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Epidermal denervation and its effects on keratinocytes and Langerhans cells

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Summary

Skin innervation has been considered to subserve sensory perception only, but several lines of evidence suggest that there are 'effector' influences of skin innervation on the immune system and keratotinocytes. In this study, we transected the sciatic nerves of rats and examined the effects of denervation on the epidermis. In normal skin, the epidermis was densely innervated by fine axons that were immunostained with several axonal markers, including neuronal ubiquitin carboxyl terminal hydrolase (protein gene product 9.5). All of the epidermal axons in the regions innervated by sciatic nerve disappeared within 24–48 h after transection of sciatic nerve, and remained absent as long as subsequent reinnervation by regenerating axonal sprouts was prevented. Denervation produced changes in both the keratinocytes and the Langerhans cells, the bone marrow-derived antigen-presenting cells of the epidermis. The thickness of epidermis decreased within 7 days. By 48 h after transection, the Langerhans cells and their dendritic processes became intensely immunoreactive for protein gene product. Protein gene product 9.5 expression on Langerhans cells remained prominent as long as skin was denervated, but disappeared with reinnervation. By reverse transcription-polymerase chain reaction, we demonstrated the presence of the transcripts for protein gene product 9.5 in epidermis, consistent with the synthesis of the protein by the Langerhans cells. We conclude that epidermal sensory fibres have novel influences on both keratinocytes and Langerhans cells of the epidermis.

Introduction

Innervation of skin by nociceptive nerve fibres is required for normal protective cutaneous sensibility. Until recently the sensory nerve fibres innervating the skin were thought to end primarily in the dermis or dermal-epidermal junction, and their penetration deep into the epidermis and between keratinocytes was considered to be exceptional (Cauna, 1959; Light & Perl, 1993). Sensitive immunocytochemical techniques have now shown that a large number of fine unmyelinated fibres normally cross the dermal-epidermal junction and end within the epidermis (Kruger *et al.*, 1985; Dalsgaard *et al.*, 1989; Karanth *et al.*, 1991; Kennedy & Wendelschafer-Crabb, 1993; McCarthy *et al.*, 1995). Among the axonal markers, immunostaining for protein gene product 9.5 (PGP), a carboxy-terminal ubiquitin hydrolase, has proved particularly sensitive in identifying small fibres (Doran *et al.*, 1983; Thompson *et al.*, 1983; Wilson *et al.*, 1988; Wilkinson *et al.*, 1989).

The epidermal fibres are presumed to have afferent nociceptive functions (Light & Perl, 1993). In addition, some of these fibres contain substance P or calcitonin gene-related peptide (CGRP) (Gibbins *et al.*, 1985; Kruger *et al.*, 1985; Gibbins *et al.*, 1987; Morris & Gibbins, 1989; Micevych & Kruger, 1992). Putative efferent functions of these fibres have recently been postulated (Stead *et al.*, 1989; Bienenstock *et al.*, 1991; Hukkanen *et al.*, 1991), and the term, 'noceffector' fibres, has been proposed (Kruger, 1991; Micevych & Kruger, 1992). For example, denervation of skin has been reported to up-regulate transcripts for nerve growth factor by basal keratinocytes (Mearow *et al.*, 1993). Another indirect indication of an efferent role is the demonstration that CGRP-positive fibres contact Langerhans cells in the epidermis, and that in tissue culture, CGRP alters antigen presentation by Langerhans cells (Hosoi *et al.*, 1993). The epidermal Langerhans cells are ramified dendritic cells of the

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epidermis of bone marrow origin. They are constitutively MHC class II-positive, and represent the major antigen-presenting cells of the skin (Agger *et al.*, 1990; de Fraissinette *et al.*, 1990).

In this study we further characterized the normal innervation of epidermis in the rat foot, and assessed the consequences of denervation on the two major cell types, the Langerhans cells and the keratinocytes, by means of immunocytochemistry and by reverse transcription-polymerase chain reaction (RT-PCR). Taken together, the results showed that denervation resulted in a rapid decrease in epidermal thickness, indicating an effect on keratinocytes, and a marked increase in PGP expression by the Langerhans cells. These findings provide direct evidence *in vivo* for the influence of epidermal fibres on both keratinocytes and epidermal Langerhans cells.

Materials and methods

Reagents

The source and characteristics of primary antibodies for immunocytochemistry are listed as follows: protein gene product 9.5 (PGP, 1:1000, UltraClone, UK), calcitonin gene-related peptide (CGRP, 1:1000, a kind gift of Dr Ian Dickerson, Miami, FL), p75-low affinity nerve growth factor receptor (p75-NGFR, 1:10, Boehringer-Mannheim, Indianapolis, IN), synaptophysin (1:10, Biogenex, San Ramon, CA), tyrosine hydroxylase (1:500, Boehringer-Mannheim, Indianapolis, IN), dopamine β-hydroxylase (1:500, Chemicon, Temecula, CA), ubiquitin (1:500, Chemicon, Temecula, CA), rat MHC Class II, Ia molecule (MRC OX-6) (1:40, Serotec, Indianapolis, IN). Other immunocytochemical reagents, including biotinylated secondary antibodies and Avidin-Biotin kit were purchased from Vector (Burlingame, CA). 3,3'-diaminobenzidine (DAB) was from Polysciences (Warrington, PA). RNA STAT-60 for RNA extraction was from Tel-Test 'B,' Inc (Friendswood, TX). Reagents for reverse transcription-polymerase chain reaction, including AMV reverse transcriptase, random primers, oligo (dT) primers, and Taq DNA polymerase were from Boehringer-Mannheim (Indianapolis, IN) except 2'-deoxynucleoside 5'-triphosphate (dNTP) from Pharmacia (Piscataway, NJ).

Nerve transection

Groups of male 8-week-old Sprague-Dawley rats (200–250 g) were used. Under pentobarbital anesthesia (45 mg kg^{-1}), the sciatic nerve on the right side was transected at the thigh level following the established procedure (Crawford *et al.*, 1995). To retard subsequent regeneration, we cut away a segment (3–5 mm) of the transected sciatic nerve. The left sciatic nerve of each animal was sham-operated as the control.

