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# Neovascularisation precedes neural changes in the rat groin skin flap following denervation: an immunohistochemical study

S. Manek, G. Terenghi, C. Shurey, H. Nishikawa, C. J. Green and J. M. Polak

*Section of Surgical Research, Clinical Research Centre, Northwick Park Hospital, Harrow, and Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital*

*SUMMARY.* **We have employed immunohistochemical techniques to study neural and vascular changes in rat skin flaps. Following partial or total denervation, flaps were studied at 4, 7 or 12 days using antisera to PGP 9.5 (panneural marker), the neuropeptides CGRP (sensory nerves) and CPON (adrenergic nerves) and an endothelial marker VWF. In partially denervated flaps, moderate increases in PGP-immunoreactive (PGP-IR) and CGRP-IR nerves and a mild increase in CPON-IR nerves in immediate surrounding skin preceded smaller increases in similar nerves around the pedicle. Following total denervation, mild increases in all nerve types at these locations were accompanied by a marked increase of these nerves in distant surrounding skin, 1–2 cm from the suture line. A gradual increase in endothelial cell staining (VWF) of blood vessels was seen in surrounding skin, flap beds and pedicles. Angiogenesis preceded flap reinnervation which occurred initially from surrounding skin, and later from the base, with sensory fibres appearing first.**

In clinical flap transfers, the problem of nerve degeneration and inadequate reinnervation can cause difficulties for the patients. Where one desires to cover a defect such as a pressure sore with flap tissue, it would be useful to know how nerves regrow into such flaps and what factors might influence their growth in order that flaps may be augmented to attain high quality functional cover. It is also desirable to have an understanding of the time course of reinnervation in order to predict the functional adequacy of a given flap in terms of neurophysiology, especially of the autonomic nervous system, and to predict the suitability of the flap for repair of a particular defect. Various studies have looked at nerve degeneration and regeneration in skin by classical methods (Davis and Kitlowski, 1934; Adeymo and Wyburn, 1957; Psillakis and Erhardt, 1972; Waris, 1978a, b; Diamond and Jackson, 1980), and several have investigated reinnervation in humans either clinically or by electrophysiological studies (Davis, 1934; Mannerfelt, 1962; Terzis, 1978; Woodward and Kenshalo, 1987). A recent report investigating the time course of reinnervation in mouse skin flaps and the distribution of nerve fibres immunoreactive for the neuropeptides calcitonin gene-related peptide (CGRP), substance P, vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) showed the importance, firstly of the base of the flap, and secondly of the adjacent skin, in the reinnervation of these flaps (Karanth *et al.*, 1990). The aim of the present study was to assess immunohistochemically the pattern of reinnervation and revascularisation and to calibrate the importance of the pedicle and surrounding skin during reinnervation in pedicled and free adipomyocutaneous flaps in rats following either partial or total denervation. We also aimed to demonstrate the role of angiogenesis in the reinnervation process. A panel of antisera was employed, including those to the general neural marker protein gene product 9.5 (PGP 9.5), the neuropeptides CGRP, a marker for sensory nerves, and C-flanking peptide of Neuropeptide Y (C-PON), a marker for adrenergic nerves, and to the endothelial marker von Willebrand factor (VWF).

## Materials and methods

### *Experimental model*

Eighteen male Lewis rats weighing 200–250 g were used in this study. These were divided into 2 main groups (n = 9) and then subdivided according to the number of days of reperfusion allowed before harvesting for examination.

### *Flap surgery*

The flaps were raised from the groin region in an oblique fashion (Nishikawa *et al.*, 1991a) on pedicles comprising the superficial epigastric artery, vein and nerve. The flaps themselves comprised the skin with its inherent muscle, the panniculus carnosus and the subcutis with its adipose tissue; hence, they were adipomyocutaneous flaps. Each flap was rectangular and measured approximately 4 × 2 cm. All flaps were sutured with 6/0 silk and no protective dressings were applied. At pre-determined times (Nishikawa *et al.*, 1991a, b), the flaps were harvested with a 1.5 cm margin of surrounding non-flap skin to include the sutured junction between the flap and non-flap skin. Subcutaneous tissue containing the pedicle was also included. Three control flaps of normal skin and subcutis of similar measurements to the experimental

flaps were also studied for determination of normal morphology, innervation, and vascularity.

