out both with and without the specific inhibitor of NEP (DL-thiorphan). The difference in the production of MNA, between membrane preparations with the specific inhibitors of NEP and those without, were attributed to NEP enzymatic activity. The enzymatic activity is reported in picomoles of MNA generated per hour per microgram of protein (Terashima *et al*, 1992).

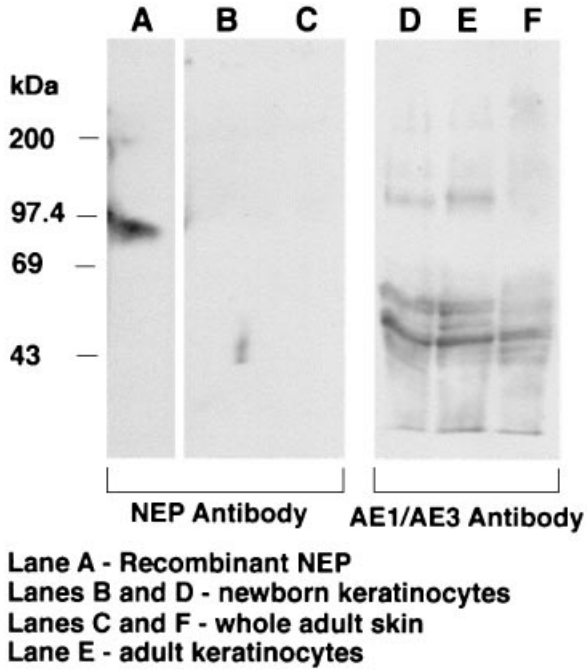

Figure 1. Western blot shows specific staining for NEP without keratin antibody contamination of the polyclonal NEP anti-serum. *Lane A* loaded with NEP shows a single positive band at approximately 94 kDa when tested with the polyclonal antibody to NEP, but showed no evidence of contamination with antibodies to keratins when tested on newborn keratinocytes (*lane B*) and whole adult skin (*lane C*). *Lanes D* (newborn keratinocytes), *E* (adult keratinocytes), and *F* (whole adult skin) stained positively for multiple keratin bands using the keratin antibodies AE 1 and AE 3. AE 1 and AE 3 did not show positive bands on lanes loaded with NEP (data not shown).

RESULTS

NEP antibody shows specificity for NEP without keratin antibody contamination We examined the specificity of the NEP antibody by western blotting. The antibody specifically interacted with recombinant NEP demonstrating a single positive band with an apparent molecular mass of approximately 94 kDa (**Fig 1**, *lane A*). Anti-sera are often contaminated with antibodies to keratin. It is thus important to confirm that the NEP anti-serum did not interact with keratins. The NEP anti-serum did not interact with proteins corresponding to keratins (40–65 kDa) in extracts from human keratinocytes or whole human skin extracts (**Fig 1**, *lanes B* and *C*). NEP was not detected by western blot in keratinocyte or whole skin extracts presumably due to low expression or the fact that NEP is primarily membrane bound and thus not extracted. As a positive control, the cytokeratin antibodies AE1 and AE3 showed binding with multiple bands in the keratin range (40–65 kDa) for cultured newborn human keratinocytes, adult human keratinocytes, and adult human skin homogenates (*lanes D*, *E*, and *F*, respectively). Thus, the NEP anti-serum specifically detects recombinant human NEP and is not contaminated with cytokeratin antibodies.

NEP is highly expressed in the brush border of the small intestine. Therefore, we stained human intestine to verify that we could detect NEP in this location. NEP immunoreactivity was highly expressed in the brush border (**Fig 2**, NEP antibody). Staining was abolished by preadsorbtion of the NEP anti-serum with recombinant NEP (**Fig 2**, control A). Thus, the NEP anti-serum detected NEP in tissue sections with specificity and at the predicted location.

IHC shows NEP staining in normal skin Immunoreactive NEP was highly expressed in the basal layer of unwounded human skin (**Fig 3**). The staining of basal keratinocytes appeared to be cytoplasmic and cell membrane associated. This pattern was consist-