Preparation of epidermal sheets

The preparation of epidermal sheets followed the procedure of Baker and Habowsky (Baker & Habowsky, 1983). Briefly, the fresh skin was kept in phosphate buffered saline (PBS) and incubated in EDTA for 2 h at 37° C. The epidermis was removed by a forceps. The quality of these preparations was assessed in preliminary studies in which the sheets were fixed, prepared as described below, and sectioned perpendicularly.

Paraffin-embedded skin sections and morphometry

For comparison of the epidermal thicknesses, animals were perfused and post-fixed in 4% paraformaldehyde overnight. Skin tissues were embedded in paraffin. Animals 1 and 2 weeks after sciatic nerve transection (duplicate at each time point) were used. Both control and denervated skin were processed at the same time. Care was taken to ensure all the tissue blocks were embedded in correct orientation, and sectioned perpendicularly. For each tissue, sequential 5 μm-sections were cut and every fifth section was stained with hematoxylin and eosin. Three sections per tissue were used for assessing the thickness of the living layers of epidermis, which was defined as the distance between the epidermo-dermal junction and the top of the outermost granular layer, and was measured with Bioquant software (R&M Biometrics, Inc., Nashville, TN) in a coded fashion. The observers were blind to the coding information of the slides. On each slide, 10–15 measurements were made with interval of 100 μm along the strip of epidermis. The data were pooled together according to two parameters: time after sciatic nerve transection and status of denervation, and expressed as mean ± SD.

Immunocytochemistry

For immunocytochemistry, animals (at least three per time point) were killed by intra-cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer. The following time points after sciatic nerve transection were examined: days 1, 2, 3, 5, 7, 10, 14, 21, 42, 56, and 84. To study the re-innervation of skin, sciatic nerves of another group of animals were crushed for 30 s at the thigh level, and killed 84 days after nerve crush, when most of the functional re-innervation was established. The skin regions innervated by sciatic nerve (Devor *et al.*, 1979; Wall & Cusick, 1984), including glabrous skin (footpads, non-pad regions of soles and the 4th and 5th toes), and pilary skin (lateral side of heels, posterior to the lateral malleolus) were fixed for another 6 h, and then changed to the buffer for storage. After thorough rinsing in buffer, the fixed skin samples were processed as described before (Hsieh *et al.*, 1994a). Briefly, they were cryoprotected with 20% glycerol in 0.1 M phosphate buffer overnight. Vertical sections (30 μm) were cut on a sliding microtome. For footpads, both transverse and vertical sections were cut. Sections from each tissue were labelled sequentially and stored with anti-freeze at −20° C. To ensure adequate sampling, every fourth section for each tissue was chosen for PGP immunostaining. The sections were treated with 0.5% Triton-X 100 in 0.5 M Tris buffer (Tris) for 30 min and processed for immunostaining. Briefly, the sections were quenched with 1% H_2O_2 in methanol, and blocked with 5% normal serum of appropriate species in 0.5% non-fat dry milk in Tris. The sections were incubated with primary antibody in 1% normal serum in Tris for 16–24 h. After rinsing in Tris, the sections were incubated with biotinylated secondary antibody made in appropriate species for 1 h, and the avidin-biotin complex for another

hour. The reaction product was demonstrated by 3,3'-diaminobenzidine (DAB).

Thin sections of immunostained vibratome sections

Paraformaldehyde-fixed footpads of rats were sectioned on a vibratome. The sections were incubated with antibody against PGP as free-floating sections. After reaction with DAB, the sections were osmicated, dehydrated in ethanol and embedded in resin (Hsieh *et al.*, 1994b). Selected areas were thin sectioned, stained with uranyl acetate, observed under a Hitachi electron microscope and photographed.

Solution-phase reverse transcription polymerase chain reaction

We performed solution-phase reverse transcription polymerase chain reaction (RT-PCR) on epidermal sheets. Rats were anesthetized and killed by cardiac puncture 2 weeks following denervation. Two 3 mm skin punches were taken from the soles of each animal. The sites of skin punch were in the centre of the sole, surrounded by footpads, and were comparable among different animals. Epidermal sheets were prepared from the skin punches as described above and quickly frozen in dry ice and stored at -70° C until use. The extraction of RNA followed the guanidine isolation method (Chomczynski & Sacchi, 1987). Epidermal sheets were homogenized with RNA STAT-60 containing guanidine thiocyanate and phenol. Total RNA was extracted with chloroform, precipitated with isopropanol, washed with ethanol, and dried to pellet with speed vacuum. The RT-PCR followed an established protocol (Wesselingh *et al.*, 1993). Briefly, the RNA pellets were resuspended with water to make cDNA by adding AMV reverse transcriptase, dNTP, oligo (dT) and random primers. Polymerase chain reaction (PCR) with 25 thermal cycles was performed by using Taq DNA polymerase. The PCR products were run on 1.2% agarose gels, and transferred to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL) overnight. The blots were hybridized with specific probes labeled with ^{32}P in a 55° C oven overnight. The hybridized blots were washed with 6XSSC (standard saline citrate), dried with 3M filter paper, and exposed to X-ray film. Appropriate positive and negative controls were included at each step: RNA extraction, cDNA synthesis and polymerase chain reaction. The nucleotide sequences of primers and probes, and their corresponding positions for protein gene product 9.5 (PGP) (Kajimoto *et al.*, 1992) in mRNA are as follows:

5'primer ATGAAGCAGACCATCGGGAAC (277–297)
3'primer GCTAAAGCTGCAAACCAAGGG (761–781)
probe GAAAGGCATTCGCCCATCGAGCTCGTAGAG (547–576)

All were designed to cross introns. The PCR product was 505 nucleotides in size.

Statistical analysis

Statistical comparison was performed by using SPSS software (Chicago, IL). At each time point, *t*-test was used to compare control and denervated groups. Any difference with $p < 0.05$ was considered statistically significant.