*Experimental protocol*

*Group 1.* Flaps were raised on their pedicles (as above) and re-sutured as autografts without transection of the neurovascular bundle, thus dividing all nervous connections from the bed and surrounding tissues but maintaining the axial nervous supply, hence achieving only partial denervation.

*Group 2.* Flaps were raised on their pedicles and their neurovascular bundles were transected. The superficial epigastric vessels were re-anastomosed leaving the nerve bundles divided, thus achieving total denervation. The flaps were then re-sutured as autografts.

The flaps and surrounding skin were harvested from 3 rats at each time point: Subgroup A (4 days), Subgroup B (7 days) and Subgroup C (12 days).

*Tissue preparation*

The flap samples were fixed by immersion in Zamboni's fluid overnight at 4°C. The tissues were then washed thoroughly in phosphate-buffered saline (PBS: 0.01 M phosphate, 0.15 M NaCl, pH 7.2) containing 15% sucrose and 0.01% sodium azide, and stored in this solution at 4°C prior to the preparation of snap-frozen blocks. Samples were taken from 5 areas in each flap (Fig. 1).

*Immunohistochemistry*

Frozen sections (10 μm thick), cut on a cryostat, were collected on poly-L-lysine coated slides for staining either by immunohistochemistry or for conventional histology with haematoxylin and eosin. Serial sections were cut and collected sequentially onto 5 slides such that every 5th section was stained for the same antigen. There were at least 4 sections on each slide.

The sections were allowed to dry for 60 min before washing in PBS containing 0.2% (v/v) Triton X-100 for 60 min at room temperature in order to make the tissue permeable to antiserum. After washing in PBS (3 × 5 min), the sections were counterstained by immersion for 30 min in 20% Pontamine Sky Blue in order to reduce the background autofluorescence. Following further washes in PBS (3 × 5 min), the sections were incubated with primary antisera applied for 16–20 h at 4°C. Antisera against PGP 9.5, CGRP, C-PON, and VWF were used in this study for immunostaining by the indirect immunofluorescence method (Table 1). After washes with PBS, the sections were incubated with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (dil 1:100 in PBS; Tago Inc, USA) for 60 min at room temperature. Following final washes in PBS (3 × 5 min), the sections were mounted in PBS glycerol (1:9 v/v) and viewed using a fluorescence microscope (Olympus AH-2).

Control skin samples were, in addition, stained with antisera to the glial tissue marker S-100 and to the neuropeptides VIP and Substance P, markers for parasympathetic and sensory nerves respectively. Since these produced largely inconclusive, often negative results, no further use was made of them in the experimental groups.

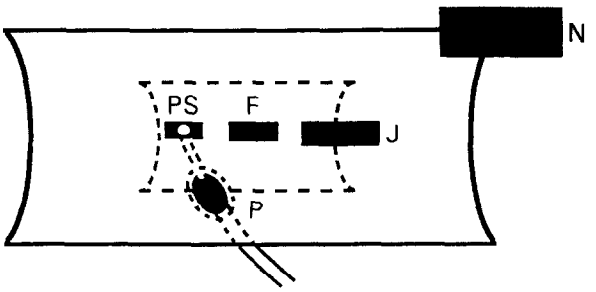


Fig. 1

**Figure 1**—A schematic representation of flap tissue (- - -) and surrounding skin to show the areas sampled for staining: N = distant surrounding skin, approximately 2 cm from the suture margin; J = junctional tissue including the suture margin, and tissues from the immediate surrounding skin and flap; F = mid-flap tissue; PS = flap tissue overlying the pedicle; P = pedicle.

**Table 1** Antisera used in this study, the dilutions used and their origins

| *Antiserum to* | *Dilution* | *Source* |
|---|---|---|
| PGP 9.5 (Human) | 1:1000 | Ultraceone, UK |
| CGRP (Syn. Rat) | 1:200 | Hammersmith Hospital |
| C-PON (Human) | 1:600 | Hammersmith Hospital |
| VWF (Human) | 1:800 | Serotec |

**Table 2** Criteria for the semi-quantitative assessment of immunoreactive nerve fibres

| *Score* | *Description* | *No. of nerve fibres/section* |
|---|---|---|
| 0 | None | 0 |
| +/− | Occasional | 1–2 |
| + | Sparse | < 10 |
| ++ | Few | 10–40 |
| +++ | Moderate | 40–100 |
| ++++ | Abundant | > 100 |