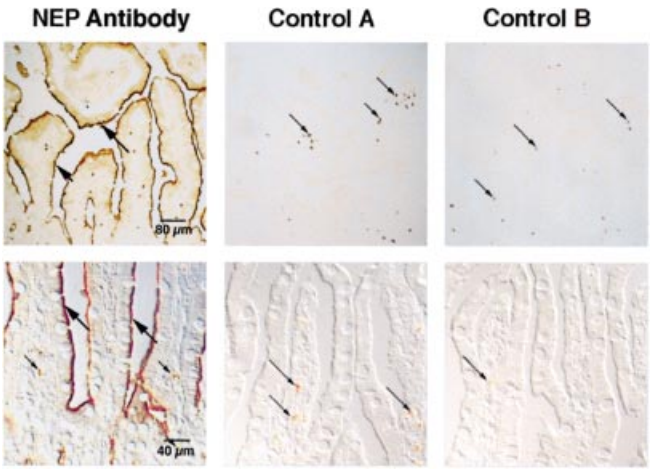

Figure 2. Intestine stains positively with NEP antibody. The strong NEP staining of the brush border of intestine (*large arrows*) was used as a positive control for antibody reactivity. *Control A*: NEP antibody preadsorbed with recombinant NEP shows no staining of the brush border. *Control B*: IHC without NEP antibody shows no staining of the brush border. *Small arrow* indicates nonspecific immunoperoxidase reactivity (possibly mast cells).

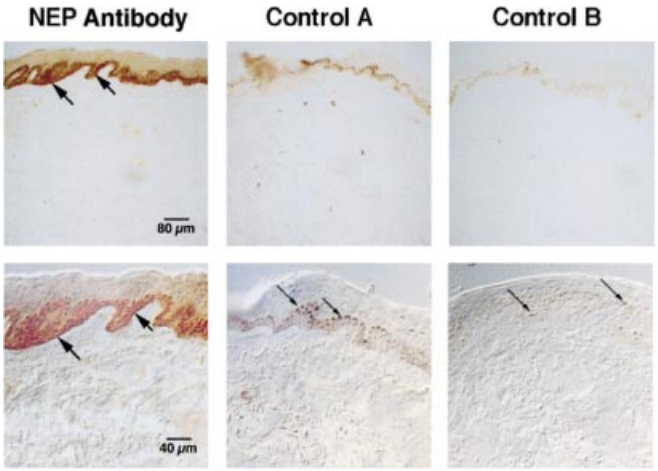

Figure 3. Unwounded human skin stains positively with NEP antibody. Low and high magnification using (differential interference contrast image capture) showing strong NEP staining in the basal layer of unwounded epidermis (indicated by *large arrows*). *Controls A* and *B* are as indicated in **Fig 2**. Small arrows indicate melanin staining in epidermis.

ent for all 14 subjects regardless of skin region (legs, thighs, arms, and sacrum). Preadsorbtion with recombinant NEP (**Fig 3**, control A) or omission of the NEP antibody (control B) abolished staining.

Other cutaneous structures noted to be stained by the NEP antiserum were cutaneous glands, particularly eccrine glands, blood vessels, and nerves (**Fig 4**). The epithelial cells of the sweat ducts were most prominently stained, but staining of the myoepithelial cells of the glands was also observed. Blood vessels showed NEP staining which was more faint than the epithelial staining in most subjects. In some subjects, there was dramatic NEP staining of the dermal vessels, particularly in the papillary dermis. Both endothelial cells and pericytes appeared stained. The large nerves of the dermis appeared stained in perineural tissue and, in a patchy pattern, within the nerve bundle itself. The nerve filaments in the papillary dermis and the epidermis showed no staining.

A biopsy from normal scalp with mild androgenetic alopecia revealed prominent NEP staining of the follicular epithelium. Using standard definitions of follicle morphology (Headington, 1984) terminal follicles had positive staining of the connective tissue sheath of the perifolliculum and the outer root sheath. The cells

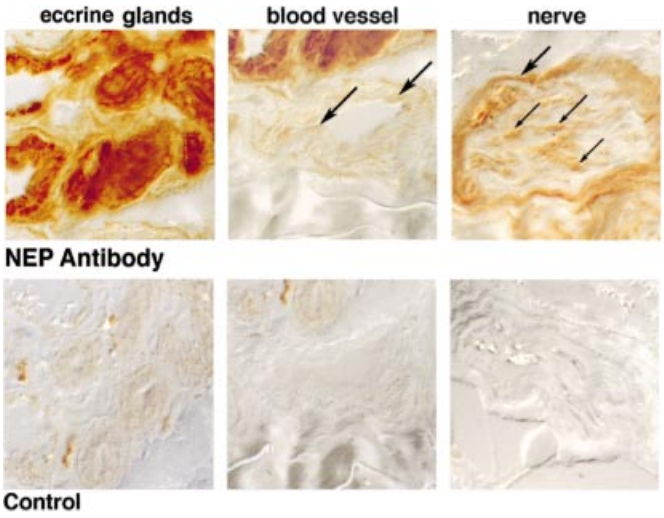

Figure 4. Eccrine glands, nerves, and blood vessels show NEP staining. Eccrine glands show strong NEP staining. The blood vessel shows a weaker NEP staining (*arrows*). The perineural sheath of a large nerve in the reticular dermis shows moderate NEP staining (*arrows*) and patchy staining is seen in the nerve bundle as well. *Controls*: NEP antibody preadsorbed with recombinant NEP.

