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Invited Review

Peripheral nerve injury and regeneration

G. Terenghi
Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead, Sussex, UK

Summary. The process of nerve regeneration has been studied extensively by traditional morphological methods, but it is only recently that has been possible to identify more precisely the contribution of different nerve subpopulations. By studying different models of nerve repair and regeneration, it is becoming apparent that other tissue components are contributing to the overall process. When muscle grafting is carried out to repair an injured nerve, the regenerating axons are migrating in parallel with Schwann cells to bridge the nerve gap. The presence of Schwann cells is essential for a successful nerve regeneration, most probably because their production of different neuronal trophic factors. This pattern is also repeated when fibronectin mats are used for nerve repair, indicating the possibility to use this new synthetic matrix for clinical application. If the target organ is analysed after nerve repair, the recovery of all nerve components is evident. However, the process occurs at different times in separate skin compartments, and the regeneration of the autonomic innervation appears to be preceded by that of the sensory nerves. When looking at cutaneous nerve regeneration following different type of injury, a common pattern of events becomes apparent. In skin flaps, nerve regeneration begins from the skin surrounding the wound edge, or from the pedicle, and sensory nerves are the first to penetrate into the flap. Angiogenesis precedes reinnervation of the flap, and initially regenerating fibres appear to be associated with newly formed blood vessels. This pattern is evident also in full-thickness wounds and in suction blisters, where only the more superficial cutaneous layer is disrupted. Furthermore, the presence of keratinocytes appears to exert a directional influence on both regenerating blood vessels and nerves, which follow the regenerating keratinocytes when re-epidermalisation is taking place. These results would indicate that there is a close relationship between nerve fibres and blood vessels during regeneration, with a substantial contribution to the process from other tissue components and soluble factors from the surrounding environment.

Offprint requests to: Dr. Giorgio Terenghi, Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead, Sussex RH19 3DZ, U.K.

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Introduction

The wound healing process following tissue injury has been divided into three main overlapping phases: inflammation, granulation tissue formation, and matrix formation and remodelling. The different events taking place during these phases have been analysed using mainly traditional morphological methods, and insight has been gained in the processes of cells proliferation, angiogenesis and tissue remodelling which take place during wound healing (Clark, 1985). Particularly, a close link with angiogenesis has been demonstrated (Arnold and West, 1991) and in the initial phases the vasculature can account for up to 60% of the repair tissue (Dyson et al., 1991), which is consistent with the large increase of tissue metabolism observed during the repair process. In the early inflammatory phase, blood vessel disruption leads to the extravasation of blood constituents and concomitant platelet aggregation. Recruited macrophages accumulate in the area and release growth factors and other active substances which contribute to the formation of granulation tissue. This is followed by angiogenesis into the wound, and, if the epithelial barrier is disrupted, by rapid keratinocyte proliferation starting from the edge of the wound. Despite the wealth of information on the general process, comparatively little is known about the changes taking place in the peripheral nervous system during wound healing. This is of particular interest as it is becoming more evident that the nervous system could modulate tissue growth and repair in different ways. For example, various neurosubstances have been found to stimulate proliferation of endothelial cells (Hegerstrand et al., 1990; Ziche et al., 1990), fibroblasts (Nillson et al., 1985) and keratinocytes (Hegerstrand et al., 1989).

Axonal regeneration has been studied extensively with histological methods (Fitzgerald et al., 1967; Orgel et al., 1972; Psillakis and Erhards., 1972; Waris, 1978; Diamond and Jackson, 1980). The development of

immunohistochemistry has made possible the recognition of different nerve subtypes, such as sensory or sympathetic fibres, using specific antibodies for neuronal markers and neurosubstances. Extensive use has been made of this technique to investigate the changes at both axonal and cellular level following sciatic nerve axotomy or ligation (or different variants of these procedures) (Baranowski et al., 1993). There is a variety of neural and endothelial peptides and markers to which antibodies are available for immunohistochemistry. For example, protein gene product 9.5 (PGP) in a pan-neuronal marker which identifies all types of nerves; the sensory neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) are used as markers for sensory fibres (Fig. 1); vasoactive intestinal peptide (VIP) is present in cholinergic fibres; neuropeptide Y (NPY), its C-flanking peptide C-PON and the enzyme tyrosine hydroxylase (TH) are present in sympathetic fibres; the endothelial marker von Willebrand factor (vWf) and the vasoconstrictor peptide endothelin 1 (ET1) are present in endothelial cells. The use of antisera to these and other markers has allowed a precise morphological mapping of the innervation and vascularisation in the skin (Wallengren et al., 1987; Karanth et al., 1991; Walsh et al., 1994), which, because of its accessibility, represents an ideal tissue for the study of wound healing and nerve regeneration in peripheral tissues. Also the combination of immunohistochemistry and image analysis quantification has allowed to define precisely these regenerative processes and, by studying different injury models, a constant pattern of events has started to become evident.