For each area examined in the flap (Fig. 1), immunoreactivity was assessed in the epidermis (including the subepidermal plexus), dermis, sweat glands, blood vessels (in all the layers), hair follicles and nerve bundles. In sections from the junctional region, immunoreactivity was studied within the normal 'non-flap' side of the suture line, the suture area itself and the 'flap' side of the suture. Examination of the pedicle itself included only blood vessels and nerve bundles. A subjective semi-quantitative assessment was carried out on each section, as shown in Table 2. A similar assessment was also made for the number of blood vessels identified by the endothelial marker VWF. Since each group contained 3 animals, an average score was calculated for the final analysis.

## Results

*Macroscopic appearance*

All flaps in both groups appeared viable and healthy. There was adequate repair at the suture margin at 4

days in Group 1 and at 7 days in Group 2. Occasionally, focal mild pallor was noticed on the flap surface, sometimes accompanied by a slight loss of hair. In Group 2, the sutured margin was often congested and bore abundant dark crusts.

*Histological appearance*

The histological features of the surrounding 'non-flap' tissue were unremarkable, with the exception of oedema and minor inflammatory cell infiltrates in the subdermal location. Granulation tissue was present in the base of a few totally denervated flaps.

The histology of junctional tissue in the partially denervated flaps (Fig. 2) was similar to that seen in a previous study (Nishikawa *et al.*, 1991a). In the totally denervated flaps there was, however, greater, more diffuse inflammation at all points with only minor repair and irregular acanthosis seen at the suture margin at day 4. The features on day 7 resembled those of the partially denervated flaps at day 4. At 12 days, both groups showed similar histology though there was greater inflammation following total denervation.

There were essentially no histological differences between the mid-flap area and the tissue overlying the pedicle. Occasional sweat glands with holocrine type secretion were seen in both areas. At 4 days there was moderate oedema in all the layers together with mild inflammation and a developing granulation tissue bed. At 7 days there was less oedema, more inflammation and a larger vascular bed, particularly in the area above the pedicle. At 12 days the vascular bed was even more pronounced.

The histological changes in the pedicle in both groups were similar to those previously described (Nishikawa *et al.*, 1991a, b).

**Fig. 2**

**Figure 2**—Junctional tissue showing complete re-epidermalisation with acanthosis at the suture margin. Haematoxylin and eosin. Magnification × 34.

**Table 3** Density of immunoreactive nerve fibres demonstrated by antisera to protein gene product 9.5 (PGP 9.5), calcitonin gene related peptide (CGRP) and C-flanking peptide of neuropeptide Y (CPON)

| | | *Group 1 (Partial denervation) days* | | | *Group 2 (Total denervation) days* | | |
|---|---|---|---|---|---|---|---|
| | *Control* | *4* | *7* | *12* | *4* | *7* | *12* |
| PGP | | | | | | | |
| Distant surrounding skin | | +++ | +++ | +++ | +++ | ++++ | ++++ |
| Immediate surrounding skin | +++ | ++ | +++ | ++++ | + | ++ | ++++ |
| Junction | | 0 | + | +++ | 0 | + | + |
| Mid-flap | | 0 | 0 | 0 | 0 | 0 | 0 |
| Skin over pedicle | | 0 | 0 | + | 0 | 0 | + |
| Pedicle | +++ | +++ | ++++ | ++++ | + | + | ++ |
| CGRP | | | | | | | |
| Distant surrounding skin | | ++ | ++ | ++ | ++ | ++ | +++ |
| Immediate surrounding skin | ++ | + | ++ | +++ | + | + | ++ |
| Junction | | 0 | + | ++ | 0 | + | + |
| Mid-flap | | 0 | 0 | 0 | 0 | 0 | 0 |
| Skin over pedicle | | 0 | 0 | +/− | 0 | 0 | 0 |
| Pedicle | ++ | ++ | ++ | +++ | + | + | + |
| CPON | | | | | | | |
| Distant surrounding skin | | ++ | ++ | ++ | + | + | ++ |
| Immediate surrounding skin | ++ | + | ++ | ++ | +/− | + | ++ |
| Junction | | 0 | +/− | + | 0 | +/− | + |
| Mid-flap | | 0 | 0 | 0 | 0 | 0 | 0 |
| Skin over pedicle | | 0 | 0 | +/− | 0 | 0 | 0 |
| Pedicle | ++ | ++ | ++ | ++ | +/− | 0 | + |