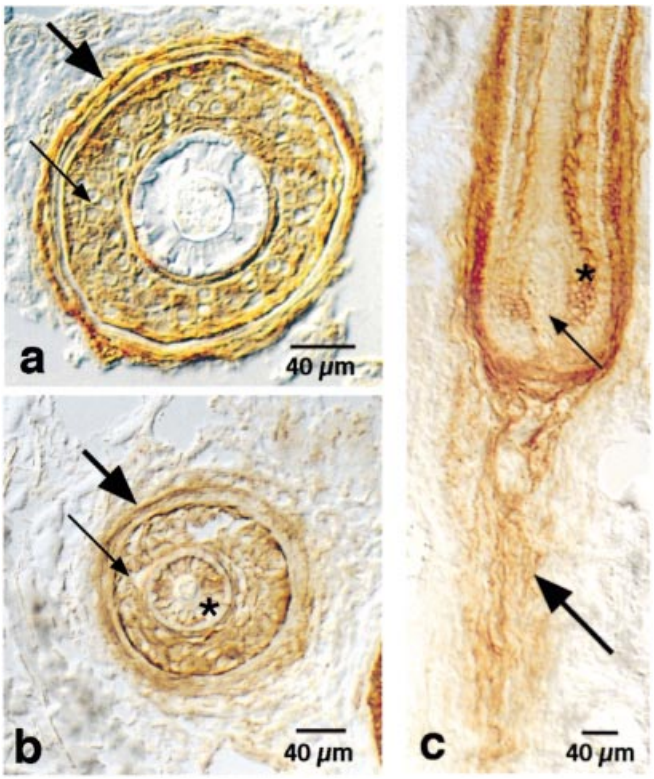

Figure 5. Hair follicles stain for NEP. (*a*) Horizontal section of terminal follicle: NEP staining of perifolliculum (*large arrow*) and outer root sheath (*small arrow*) (*b*) Horizontal section of vellus follicle. NEP staining of the perifolliculum (*large arrow*), outer root sheath (*small arrow*), and Huxleys' layer of the inner root sheath (*), and (*c*) vertical section of a follicle. NEP staining of the hair matrix (*), dermal papillae (*small arrow*), and follicular stela (*large arrow*).

of the inner root sheath, including both Henle's and Huxley's layer, were not immunoreactive for NEP. Vellus follicles also demonstrated NEP staining in the connective tissue sheath and the outer root sheath, but in contrast to the terminal follicles, showed positive staining of Huxley's layer of the inner root sheath. This differential staining pattern is demonstrated in terminal (**Fig 5*a***) *versus* vellus