Nerve regeneration in skin flaps

The use of flap tissue may be a desirable option for clinical tissue repair. In order to obtain a fully functional flap, it is important to know the pattern of reinnervation and whether any factors may specifically improve such process. Indeed, nerve degeneration and inadequate reinnervation in myocutaneous flaps can result in poor functional recovery, consistently with the observations that biopsies from patients with improved sensory recovery showed an increased nerve density (Turkof et al., 1993). Ultrastructurally these fibres are mostly unmyelinated and generally associated with blood vessels (Turkof et al., 1993). Furthermore, the axons tend to regenerate along empty neurilemal sheaths, generally starting from the margin of the flap, with a lesser component from the wound bed (Terry and Harkin, 1957; Nathaniel and Pease, 1963; Thomas, 1966; Jurecka et al., 1975; Diamond and Jackson, 1980).

The importance of the pedicle and the surrounding skin can be paramount in the reinnervation and revascularisation of the flap, as shown on experimental flap models, which were used to assess the influence of partial and total denervation, and of transient or prolonged ischemia, on the flap survival. An initial depletion of all nerve types was observed in the skin surrounding a musculo cutaneous flap (Nishikawa et al., 1991). After a week, an increase of PGP and CGRP-immunoreactive fibres was evident at the margin of the flaps, and this progressed throughout the time course investigated. Interestingly, soon after grafting there was a noticeable and gradual increase of small and medium-sized blood vessels, which preceded the nerve fibre regeneration (Manek et al., 1993a). A similar time-course of event was observed also using a different model, the prefabricated muscle flaps, clearly indicating that angiogenesis precedes innervation (Kostakoglu et al., 1994). Denervation and reinnervation of flaps did not seem to have any influence on the revascularisation process, although transient ischemia might enhance vascular growth from the flap bed (Cohen, 1979).

If the flap was affected by mild ischemia, the sequence of event was similar, although delayed in time and the reinnervation process did not take place until about 12-14 days. When the flap was totally denervated by transecting the neurovascular bundle of the pedicle, the initial reinnervation took place largely from the surrounding skin, and the pedicle played a lesser part in the repair process (Manek et al., 1993a). However it has been suggested that after nerve implantation reinnervation is mainly derived from the nerve trunk, rather than the skin surrounding the flap (Fig. 2) (Kostakoglu et al., 1994). In all cases CGRP-immunoreactive sensory fibres were the first recognisable subpopulation to penetrate into the flap, consistently with the finding that nociception returns early to transplanted skin (Rivers and Head, 1908; Sharpley-Schafer, 1928). This early appearance of CGRP might also explain its possible influence on nerve regeneration, either directly as neurotrophic agent (Dennis-Donini, 1989), or indirectly by mediating either an increased blood flow (Kjartansson et al., 1987, 1988; Knight et al., 1990) or the healing and repair process (Kjartansson et al., 1987). Furthermore, a selective depletion of sensory nerves affect adversely the survival of cutaneous flaps (Kjartansson and Dalsgaard, 1987).

At the early stages, around the flap there was dense granulation tissue, from which early neovascularisation can developed as early as at 24 hours post-wounding (Phillips et al., 1991). The development of early granulation tissue also preceded the repair of other tissue components, such as epidermis and dermis (Nishikawa et al., 1991), and it has been suggested that the cellular component of the inflammation, such as macrophages, could contribute to the reinnervation process, possibly through the release of cytokines and trophic factors (Hall, 1989; Griffin et al., 1993). Hence, it appears that three main factors could contribute to the reinnervation process of the flap: the direct response to denervation, the indirect effects of factors produced during inflammation, and the neovascularisation process.