**Table 4** Density of blood vessels demonstrated by endothelial cell staining with antiserum to von Willebrand factor

| | | *Group 1 (Partial denervation) days* | | | *Group 2 (Total denervation) days* | | |
|---|---|---|---|---|---|---|---|
| | *Control* | *4* | *7* | *12* | *4* | *7* | *12* |
| Distant surrounding skin | | +++ | +++ | ++++ | +++ | +++ | ++++ |
| Immediate surrounding skin | ++ | +++ | ++++ | ++++ | +++ | +++ | ++++ |
| Junction | | +++ | ++++ | ++++ | +++ | ++++ | ++++ |
| Mid-flap | | +++ | +++ | +++ | +++ | +++ | +++ |
| Skin over pedicle | | +++ | ++++ | ++++ | +++ | +++ | ++++ |
| Pedicle | ++ | +++ | ++++ | ++++ | ++ | +++ | +++ |

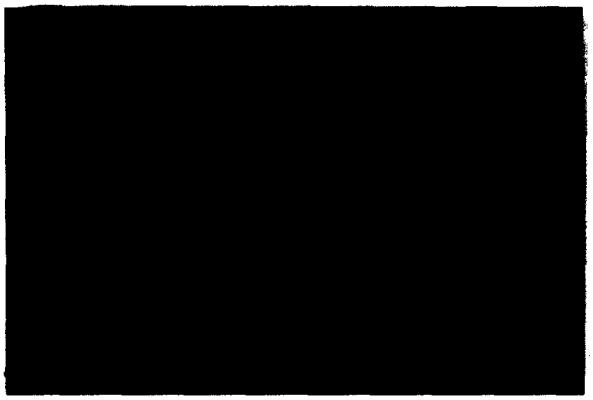

Fig. 3

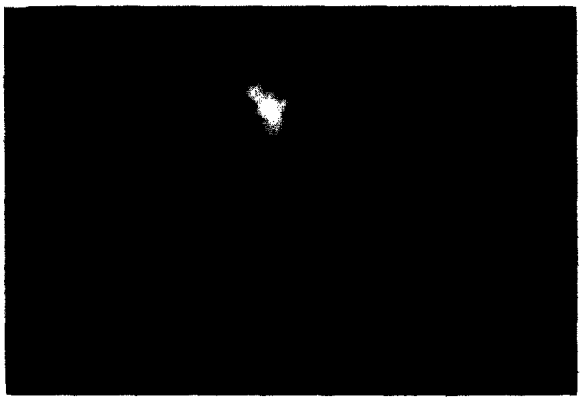

Fig. 4

Fig. 5

**Figure 3**—Control rat skin stained with antiserum to PGP 9.5 to show normal distribution of IR nerve fibres: E = epidermis; D = dermis; H = hair follicles. Magnification × 180. **Figure 4**—Control rat skin stained with antiserum to CGRP to show normal distribution of IR nerve fibres: E = epidermis; D = dermis. Magnification × 275. **Figure 5**—Control rat skin showing normal, mild density of blood vessels in the subdermis. P = panniculus carnosus. Stained with antiserum to VWF. Magnification × 180.

*Immunohistochemistry*

The following results are summarised in Tables 3 and 4.

*Normal distribution*

With PGP 9.5, a moderate number of nerve fibres were seen in the epidermis and dermis with a few fibres around hair follicles and blood vessels (Fig. 3). Nerve bundles were present in the dermis, subdermis and the pedicle and were closely associated with the panniculus carnosus. A major subpopulation of the PGP-IR nerve fibres were immunoreactive for CGRP (Fig. 4). CPON staining revealed a sparse innervation around blood vessels. VWF endothelial cell staining showed a moderate density of small and medium-sized blood vessels, particularly in the subdermis (Fig. 5). Larger vessels were observed in the pedicle tissue.

*Experimental groups*

*Surrounding skin*

*Group 1.* Immunostaining of nerve fibres in distant surrounding skin, approximately 1–2 cm from the suture line, showed no significant deviation from the control at any time point studied.