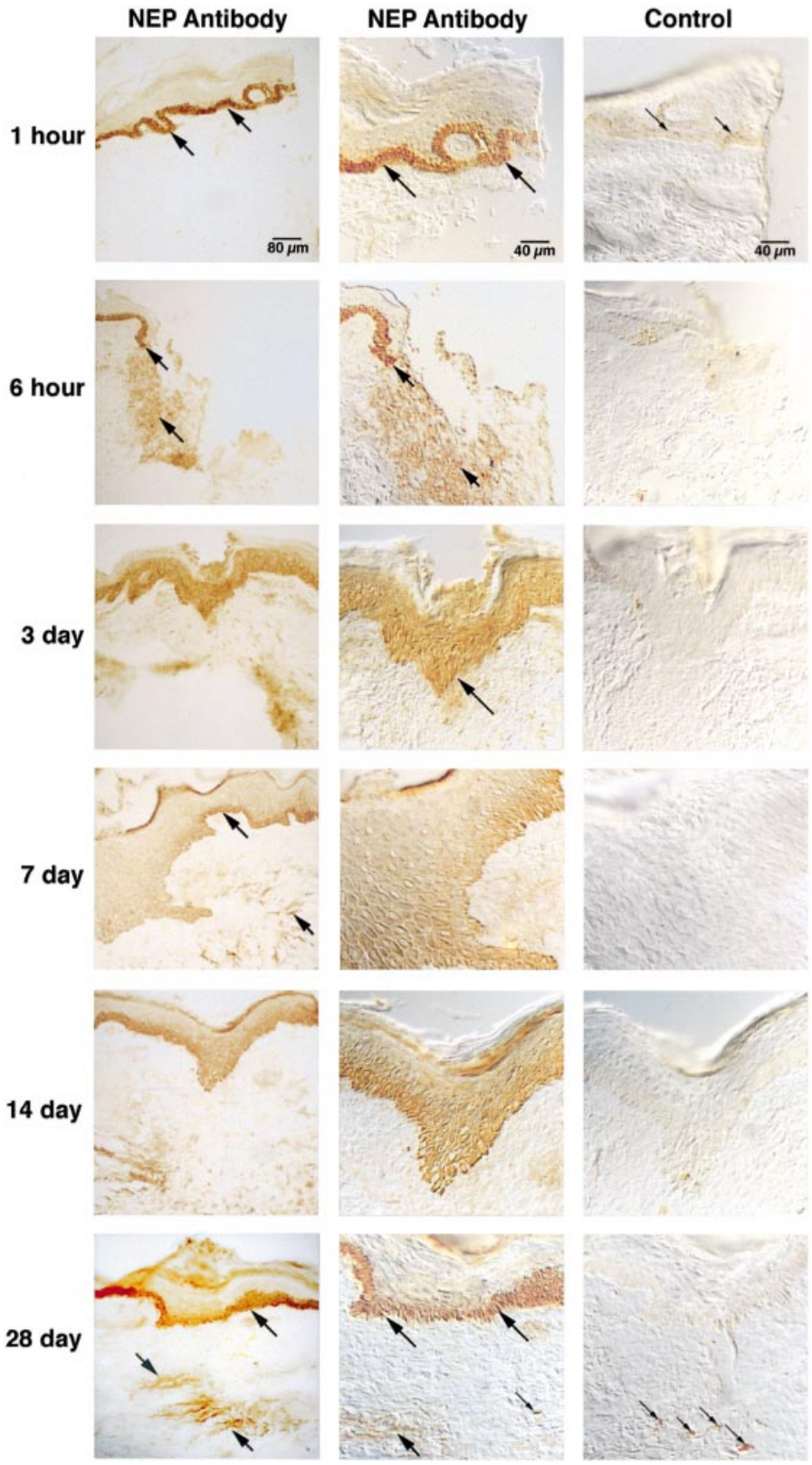

Figure 6. Partial thickness human wounds show changes in the distribution of NEP staining from 1 h to 28 d after wounding. One hour wounds show a freshly cut wound edge with no epidermal migration and no staining of the wound matrix but positive staining in the basal cells of the epidermis adjacent to the wound (*arrows*). The *arrowhead* indicates the cut edge of the wound. Six hour wounds show no migrating wound epidermis, but positive staining in the wound matrix and positive staining in the basal cells of the epidermis adjacent to the wound. Three day wounds and 7 d show diffuse NEP staining throughout the wound epithelium, in the epidermis adjacent to the wound ("transition epithelium") and in the wound matrix. Fourteen day wounds show diffuse staining throughout the wound epithelium and wound matrix; however, the epidermis adjacent to the wound shows primarily basal cell staining. Twenty-eight day wounds show wound matrix staining and basal staining of the wound epidermis, and epidermis adjacent to the wound. Control IHC without primary antibody.

follicles (**Fig 5*b***) on horizontal sections. The NEP staining pattern appeared to be the same in anagen and telogen follicles. Vertical scalp sections revealed strong NEP staining of the hair matrix cells, the dermal papillae and follicular stela (**Fig 5*c***).

IHC shows NEP staining in wounds We then examined NEP expression in experimental human wounds. The pattern of IHC observed in wound tissue was consistent over all subjects studied (**Figs 6** and **7**). In 1 h wounds, the basal pattern of NEP staining extended to the cut edges of the wound. In 6 h wounds, the NEP basal expression was present at the cut edge of the wound as well as in the wound matrix. After 24 h, NEP staining of the epithelium was noted on the migrating tongue of epithelium as well as in the provisional wound matrix. In 3 d wounds, NEP staining occurred