Muscle graft for nerve repair

The degeneration of nerve terminals that can be seen

after axonal nerve damage has been clearly illustrated in experiments where injection of *Mycobacterium leprae* into the tibial nerve was used to produce a localised lesion. The nerve developed a granuloma and lesions with features similar to those found in leprosy patients, which resulted in degeneration of nerve terminals in the skin (de Blaquiere et al., 1994a). If the granuloma is excised, the nerve damage can be repaired by insertion

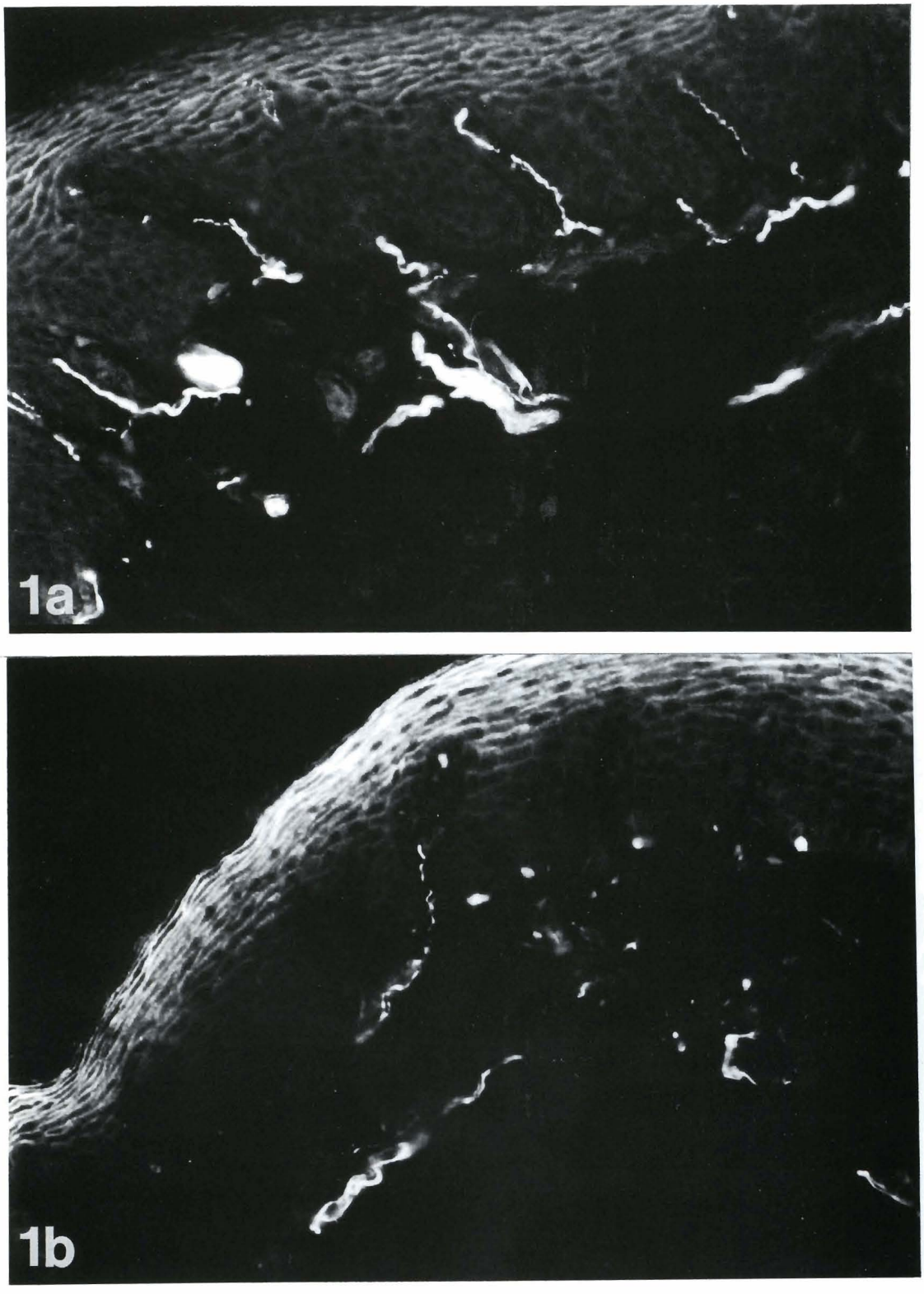

Fig. 1. A. Immunostaining of PGP in normal skin of rat footpad, showing the abundant distribution of nerve terminals and nerve bundles in the epidermis and papillary dermis. **B.** A similar distribution is seen for CGRP-immunoreactive nerves, although these show a lower density that PGP-immunoreactive nerves. Indirect immunofluorescence method.

of a denatured muscle graft, which facilitate nerve regeneration (Glasby et al., 1986, 1991).