Results

CHANGES IN HISTOLOGY OF THE DENERVATED EPIDERMIS

The normal rat footpad had the expected histologic

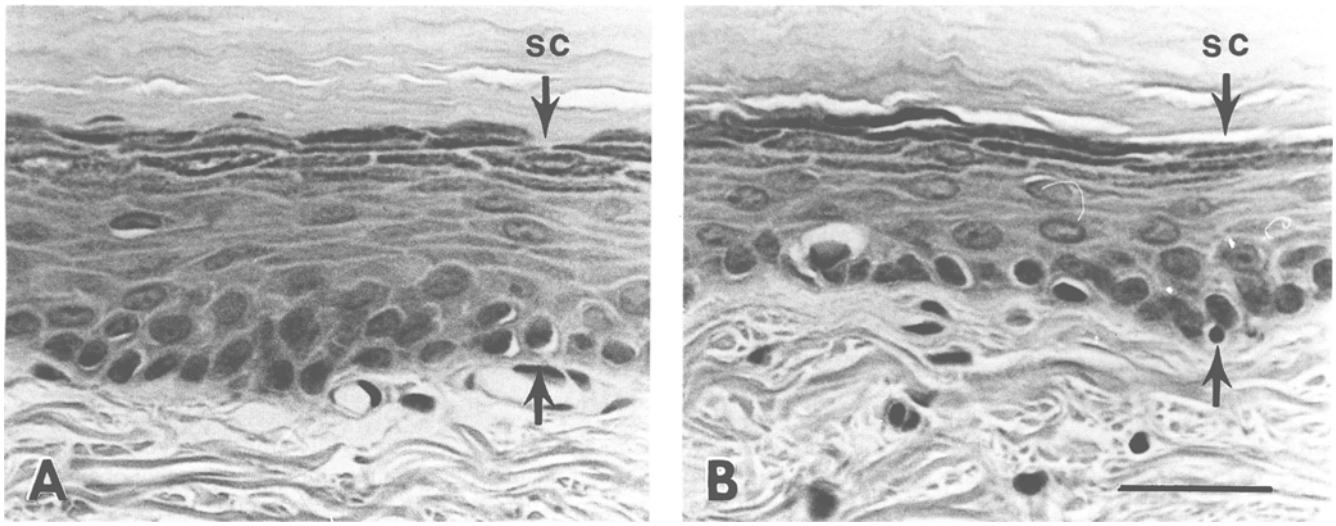


Fig. 1. Gross appearance of normal and denervated skin. The rat skin was embedded in paraffin and stained with hematoxylin and eosin. Shown in (A) is an example of normal skin. The organization of epidermis include living layers (indicated by the opposing arrows): the basal layers and the granular layers. Outside these are stratum corneum (sc). The basal cells possess proliferation potentials, and have a high nuclear to cytoplasm ratio. During the process of differentiation, the keratinocytes move upwards, appear flattened in the granular layers, and finally become anucleated in stratum corneum. (B) This skin section is from the same animal 2 weeks after denervation. The overall organization of epidermis includes the basal layers, granular layers, and stratum corneum, and is similar to that of normal epidermis except that the living layers (inside the opposing arrows) are thinner that of the normal ones. Scale bar = 10 μm.