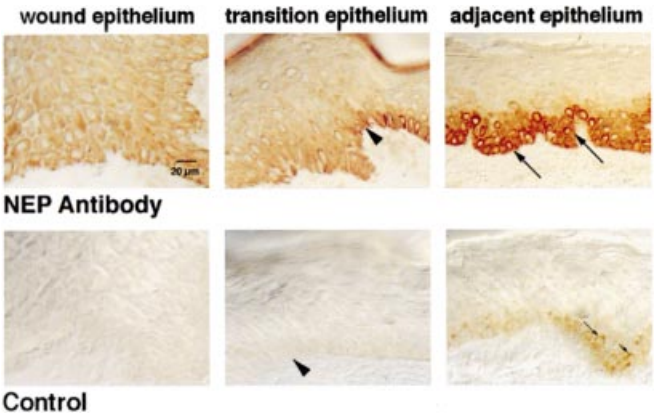

Figure 7. Higher magnification of the epidermis in a 7 d wound shows a transitional pattern of staining for NEP. NEP staining is diffuse throughout the wound epithelium and in the "transition epithelium" between wound and adjacent unwounded epithelium. It appears to resume basal staining adjacent to the "transition epithelium" (*arrowhead*). The epidermis beyond the "transition epithelium" shows basal cell staining (*small arrows*). *Arrowhead* in control "transition epithelium" indicates comparable region as in NEP antibody panel. *Small arrows* in control adjacent epithelium indicates melanin staining.

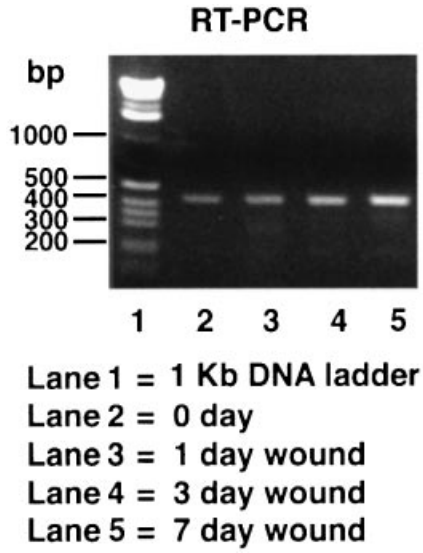

Figure 8. NEP mRNA is detected in skin and wounds by reverse transcriptase–PCR. *Lanes 2–5* show an approximately 400 bp product corresponding to the expected NEP reverse transcriptase–PCR product of 408 bp (base pairs 903–1311).

throughout the full thickness of the epidermis distinct from the basal pattern observed in unwounded epidermis. This full thickness pattern of epidermal staining extended for approximately 30–50 cells lateral to the wound margin in a region that we called the "transition epithelium." In 7 d wounds (**Fig 7**), the hypertrophic wound epithelium expressed NEP throughout the epidermis and the pre-existing wound margin showed expression of NEP throughout the epidermis of the "transition epithelium." The basal pattern was observed lateral to the "transition epithelium." By 14 d post-wounding, the new wound epithelium still appeared to have increased expression of NEP above the basal layer. After 28 d, the basal pattern of NEP staining was re-established, although in some wounds the eschar was not detached above the wound.

At each time point after 6 h, there was NEP staining within the dermal wound bed that appeared to be largely noncellular. The NEP staining initially appeared to be in the provisional wound matrix and later, bound to pre-existing dermal collagen and elastin. It is noteworthy that the 14 d and 28 d wounds had a considerable number of fibroblasts in the papillary dermis. These cells, known to be capable of expressing NEP, showed no significant staining.

NEP mRNA is detected in normal skin, wounds, and human keratinocytes by reverse transcriptase–PCR NEP mRNA was detected by reverse transcriptase–PCR in normal skin (day 0) and from day 1, 3, and 7 wounds (**Fig 8**). A 408 bp cDNA product was produced. DNA sequencing of the PCR product demonstrated that it is identical with the known cDNA sequence for NEP (base pairs 903–1311) (Malfroy *et al*, 1988). To confirm that NEP is expressed by keratinocytes we used reverse transcriptase–PCR to amplify a 313 bp cDNA product from subconfluent keratinocytes and from confluent keratinocytes. DNA sequencing of the PCR product demonstrated that this product is identical with the known cDNA sequence for NEP (base pairs 461–774) (Malfroy *et al*, 1988) (**Fig 9**). Levels of glyceraldehyde-3-phosphate dehydrogenase expression were used in all reverse transcriptase–PCR experiments to confirm loading amounts were equivalent (data not shown).