*Group 2.* The distribution of PGP-IR and CGRP-IR nerve fibres varied at all time courses in comparison to control tissue, with a mild increase at day 4 and a moderate increase at days 7 and 12 in all areas (Fig. 6A, B). CPON staining showed a slight depletion of nerve fibres around blood vessels up to day 7, followed by a full recovery at 12 days.

In both groups, VWF staining showed a progressive increase in angiogenesis from day 4 onwards with the presence mainly of small vessels correlating with developing granulation tissue.

*Junctional tissue*

*Group 1.* An initial slight depletion of all nerve fibres in the immediate surrounding skin was most marked within 0.5 cm of the suture margin. By day 7, a slight increase in PGP-IR and CGRP-IR nerve fibres was noted in all layers with occasional haphazard distribution and nerve sprouting. A moderate increase of both PGP-IR and CGRP-IR nerve fibres was observed at 12 days. Despite established neo-epidermalisation across the suture margin with underlying granulation tissue and fibrosis as early as 4 days, no nerves were seen in the fibrotic tissue until day 7, when occasional PGP-IR and CGRP-IR nerve fibres were noted (Fig. 7). By day 12, a normal distribution of nerves, similar to control skin, was seen in the acanthotic neo-epidermis (Fig. 8), though still fewer than normal

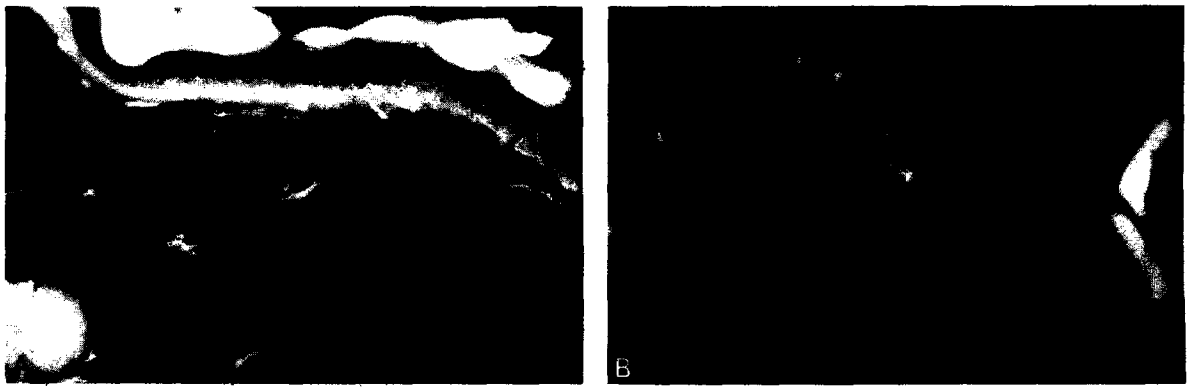


Fig. 6

**Figure 6**—(A) Group 2 (totally denervated) flap harvested at 12 days. There is a moderate increase in PGP-IR nerve fibres in surrounding skin, 1–2 cm away from the junction. Magnification × 275. (B) Same flap as (A) showing a moderate increase in CGRP-IR nerve fibres in the surrounding skin. Magnification × 275.

Fig. 7

Fig. 8

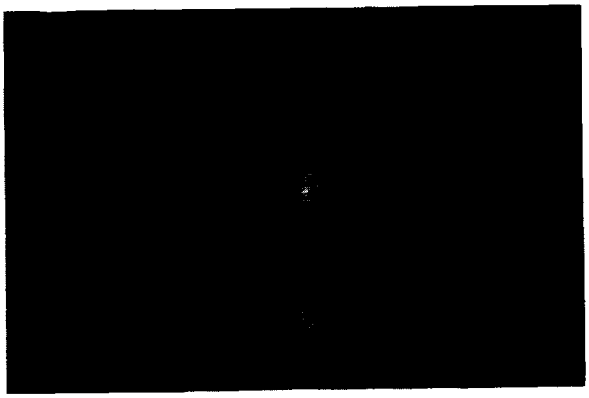

Fig. 9

**Figure 7**—Junctional tissue of Group 1 (partially denervated) flap at day 7 showing occasional IR nerve fibres in the repaired, fibrotic dermis of the suture margin. Stained with antiserum to PGP 9.5. Magnification × 180. **Figure 8**—Group 1 (partially denervated) flap showing reinnervation by PGP-IR nerve fibres of an acanthotic neo-epidermis at the suture margin on day 12. Magnification × 180. **Figure 9**—Mid-flap area of a Group 1 (partially denervated) flap at 12 days. There are no immunoreactive nerve fibres seen: E = epidermis; D = dermis. Stained with antiserum to PGP 9.5. Magnification × 34.