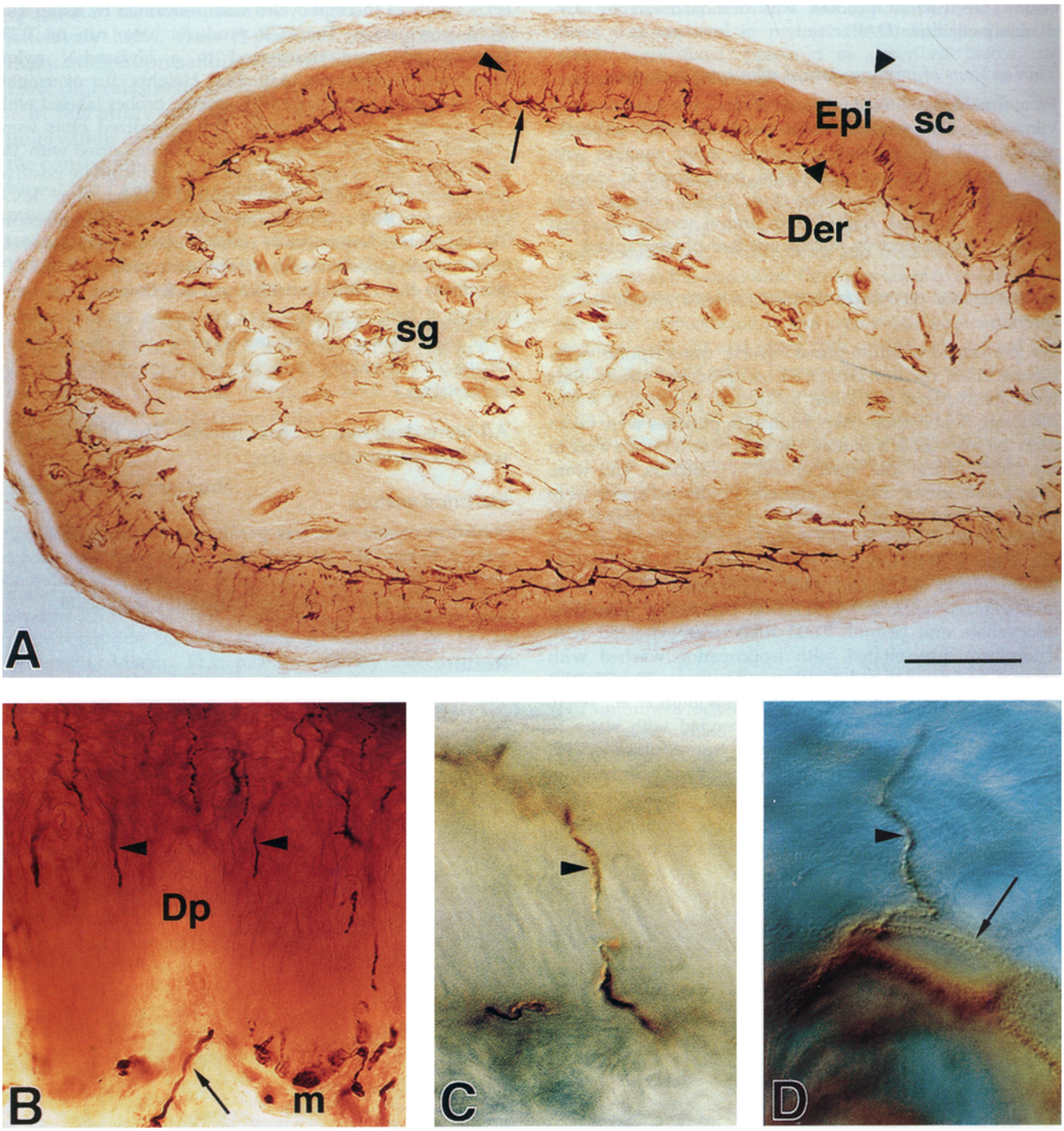


Fig. 2. Normal innervation of rat footpad. The rat footpad was immunocytochemically stained with the antibody against protein gene product 9.5 (PGP) (A,B), calcitonin gene-related peptide (CGRP) (C), and p75 low-affinity nerve growth factor receptor (p75-NGFR) (D). (A) In this lower-magnification image, the opposing solid triangles indicate the extent of epidermis (Epi). Inside this layer of skin is dermis (Der). The subpapillary dermal nerves (arrow) usually parallel the border of basal epidermis and penetrate vertically into epidermis (arrowhead), sometimes ending in the stratum corneum (sc). Axons innervating sweat glands (sg) were immunostained with PGP. (B) Several PGP-immunoreactive axons (examples identified by arrowheads) run perpendicularly to the epidermis. The arrow indicates a dermal axon. The Merkel cell (m) in the basal epidermis was also immunostained with PGP. (DP = dermal papillae.) (C) A CGRP-positive axon (arrowhead) penetrating the epidermis. (D) The arrowhead indicates an axon positive for p75-NGFR in epidermis. p75-NGFR immunoreactivity is also present on the membrane of basal keratinocytes (arrow). In the area where the dermal nerve fibres pass by the basal surface of keratinocytes and enter the epidermis, the staining for p75-NGFR is exaggeraated. Scale bars, A = 600 μm; B = 30 μm; C and D = 40 μm.

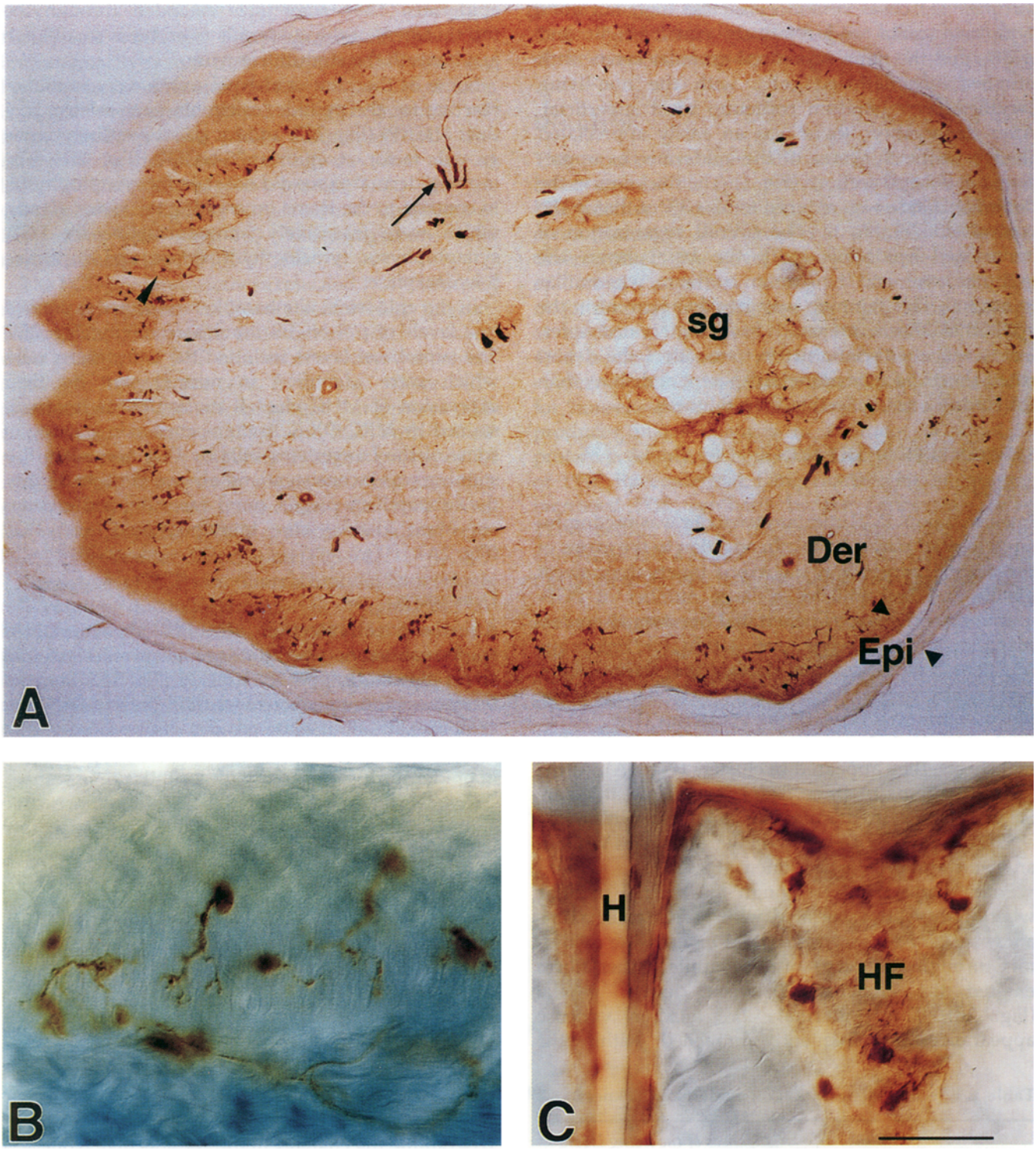


Fig. 3. Denervated skin immunostained with protein gene product 9.5 (PGP). (A) In the epidermis (Epi, bounded by the two opposing solid triangles), the epidermal axons have disappeared, but prominent PGP-positive cells (arrowhead) are present in the epidermis. In the denervated dermis (Der), PGP-immunoreactive subpapillary dermal axons and axons innervating sweat gland (sg) disappear. The cells in denervated dermal nerve trunks (arrow) are stained with PGP. These cells at higher resolution proved to be denervated Schwann cells. (B) In the epidermis of glabrous skin, these PGP-immunoreactive cells have prominent dendritic processes. (C) In denervated hairy skin, demonstrated by the hair shaft (H), and hair follicle (HF), epidermal axons have disappeared, but prominent dendritic cells are present along the hair follicle. Scale bars, A = 600 μm; B and C = 40 μm.

features: the basal keratinocytes had high nucleus/cytoplasm ratios and relatively equal height and width, and the more superficial granular layers with progressively flattened cells, with the non-living cornified layer (stratum corneum) most superficial. After denervation, there was an easily appreciated change in the thickness of the epidermis, as appreciated in vertical sections (Fig. 1). The thickness in the denervated side was reduced to 57–63% of that in the control side (Table 1). The overall organization of denervated epidermis, with the basal layer, the granular layer, and the stratum corneum, was similar to normal. This thinning was grossly apparent in the epidermal sheets prepared from denervated soles. One week after sciatic nerve transection, the denervated sheets became more transparent and shiny than the control ones, reflecting their thinness.