NEP mRNA is detected in wounds, human keratinocytes, HDF, and HDMEC by northern blot analysis NEP expression in 3 d and 7 d wounds was also documented by northern blot analysis (data not shown). The mRNA detected using a 401 bp RNA antisense probe (base pairs 945–1346) produced exactly the same size band 3.1 kb as observed for the cDNA probe used for the human keratinocytes, HDF and HDMEC experiments (**Fig 10**). Normal skin and day 1 wounds were not tested because less than 20 μg of total RNA was extracted. We were unable to detect NEP mRNA by northern analysis with 10 μg of total RNA in the 3 d and 7 d wounds.

We used northern blotting to examine the relative expression of NEP mRNA of skin derived cells in culture. As indicated in **Fig 10**, NEP mRNA was detected using an 860 bp cDNA probe (base pairs 101–961) in all three cell lines by a single band at 3.1 kb. Our studies indicated that of the cell types, HDF by far expressed the highest level of NEP mRNA compared with human keratinocytes and HDMEC. The addition of 100 nM SP for 3 h resulted in a modest increase in HDF NEP mRNA but little or no change in human keratinocytes or HDMEC NEP mRNA expression. Thus, these data demonstrate that NEP mRNA is expressed in normal human epidermal and dermal cells.

NEP enzymatic activity is present in keratinocytes To confirm than NEP expressed by keratinocytes is biologically active we measured NEP activity using a fluorescent substrate degradation assay. Homogenized human keratinocytes demonstrated NEP enzymatic activity of 37.76 pmol of MNA per h per μg. The activity is specific for NEP as DL-thiorphan inhibited the enzymatic activity. Whereas this activity is clearly above baseline, it represents only approximately 1/20 the activity which has been measured in jejunal mucosa using the same method (Bunnett *et al*, 1993).

DISCUSSION

In this paper we have documented that NEP is expressed in the epidermis by keratinocytes. Several lines of evidence were used to document that keratinocytes express NEP. First, NEP was detected by IHC in the epidermis of normal human skin and wounds. Second, both reverse transcriptase–PCR and northern blot methods demonstrated that cultured keratinocytes transcribe NEP mRNA. Third, an enzymatic assay showed specific NEP activity in cultured keratinocytes, although at a lower level than previously measured for jejunum using the same method (Bunnett *et al*, 1993).

It is not surprising that NEP is expressed by epidermis and epidermal appendages. The skin is richly innervated and these epidermal structures have been shown to contain thin nerve fibers (Hilliges *et al*, 1995) which immunolabel for neuropeptides (Levy *et al*, 1992; Hosoi *et al*, 1993). NEP is the major SP degrading enzyme and it degrades other neuropeptides, such as calcitonin gene related peptide, known to play an important part in cutaneous neuroinflammation (Katayama *et al*, 1991). Other densely innervated

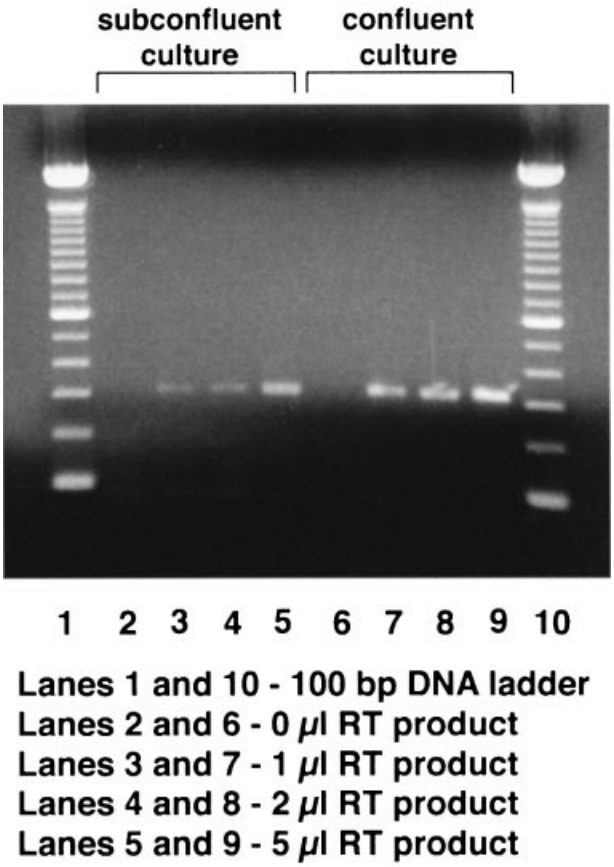

Figure 9. NEP mRNA is detected in cultured human keratinocytes by reverse transcriptase–PCR. *Lanes 2 and 6* show no PCR product as expected. All other lanes show an approximately 300 bp product corresponding to the expected NEP reverse transcriptase–PCR product of 313 bp (base pairs 461–774).