PGP-IR, CGRP-IR and CPON-IR nerve fibres were present in the underlying fibrotic dermis. VWF staining revealed a gradual increase in vascularity, particularly of small and medium-sized blood vessels from day 4 onwards, this being mainly in the bases of the suture margin and surrounding skin. In the flap tissue immediately adjacent to the suture margin, there was a gradual depletion of all nerve fibre types with no innervation seen at 12 days (Fig. 9). Blood vessels outlined by endothelial cell staining (VWF) were present in the base by day 12.

*Group 2.* A mild depletion of all nerve fibre types in the immediate surrounding skin was maintained until day 7, followed by only a small increase in nerve fibres by day 12. This late increase in nerve fibres, associated with the moderate increase found early in distant surrounding skin, was seen right up to the repaired suture margin though no reinnervation of the repaired dermis and epidermis was seen at any time interval. As with Group 1, in the flap tissue immediately adjacent to the suture margin, there was a gradual depletion of all nerve fibres until they were completely absent by

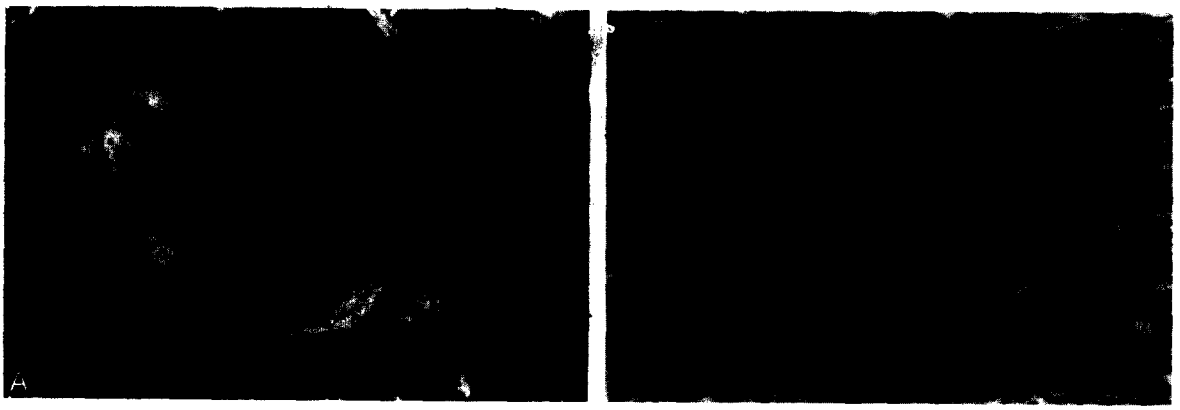

Fig. 10

**Figure 10**—(A) Mid-flap area of a Group 1 (partially denervated) flap at 12 days. Staining with antiserum to VWF shows an increase in vascularity with some ectatic blood vessels in the base, outlined by endothelial cells. Magnification × 180. (B) Mid-flap area of a Group 1 flap at 12 days showing the presence of granulation tissue in the base. Haematoxylin and eosin. Magnification × 180.