NERVE FIBRES OF THE SKIN

Normal

In normal skin, PGP immunoreactivity was predominantly located within axons. In addition to the axons of the dermal nerves and the axons innervating sweat glands and hair follicles, a large number of PGP-immunoreactive axons penetrated the dermal-epidermal junction and were seen within the epidermis (Fig. 2A). Vertical sections showed that these epidermal axons emerged from the superficial dermal nerve plexuses running beneath the epidermis (Fig. 2A). In PGP-stained whole mounts prepared from the epidermal sheets, they appeared in clusters (Fig. 4A). At the EM level, some of these axons were ensheathed by Schwann cells in dermis (Fig. 5A), but within the epidermis they passed between keratinocytes without any Schwann cell ensheathment (Fig. 5B).

Some axons passed through the dermal papillae where they were associated with perivascular axons and papillary sensory fibres, and entered the epidermis through the dome of the papilla. Other fibres entered the epidermis between dermal papilla (Fig. 2B). The epidermal fibres had a varicose appearance and frequently branched within the epidermis. They sometimes extended through the full thickness of the epidermis to end in a small knob just beneath the stratum corneum.

Some of the intra-epidermal axons were immunoreactive for the peptide, calcitonin gene-related product (CGRP) (Fig. 2C), and for p75-low-affinity nerve growth factor receptor (p75-NGFR) (Fig. 2D). The intra-epidermal axons did not stain for synaptophysin, in contrast to the axons innervating the sweat glands, which were intensively immunoreactive for PGP, synaptophysin, tyrosine hydroxylase, and dopamine β-hydroxylase.

In the epidermis, the basal keratinocytes expressed low levels of p75-NGFR immunoreactivity (Fig. 2D). The suprabasal layers included the Langerhans cells, which were identified in normal skin by their expression of MHC class II molecules, as immunostained by MRC OX-6. In the normal skin staining of some of the Langerhans cells by PGP was seen; this staining was inconsistent and always faint. In dermis, the subpapillary plexus, nervi vasorum, and endings on sweat glands were immunostained with PGP (Fig. 2A).

Denervation

After nerve transection, the PGP-immunostained epidermal axons and the axonal profiles around sweat glands disappeared quickly (Fig. 3A). Some of the intra-epidermal axons were still present 24 h after axotomy, but all of these profiles disappeared within 48 h. The staining for CGRP, and for axonal p75-NGFR in epidermis disappeared with a similar time course.

For a brief period after the PGP-immunoreactive epidermal axons disappeared, PGP immunoreactivity remained in the dermal nerve fibres, but the staining became progressively segmented and discontinuous, forming globular chains reflecting Wallerian degeneration. By 1 week after transection, all PGP-immunoreactive axons had disappeared from dermis. The PGP-immunoreactive epidermal axons remained absent as long as the skin remained denervated. The p75-NGFR immunoreactivity in basal keratinocytes and around sweat glands slightly increased after denervation.

Reinnervation

To examine the changes in intra-epidermal axons and Langerhans cells during reinnervation of the skin, we extended our observation at various time points after sciatic nerve transection. In some regions by 12 weeks after axotomy (the longest time examined), PGP-immunoreactive nerve fibres appeared in epidermis and around sweat glands, but were much thinner and varicose than normal (Fig. 6A and B). In the reinnervated skin, the intensity of p75-NGFR immunoreactivity around basal keratinocytes and in nerve trunks was indistinguishable from the control.

Table 1. Effects of denervation on epidermal thickness.

	Thickness of the living layers of epidermis (μm)*	
Days after denervation	*Control*	*Denervated*
7	64 ± 12	37 ± 12**
14	60 ± 10	38 ± 11**

* The measurement was based on 5 μm sections stained with hematoxylin and eosin, and expressed as mean ± SD. The results were from two animals at each time point. Detailed procedures of sampling were described in Materials and Methods.

** $p < 0.01$ by t-test.

CHANGES IN LANGERHANS CELLS WITH DENERVATION

Pari passu with the disappearance of the PGP-immunoreactive axons from the epidermis, prominent PGP immunoreactivity appeared in the Langerhans cells of the epidermis (Figs 3 and 4B). That the PGP immunoreactivity was within Langerhans cells was confirmed by the presence within the same cells of both PGP-immunoreaction product and Birbeck granules, the specific ultrastructural markers of Langerhans cells (Fig. 5C). The Langerhans cells remained negative for p75-NGFR.

The PGP-immunoreactive axons and PGP-immunoreactive Langerhans cells were reciprocally present. Thus, in the area where PGP-immunoreactive axons were present, the PGP immunoreactivity was absent or faint in Langerhans cells. In the area where PGP-immunoreactive axons were absent, Langerhans cells were strongly immunostained with PGP, both in their cell bodies and their elaborate dendritic processes. In certain area, where new axons growing into the epidermis approached Langerhans cells, both axons and some Langerhans cells could be visualized with PGP 12 weeks after denervation (Fig. 6A).

The source of PGP immunoreactivity in Langerhans cells could be due to local synthesis, or uptake of PGP by Langerhans cells. To address this issue, we performed reverse transcription-polymerase chain reaction (RT-PCR). By RT-PCR, PGP transcripts were detectable in both normal and denervated epidermis with more abundance in denervated one (Fig. 7). This finding suggest that local synthesis of PGP contributes to the PGP immunoreactivity in the Langerhans cells of the denervated epidermis.