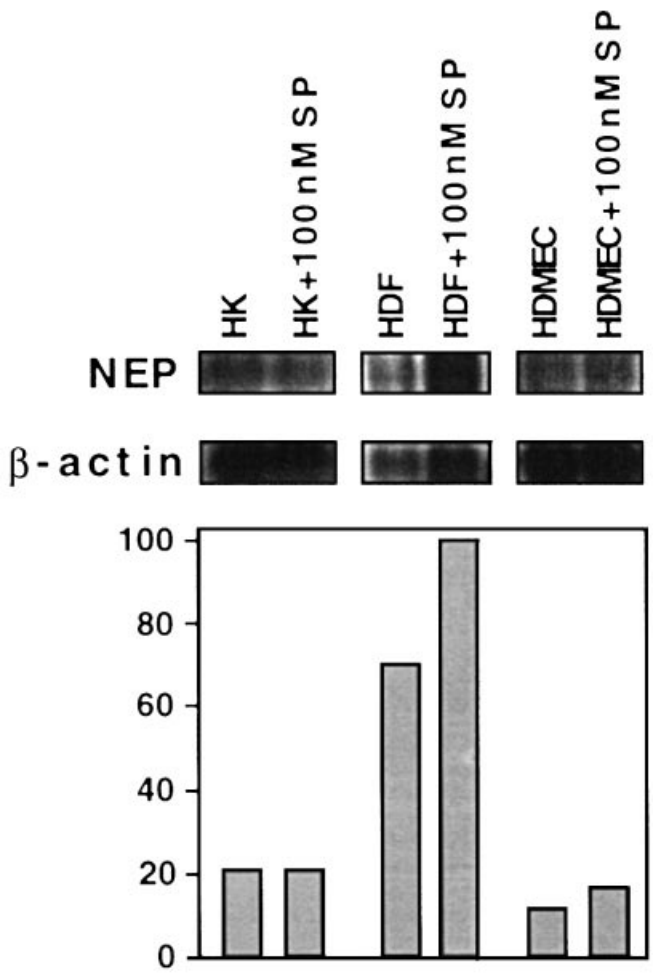

Figure 10. NEP mRNA is detected by northern blot analysis in human keratinocytes, human dermal fibroblasts (HDF), and human dermal microvascular endothelial cells (HDMEC). NEP mRNA expression is demonstrated in human keratinocytes, HDF and HDMEC derived from human foreskins. NEP mRNA expression (3.1 kb) was measured by northern blot analysis with or without the addition of 100 nM SP for 3 h to cultured human keratinocytes, HDF and HDMEC. Actin mRNA levels were measured to determine relative loading of RNA. The height of the bars is based on the percentage expression of NEP mRNA relative to β-actin.

epithelial structures such as nasal mucosa (Ohkubo *et al*, 1994), pulmonary epithelium (Johnson *et al*, 1985; Borson, 1991), and intestinal mucosa (Bunnett *et al*, 1993) have been shown by IHC and/or *in situ* hybridization methods to express NEP. In support of our results, previous studies of whole skin homogenates have shown NEP enzymatic activity in mice (Paus *et al*, 1994), guinea pigs (Iwamoto *et al*, 1989; Rubenstein *et al*, 1990), and humans (Bou-Gharios *et al*, 1995) but the anatomic localization of this activity was not described. Bou-Gharios *et al* (1995) showed NEP enzyme activity in human fibroblasts taken from patients with scleroderma but not from tissue sections by IHC. We also detected NEP in fibroblasts by northern blotting but did not observe staining by IHC in normal skin or wounds. One possible explanation for the difficulty in NEP localization by IHC is that the molecule is extensively glycosylated. NEP expressed by different cells may be glycosylated differently (Braun *et al*, 1984) and glycosylation may influence epitope recognition by IHC. In our studies of wounds, one must invoke such an explanation to account for the observation that the fibroblasts in the wound bed do not stain by IHC while keratinocytes and endothelial cells do stain. All three cell lines in culture can be demonstrated by northern blot analysis to express mRNA for NEP and, in our studies, fibroblasts expressed the highest level. Alternatively, cell culture may differ from the wound environment regarding stimulation of NEP expression in fibroblasts.