Following muscle graft, functional motor and sensory recovery were observed by 11 weeks, soon after an increased conduction velocity was observed. The number and diameters of myelinated fibres increased steadily throughout the time course, but the maximum recovery of myelinated fibres reached only one third of the control nerve (de Blaquiere et al., 1994b). When quantitative immunohistochemistry was carried out on the foot-pad skin, the nerve degeneration due to the nerve lesion was followed, after grafting, by a gradual re-appearance of all nerve types (Santamaria et al., 1994). The distribution pattern of the immunoreactive fibres in the reinnervated skin was similar to that of control tissue, suggesting that the reinnervation process might follow existing pathways of degenerated nerves (Terry and Harkin, 1957; Nathaniel and Pease, 1963; Thomas, 1966; Jurecka et al., 1975; Diamond and Jackson, 1980). However, the reinnervation process occurred differentially in separate skin compartments.

Immunoreactive nerve bundles appeared in the deep dermis soon after grafting, possibly because they represent the initial pathway of nerve regeneration. CGRP and SP-immunoreactive sensory nerves were the first to appear in epidermal and subepidermal layers, reaching a normal level of distribution at the end-point of the experiment (Santamaria et al., 1994), which is consistent with the normal response to sensory tests (de Blaquiere et al., 1994b). Recovery of autonomic nerves around blood vessels and sweat glands took longer, and in particular the vessels still showed a low density of innervation by the time the epidermal reinnervation was complete (Fig. 3). The early appearance of sensory nerves is consistent with finding seen during development, where CGRP-immunoreactive nerves are the first to be detected in foetal skin at a gestational time when there are early foetal responses to external stimuli (Terenghi et al., 1993).

Muscle grafting procedure has been used in patients with nerve lesion due to leprosy (Pereira et al., 1991) or to accidental damage (Norris et al., 1988; Calder and Norris, 1993). The results have been encouraging, with some improvement of sensory perception, indicating the potential of this surgical approach to nerve repair. However, there are limitation in this use of these grafting methods, because of the rather short gap length that can be bridged. The regeneration power of the nerve in the absence of Schwann cells is reduced (Hall, 1986), and our studies have shown that axons and Schwann cells co-migrate in muscle grafts (Calder et al., 1994). In more recent experiments, an intermediate depot of Schwann cells was created in a graft by dividing the denatured muscle in equivalent lengths and by suturing between the two halves of the muscle a small segment of the distal nerve. Despite lengthening the graft and increasing the number of anastomosis, this technique enhanced nerve regeneration over the longer nerve gaps, with Schwann cells migrating in both direction from the interposed nerve segment into the muscle graft (Calder and Green, 1995). This and similar experiments (Maeda et al., 1993) suggests that nerve regeneration can be