Discussion

This study confirmed the extent of the normal innervation of the epidermis by fine non-ensheathed PGP-positive axons running between the keratinocytes (Kennedy & Wendelschafer-Crabb, 1993) and demonstrated the novel effects of denervation on epidermal Langerhans cells and keratinocytes. The major findings include: first, the rapid disappearance of epidermal fibres after nerve transection (within 48 h); second, the early reduction in the height of the epidermis and in the relative abundance of transcripts for 'housekeeping' genes, suggesting that denervation produces major changes in keratinocyte number, protein economy, or both; and finally, the expression of PGP by epidermal Langerhans cells, suggesting increased PGP synthesis by the Langerhans cells. These results indicate the sensory innervation of the epidermis has a richer spectrum of interactions with the epidermal cells than previously recognized.

Normal skin innervation

The present observations confirmed the great abundance of nerve fibres in epidermis. Intraepidermal axons were first suggested by Langerhans over 100 years ago (Langerhans, 1868), but subsequently often

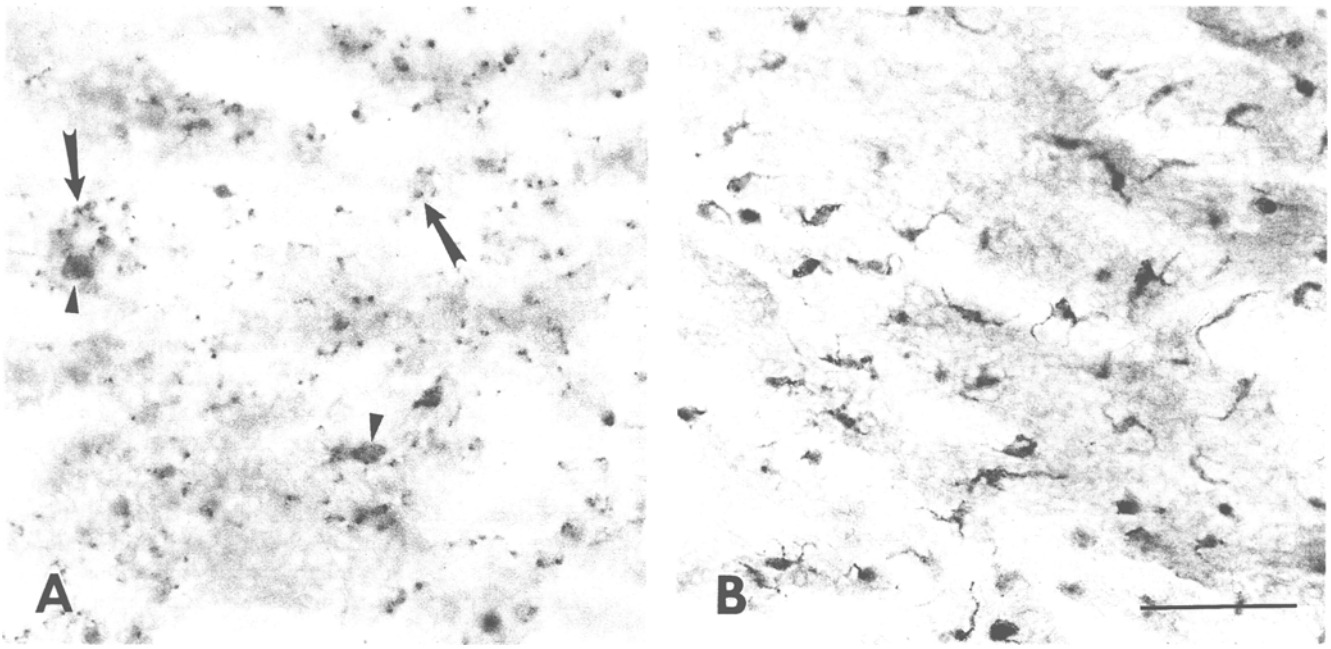
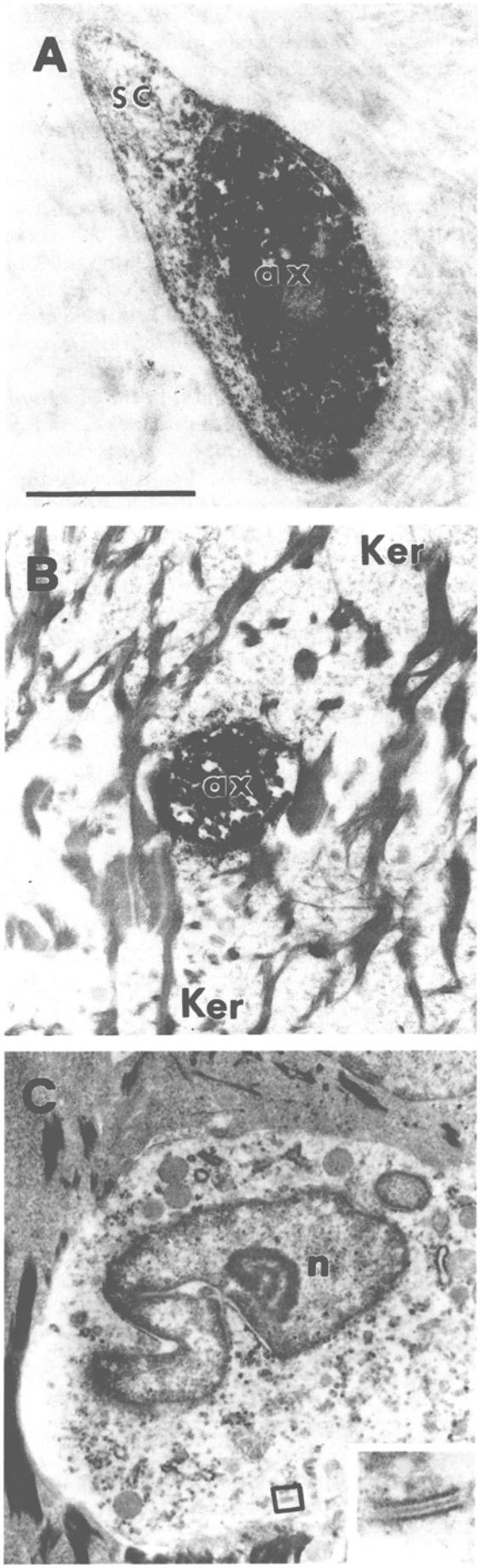