An explanation for localization of NEP to the basal layer of keratinocytes is not immediately apparent. Nerve filaments have been observed throughout the epidermis (Hilliges *et al*, 1995). NEP staining in a basal pattern has, however, been previously described in nasal mucosa (Ohkubo *et al*, 1994) and the lung (Nadel and Borson, 1991). The explanation may reside with localization of neuropeptide receptor expression, although this has not been studied in skin. The tachykinin receptors and NEP are coexpressed in many tissues, including the central nervous system (Waksman *et al*, 1986), the airways (Kummer *et al*, 1990; Kummer and Fischer, 1991), and the gastrointestinal track (Bunnett *et al*, 1993). Epithelial cells transfected with cDNA encoding NEP and/or SP receptor (SPR), effectively downregulate the interaction between SP and SPR only when NEP is colocalized on the same cell as SPR (Okamoto *et al*, 1994). Competition between receptors and degradative enzymes for neuropeptides may best explain the pattern of location for NEP observed in the epidermis. We hypothesize that the SPR is concentrated on the same cells that express NEP in the epidermis, but this has not yet been studied. Naturally, as noted above, we must also consider the possibility that the differential IHC staining in the epidermis and in wounds may be related to differential glycosylation of the NEP protein with masking of the antigenic site under certain conditions.

The hair follicles deserve separate mention. In 1994 Paus *et al* demonstrated hair growth induction by SP in mice during the telogen phase of the hair cycle. They also showed hair cycle-dependent activity of NEP. Our work demonstrates the presence of NEP in human follicles. Although we were not able to detect any difference in staining pattern of telogen *versus* anagen follicles

in this limited sample, the differential staining of inner root sheath in terminal *versus* vellus follicles and the staining of follicular stela is intriguing and deserves further study. One could speculate that different follicles might be induced or maintained, at least in part by neural regulation.

In wound healing, one would expect neuropeptides to be one of the earliest signals present in the wound environment. The cells that mediate the cutaneous repair process including keratinocytes, fibroblasts, endothelial cells, and inflammatory cells are responsive to SP (Ansel *et al*, 1996). The presence of NEP in the wound environment would downregulate the trophic effects of neuropeptides on the cells in the wound.

The full thickness staining pattern of the new wound epithelium and the adjacent "transition epithelium" at the pre-existing wound margin is unexplained. One could speculate about the importance of these cells in the secretion of NEP in the wound environment or possibly, the coexpression of NEP in cells with upregulated neuropeptide receptor expression.

At 1 h after an incisional wound, no change in the pattern of expression of NEP is seen at the epithelial wound margin and no NEP staining is present in the provisional wound matrix. By 6 h, only the basal cells express NEP in the epidermis but staining is also present in the provisional wound matrix. It has been previously shown that human fibroblasts in culture take approximately 1 h to begin synthesizing mature NEP (Lorkowski *et al*, 1987). NEP staining is consistently observed in the wound matrix after 6 h and it appears to be largely associated with extracellular matrix. While NEP is generally thought of as a membrane bound metalloendopeptidase, it has been shown to be secreted in certain physiologic fluids such as plasma, cerebrospinal fluid, amniotic fluid, and seminal plasma (Spillantini *et al*, 1990). The concentration of NEP seems to increase during inflammatory processes in the synovial fluid (Appelboom *et al*, 1991) and in the respiratory secretions in sarcoidosis and adult respiratory distress syndrome (Johnson *et al*, 1985). Frog skin has been shown to secrete a 100 kDa metalloendopeptidase, distinct from NEP, which is thought to be a prototype of the metalloendopeptidase family (Carvalho *et al*, 1992).

In summary, we have demonstrated the localization of NEP expression in human skin and in partial thickness cutaneous wounds. We speculate that neuroinflammation may play an important part in wound healing. Patients with diabetes[1] or spinal cord injuries[2]exhibit a decreased number of neuropeptide-containing nerves and show impaired wound healing. It will be of interest to study expression of NEP, neuropeptide, and neuropeptide receptors in such patients. Likewise, it will be of interest to study these mediators of neuroinflammation in a variety of inflammatory skin diseases such as psoriasis, atopic dermatitis, and hair disorders.

NIH RO1 AR 43006–001, the Odland Endowed Chair and a Warren G. Magnuson Scholarship supported this research. We thank Dr. Phil Fleckman for cultured keratinocytes, Robert Underwood, Lara Muffley, Dr. Eleanor Letran, and Jing Shan for assistance in sample collection, and Linda Foy for technical assistance with molecular biology.

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