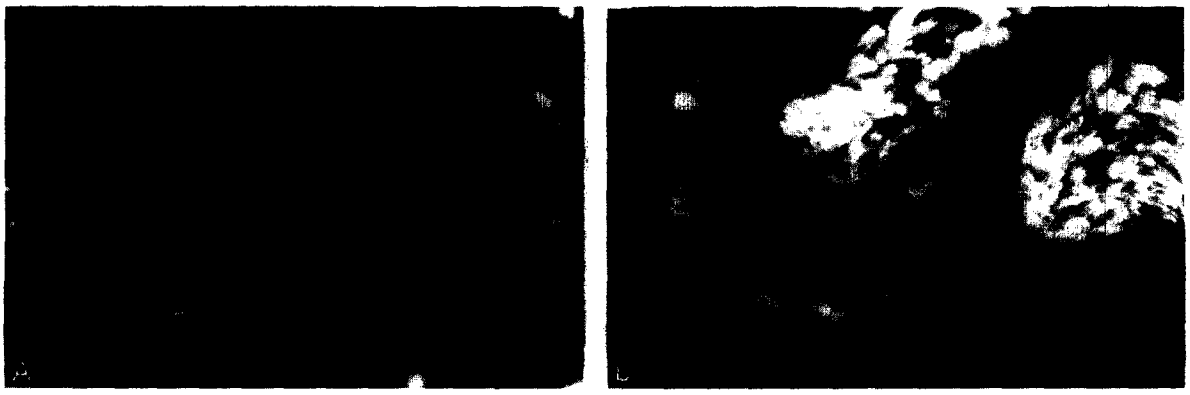

Fig. 11

**Figure 11**—(A) Pedicle of a Group 2 (totally denervated) flap harvested at 7 days. There is depletion of PGP-IR nerve fibres and loss of immunoreactivity in nerve bundles. Magnification × 180. (B) Pedicle of a Group 2 flap harvested at 12 days. There are abundant PGP-IR nerve fibres together with normal immunoreactivity in a nerve bundle. Magnification × 180.

day 12. Vascular changes in junctional tissue were similar to Group 1.

*Flap tissue*

*Group 1.* In the mid-flap area, there was a gradual depletion of nerves with a total absence noted by 7 days. The tissue overlying the pedicle showed re-innervation in the base, represented by small nerve bundles and individual nerve sprouts and fibres at 12 days. VWF staining revealed a normal number of small and medium blood vessels throughout the flap area at day 4. However, a gradual but small increase was seen up to day 12, reflecting the presence of granulation tissue in the base of the flap with some ectatic blood vessels (Fig. 10A, B).

*Group 2.* Only a few isolated PGP-IR nerve fibres were present in the base of the skin above the pedicle, with none in the mid-flap area. The increase in the number of blood vessels seen in Group 1 was also less pronounced, particularly in the mid-flap area.

*Pedicle*

*Group 1.* A slightly denser distribution of nerve fibres and bundles compared to control tissue was observed without any significant initial depletion. Moderate angiogenesis was evident, with proliferating endothelial cells and budding capillaries.

*Group 2.* A marked depletion of nerve fibres and bundles, particularly around the anastomosed blood vessels, was found up to and including day 7 (Fig. 11A). By day 12, isolated PGP-IR nerve fibres had appeared throughout the pedicle tissue, approximately matching the density and distribution found in control tissue (Fig. 11B). There was a similar amount of vascularity to Group 1 but only at a later stage. Initially, however, it was considerably less and more haphazard.

## Discussion

This study has looked at the oblique groin flap, comprising adipomusculocutaneous tissue, in two different situations. Study of Group 1 (partially denervated) flaps enabled assessment of the importance of the surrounding skin relative to the pedicle as a source of flap reinnervation. Study of Group 2 (totally denervated) flaps allowed assessment of further effects following transection of the nerve

supply from the pedicle. The time course of 4, 7 and 12 days allowed study of the early, intermediate and advanced changes respectively. By 12 days, complete wound healing could be expected (Nishikawa *et al.*, 1991a).

Complete depletion of immunoreactivity from nerve fibres to any antiserum did not necessarily imply total degeneration of nerves since levels of neurotransmitter substances at the point of study may have been below the limits of detection of the antiserum or the techniques employed. We have demonstrated the usefulness of PGP 9.5 as a neural marker for investigation of cutaneous nerve regeneration, similar to a study in humans (Dalsgaard *et al.*, 1989).

The minor inflammation and oedema in skin surrounding partially denervated flaps, with accompanying early acanthotic neo-epidermalisation across the suture margin, have been noted previously (Nishikawa *et al.*, 1991a, b). This acanthosis may have been responsible for specific patterns of reinnervation.