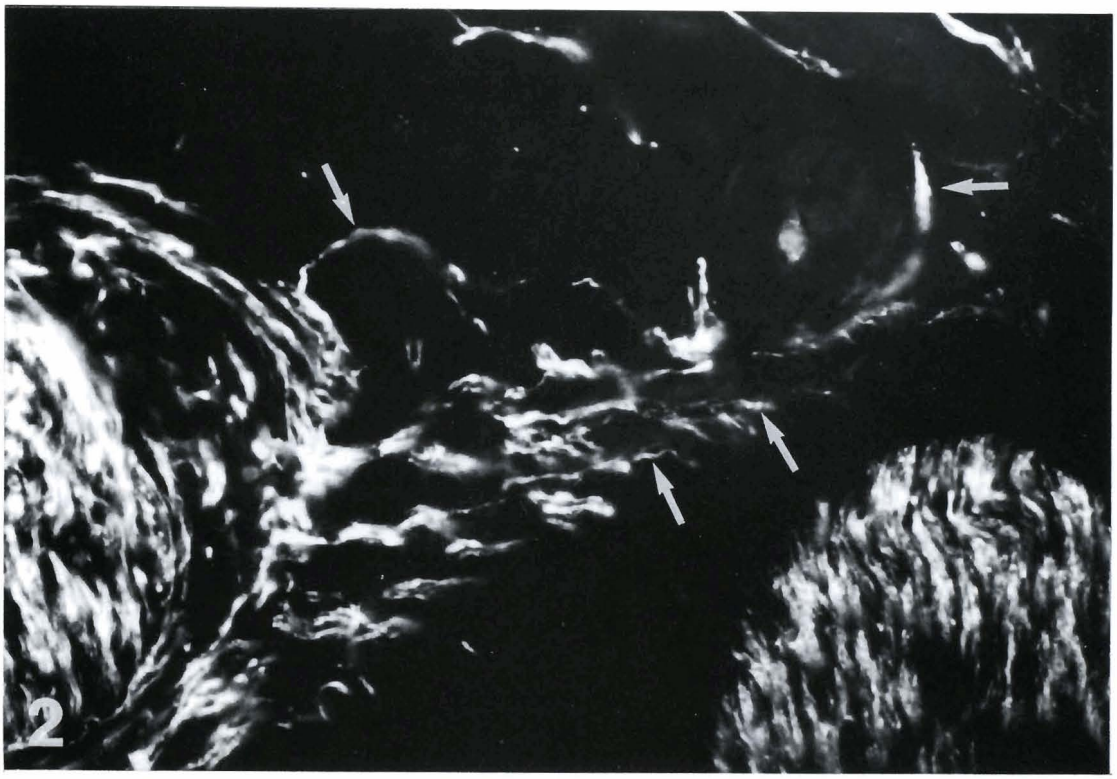

Fig. 2. At an early stage of the re-innervation process, PGP immunostaining shows nerve fibres sprouting from large nerve bundles (arrows), which originate from the nerve implanted into a musculocutaneous flap. Indirect immunofluorescence method.

enhanced by the presence of Schwann cells, which are also known to be a source of trophic factors (Korsching, 1993).

Alternative methods for nerve repair have made use of synthetic materials (Mackinnon and Dellon, 1990a,b), but with limited success. Recently, a new grafting material, fibronectin, has been used with success. Fibronectin is a naturally occurring protein found in blood plasma, and it can be extracted and lyophilised to form mats of oriented fibres, which can be used as grafts for nerve repairs (Ejim et al., 1993). Initial experiment have yielded encouraging results, showing regeneration of fibres and penetration of Schwann cells through fibronectin. The fibronectin mats were grafted into short nerve gaps, and samples of the nerve and conduit were collected at different time points after grafting. Staining for CGRP and other neural markers was used to assess the rate and volume of axonal regeneration within the graft, and quantification of the results showed that fibronectin supported regeneration in similar quantity to that seen in denatured muscle grafts (Fig 4) (Whitworth et al., 1995). Long-term experiments are ongoing to assess the reinnervation and functional recovery of target tissues after fibronecting grafts, but it is apparent that this material can offer a suitable alternative for nerve repair.

Wound healing and nerve regeneration

In the suction blister injury, the epidermis is detached from the dermis with disruption of neural and vascular supply within the superficial cutaneous layers (Hertle et al., 1992). Healing starts with the regeneration of the keratinocyte layer from the surrounding undamaged edges of the skin, and continues with its growth towards the centre of the blister. Using immunostaining for vWf and ET1, a pattern of vascular changes was seen in the papillary dermis with an initial increase during the reactive phase, followed by a sharp decrease signalling injury. The start of tissue repair was