Fig. 4. Protein gene product 9.5 (PGP) immunoreactivity in epidermal sheets. The epidermal sheets were prepared as described in Materials and Methods, and immunostained with the antibody against PGP. (A) In control epidermis, PGP-positive axons appear as small knobs, and sometimes are in clusters (arrows). A few Langerhans cell bodies (arrowheads) are faintly stained. (B) In denervated epidermis, the small knobs representing epidermal axons have disappeared, and the Langerhans cells with their prominent dendritic processes are darkly immunostained for PGP. Scale bar = 16 μm.



dismissed as artifactual or rare (Arthur & Shelley, 1959; Cauna, 1959). The recent recognition of the extent of epidermal innervation has resulted from the development of sensitive immunocytochemical methods (Silverman & Kruger, 1989; Karanth *et al.*, 1991; Kennedy & Wendelschafer-Crabb, 1993; McCarthy *et al.*, 1995). The epidermal axons are only rarely visualized with silver stains (Ridley, 1969) or with anti-neurofilament antibodies, presumbly reflecting the relative small number of neurofilaments in these very fine fibres (Hoffman *et al.*, 1984). In contrast, protein gene product 9.5 (PGP), provides a highly sensitive marker for all axons, including the fine unmyelinated fibers terminating in the epidermis. Protein gene product 9.5 is an abundant, predominantly neuronal form of ubiquitin C-terminal hydrolase, a cytosolic enzyme that removes ubiquitin (Wilkinson *et al.*, 1989). It is transported within the slow component (SC_b) of axonal transport (Bizzi *et al.*, 1991). In spite of the abundance of this form of ubiquitin hydrolase in normal cutaneous nerves, we did not identify immunostaining for ubiquitin in axons, and the neurobiological significance of the large amount of PGP within the axons remains unknown.

One or both of the neuropeptides, CGRP and substance P, was present in a proportion of the epidermal axons (Kruger *et al.*, 1985; Silverman & Kruger, 1989), but these markers stained many fewer axons than seen with PGP. The nonpeptidergic PGP-positive epidermal fibres may arise from the lectin-positive sensory neurons described by Silverman and Kruger (1990). In addition, p75-NGFR was identified immunocytochemically on some of the epidermal fibres. This finding was expected, because these fibres are nerve growth factor (NGF)-dependent, and fail to develop on the absence of NGF (Crowley *et al.*, 1994), the high-affinity *trkA* receptor (Smeyne *et al.*, 1994) or, p75-NGFR (Lee *et al.*, 1992). The absence of ensheathing Schwann cells in epidermis presumably accounts for the unusually clear demonstration of p75-NGFR in the intra-epidermal axons. In unmyelinated fibers within the nerves and dermis, some p75-NGFR immunostaining is known to be localized in the

Figure 5. Ultrastructural correlates of protein gene product 9.5 (PGP)-immunoreactive profiles in skin. Immunostained vibratome sections of control footpads were thin-sectioned to correlate with the light microscopic observations. (A) In the dermis, the PGP-immunoreactive axon (ax) is ensheathed by a non-myelinated Schwann cell (sc). (B) In the innervated epidermis, a PGP-immunoreactive axon (ax) is devoid of Schwann cell ensheathment as it passes between keratinocytes (ker) with the electron-dense keratohyalin. (C) In the denervated epidermis, PGP immunoreactivity appears in a Langerhans cell. The Langerhans cell is recognized by its indented nucleus (n) and Birbeck granules (inset). Scale bar, A and B = 1 μm, and C = 2.25 μm.

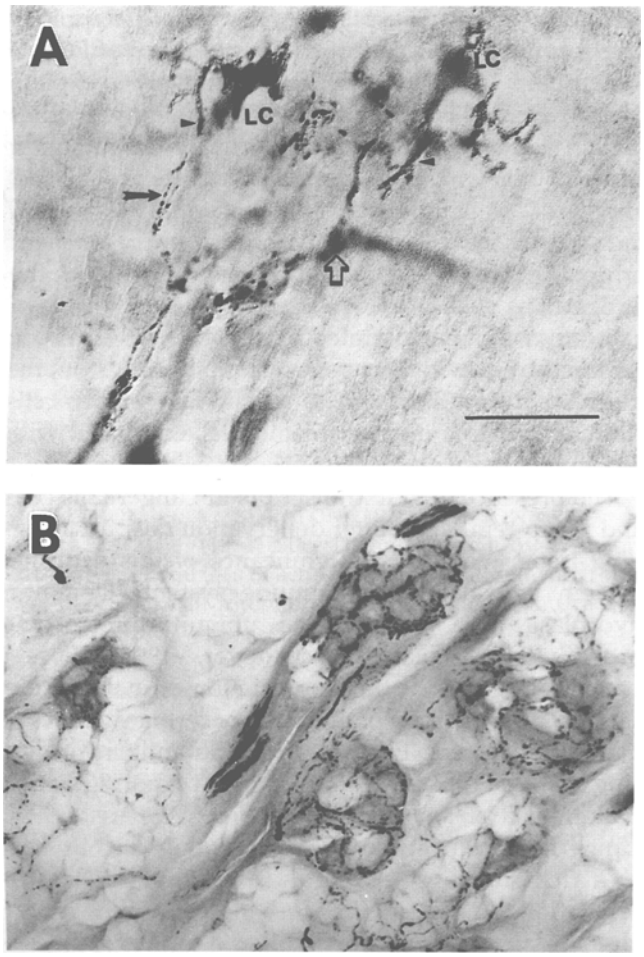


Figure 6. Presence of protein gene product 9.5 (PGP)-immunoreactivity in skin being re-innervated (12 weeks after denervation). (A) In re-innervated skin, the regenerating axons with the typical varicose appearance (arrows) approach the epidermis from the parent axons in dermis (empty arrow). In this area, both PGP-positive axons and Langerhans cells (LC) with their dendritic processes (arrowheads) are present. (B) In the dermis, there appear fibres re-innervating sweat glands. Some of these fibres are thin and knobby. Scale bar = 30 μm.