Following both forms of denervation, it was evident that the initial reinnervation in flaps took place largely from the surrounding skin, where a considerable increase in PGP-IR and CGRP-IR nerve fibres occurred, and to a lesser extent, and slightly later, from the pedicle neurovascular bundle. This early contribution from the surrounding skin was more pronounced in totally denervated flaps. It is possible that ingrowth of nerve fibres from the surrounding skin proceeded along degenerate neural channels within the denervated flap tissue (Diamond and Jackson, 1980). Similar nerve fibre regeneration was observed from the edges in necrotising myocardial injuries (Vracko *et al.*, 1990). The late reinnervation from the pedicle was in contrast to the study in mouse skin flaps (Karanth *et al.*, 1990) where reinnervation from the base of the flap took place prior to that from the margins. Our present study has illustrated a twin source of reinnervation, whereas others have shown origins of nerve regeneration in the base alone (Fitzgerald *et al.*, 1967) or solely from the margins (Waris, 1978a).

In flap tissue there was a slow but gradual degeneration of all nerves, concomitant with a gradual increase in vascularity. Our findings of dense granulation tissue correlate with a study of the pattern and initiation of angiogenesis in wound healing in rats, where the elaborate network of capillaries started with budding as early as 72 hours and continued up to 14 days (Phillips *et al.*, 1991). In our study, angiogenesis appeared to play an important role in the reinnervation process. The development of granulation tissue seen consistently before any nerve regeneration suggested angiogenesis preceded reinnervation. A similar role for endothelial cells was seen in a study of peripheral nerve regeneration using a silicon chamber (Varon and Williams, 1986). It is possible that the earlier and greater increase in vascularity around partially denervated flaps led to earlier reinnervation at the junction. Angiogenesis around totally denervated flaps appeared to be delayed, possibly contributing to slower reinnervation at the junction in these flaps. Nerve regeneration in the pedicles of totally denervated flaps was absent until 12 days, probably because larger nerves needed to regenerate following their transection. The skin above the pedicle had a spatial advantage over the mid-flap tissue through being closer to the neurovascular supply, resulting in slightly different patterns of reinnervation and revascularisation.

CGRP appeared to influence the reinnervation process, possibly directly by its neurotrophic actions (Dennis-Donini, 1989) or indirectly through mediating increases in blood flow (Kjartansson and Dalsgaard, 1987; Kjartansson *et al.*, 1988; Knight *et al.*, 1990; O'Halloran and Bloom, 1991), or through its role in healing and repair (Kjartansson and Dalsgaard, 1987). A recent study found CGRP-IR fibres in hairy foetal tissue, within 2 weeks of transfer into the anterior eye chamber of an adult rat (Katoh *et al.*, 1991). Both this report and our findings suggest an important role for CGRP in reinnervation.

The changes in totally denervated flaps suggested that cellular components in chronic inflammation aided the reinnervation process from a distant source, possibly mediated by cytokines and mitogens via Schwann cells and macrophages (Hall, 1989). However, in the partially denervated flaps, chronic inflammation was never sufficient to elicit these distant long-term effects. On the other hand, various factors such as acute inflammation, oedema, necrosis, fibrosis and the presence of suture material, might have inhibited the reinnervation process to some extent, while simultaneously enhancing the degeneration process within and around the flap.

The overall pattern of reinnervation, although observed only in its early stages, appeared similar in parts to that previously seen in rat and rabbit models using other methods of investigation (Diamond and Jackson, 1980). However, it was evident that nerve regeneration was slow compared to repair of other tissues such as the epidermis, dermis and blood vessels. The total time course of the present study has been insufficient to show in either group the final effects of the observed increase in nerves in both the distant and immediate surrounding skin on flap innervation.

In conclusion, it is hypothesised, on the basis of this study, that three factors are involved in initiating reinnervation: first, the direct response to denervation; second, the indirect effects of factors produced during inflammation; third, and possibly most importantly, neovascularisation.

## Acknowledgements

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## The Authors

**S. Manek, BSc, MBBS,** Pathologist, Section of Surgical Research, Clinical Research Centre, Northwick Park Hospital, Watford Road, Harrow, Middlesex HA1 3UJ.

**G. Terenghi, MSc, PhD,** Lecturer, Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital.

**C. Shurey, AIAT, MIScT,** Scientific Officer, Section of Surgical Research.

**H. Nishikawa, MA, MB, FRCS,** Research Surgeon, Section of Surgical Research.

**C. J. Green, PhD, DSc(Med), FRCVS, FRCPath, FRCS(Hon),** Head, Section of Surgical Research.

**J. M. Polak, DSc, MD, FRCPath,** Head, Department of Histochemistry.

Requests for reprints to Dr S. Manek.

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