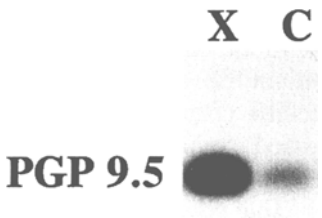


Figure 7. Transcripts of protein gene product 9.5 (PGP) in epidermis by reverse transcription-polymerase chain reaction (RT-PCR). RNA was extracted from two epidermal sheets of 3 mm in diameter, and reversed transcribed. Polymerase chain reaction was performed with the first strand cDNA for PGP according to the primers listed in Materials and Methods. After agarose gel electrophoresis, the PCR products were transferred to nylon membrane and hybridized with specific probes. Lane designation: **X** for denervated epidermis, and **C** for epidermis on the control side from the same animal. This experiement has been repeated on three animals; all showed the same patterns, and shown here was one of the three sets of Southern blotting. Each lane reflected the abundance of the PGP transcripts from two epidermal sheets of the same area with probably different volume (i.e. the epidermal volume may be different because of the difference in epidermal heights).

ensheathing Schwann cells (Ribeiro-da-Silva *et al.*, 1991) and surrounding perineurial cells (Ribeiro-da-Silva *et al.*, 1991), so that any p75-NGFR expression within the axon is obscured. In the epidermis there are no ensheathing cells; instead, the non-ensheathed axons run between adjacent keratinocytes.

The pattern of degeneration in transected cutaneous nerve fibres

The PGP-immunoreactive epidermal profiles disappeared after sciatic nerve transection, and re-appeared when reinnervation of the foot was allowed. Within 24 h after axotomy, many of the axonal profiles were gone, and all of the intra-epidermal axons disappeared within 48 h. In young rodents, disintegration of the axonal cytoskeleton is known to take place along the whole length of the sciatic nerve fibres within 48–72 h after axotomy (Griffin & Hoffman, 1993). The present data suggest that degeneration of the terminal parts of these axons may occur earlier than their parent axons. A similar phenomenon of early terminal degeneration applies to motor nerve endings in muscle (Miledi & Slater, 1970) and to degeneration of the primary sensory axons in the gracile nucleus (George & Griffin, 1994). Quantitation of epidermal axons in skin biopsies is currently being evaluated as a tool for assessment of the unmyelinated fibre involvement in human sensory neuropathies (McCarthy *et al.*, 1995).

Effects of epidermal innervation on keratinocytes

The prompt thinning of the epidermis in glabrous skin after denervation is an intriguing observation, but sensory denervation is known to produce thinning of the epithelium in some experimental systems. For example, sensory deprivation of tongue following glossopharyngeal nerve transection results in thinning of the circumvallate papillae in the epithelium of the tongue (Guth, 1957, 1958, 1963). Similarly, denervated taste buds atrophy (Guth, 1957) and in hairy skin, the Merkel cells, the mechanoreceptor cells at the epidermal-dermal junction, decrease in number (English, 1977). The present data also confirmed similar observation made by Svetikova and Chumasov (1987) and extend the list of epithelial changes predicted by denervation.

Keratinocytes are a continous renewing population: they proliferate in the basal layer, differentiate during their displacement upwards, and finally lose their nuclei and become cornified in the most superficial layers. Decreased renewal, shorter survival, or increased death of specific populations are among the mechanisms that could be involved. Alternatively, it is conceivable that the keratinocyte cytoskeletal proteins could be down-regulated, resulting in smaller keratinocytes. These mechanisms are not mutually exclusive and more than one could contribute to the thinning of epidermis. In any event, the present results are consistent with an important 'efferent' function of the epidermal afferent fibres.

Effects of denervation on the Langerhans cells

Protein gene product 9.5 is a novel and useful marker for assessing axon-Langerhans cell interactions during nerve fibre degeneration in skin. Two recent reports suggested the possibility of interactions between epidermal innervation and Langerhans cells (Hosoi *et al.*, 1993; Rice *et al.*, 1993). In human skin, Hosoi (1993) demonstrated that the CGRP-positive epidermal axons tend to cluster around Langerhans cells. By confocal microscopy and EM these axons appeared to contact the Langerhans cells (Hosoi *et al.*, 1993). The sites of contact contained vesicular profiles. That the Langerhans cells might respond to CGRP was suggested by the observation that their ability to present antigen to lymphocytes in culture was influenced by CGRP (Hosoi *et al.*, 1993). Another study found PGP immunoreactivity in the rat mystacial skin after denervation, and suggested that it was likely to be located in Schwann cells and Langerhans cells (Rice *et al.*, 1993).

As PGP-immunoreactive axons disappeared from epidermis after nerve transection, we found that immunoreactivity for PGP increased in the Langerhans cells of the epidermis. In principle, these immunoreactive profiles could reflect phagocytosis of axonal constituents, cross-reactivity of the antibody with newly expressed proteins, or new synthesis of PGP by Langerhans cells. The presence of increased PGP transcripts in epidermal sheets suggests from denervated skin that the protein is likely to be synthesized by Langerhans cells. We cannot exclude the possibility that uptake of axonal PGP protein might contribute to the prompt appearance of PGP in Langerhans cells. However, this is unlikely to explain the presence of PGP 3 months after axotomy. Studies of skin depleted of Langerhans cells by UV radiation showed renewal from dermal dendritic cells, and analyses of skin grafts indicated that within 3–7 weeks, the Langerhans cells in the epidermis were of recipient origin (Katz *et al.*, 1979; Krueger *et al.*, 1983). Thus, the Langerhans cells present in epidermis at the time of axotomy will have migrated to lymph nodes and been replaced by new cells entering the epidermis from the dermis within a few weeks, yet the Langerhans cells staining remains intense for months, consistent with *de novo* synthesis of PGP.

The presence of PGP transcripts in Langerhans cells and its up-regulation after denervation raise an intriguing issue: what is the significance of this neuronal protein in cells of the immune system? Ubiquitin hydrolase is a part of the ubiquitin-proteasome proteolytic pathway (Ciechanover, 1994). Recent studies suggest that the transduction of extracellular signals utilizes this pathway to exert a variety of biological process. Examples include antigen processing (Goldberg, 1994) and transcription regulation (Palombella *et al.*, 1994; Thanos & Maniatis, 1995). Langerhans cells are antigen-presenting cells as well as a rich source of cytokines in epidermis. The up-regulation of PGP in Langerhans cells may reflect altered antigen processing or altered mRNA synthesis in Langerhans cells after denervation. An attractive hypothesis is that denervation may change the behaviors of Langerhans cells and that altered cytokines or other Langerhans cell products may in turn alter keratinocyte survival, size, or both.

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