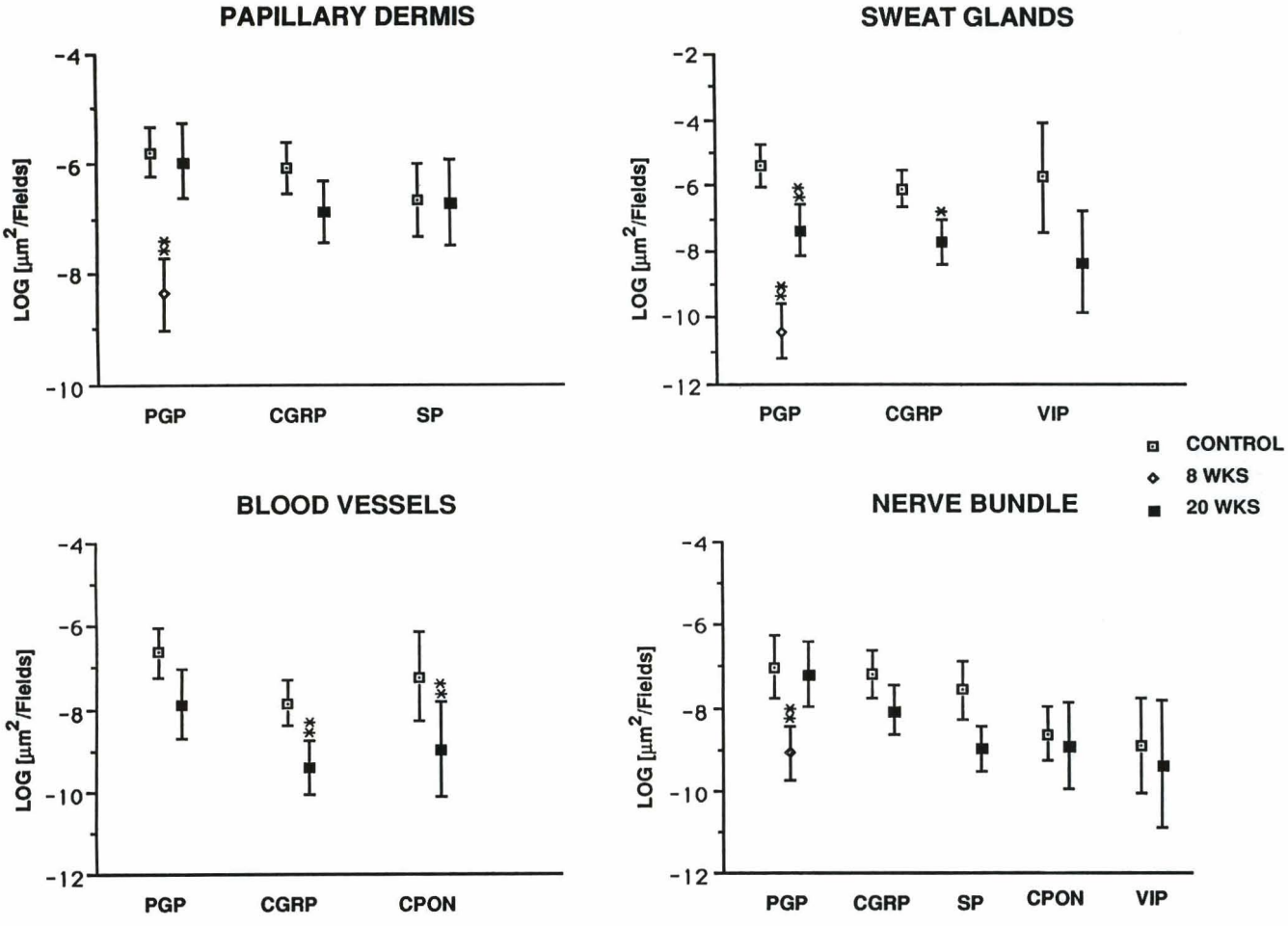

Fig. 3. Diagram showing the values of immunoreactive area (μm^2) for PGP, CGRP, SP, VIP and CPON staining in different skin compartments of the guinea pig foot pad. Quantification was carried out on tissues from controls and from animals at different times after muscle graft repair. In most cases, no immunoreactivity could be measured at 8 weeks after grafting, but there was a recovery at 20 weeks. This varied according to the peptide and the skin area. *: $p<0.005$; **: $p<0.01$.

indicated by a second more gradual increase which was seen starting at 23 hours from the edge of the blister, spreading towards the centre of the blister in parallel with the re-epidermalisation process (Fig. 5) (Gu et al., 1994). It has been reported that during wound healing the keratinocytes becomes activated very rapidly, producing both angiogenic cytokines and neurotrophic factors (McKay and Leigh, 1991; Antoniades et al., 1993), hence acting as a potential trigger for vascular and neural regeneration in the dermis. Quantification of the vascular changes indicated that the initial increase of vWf was due to enlarged vessel rather than to a numerical change, which is consistent with the vasodilatation described in the initial inflammatory phase of wound healing (Clark, 1985). It has been shown that capillary bud formation starts at 24 hours after injury (Phillips et al., 1991), which is consistent with the second increase of immunostaining. This is followed by the tissue remodelling phase, which coincides with the formation of a network of anastomosis (Phillips et al., 1991).

An increase of PGP- and CGRP-immunoreactive fibres was also found during the first 6 hours from blistering, followed by a decrease of immunoreactivity

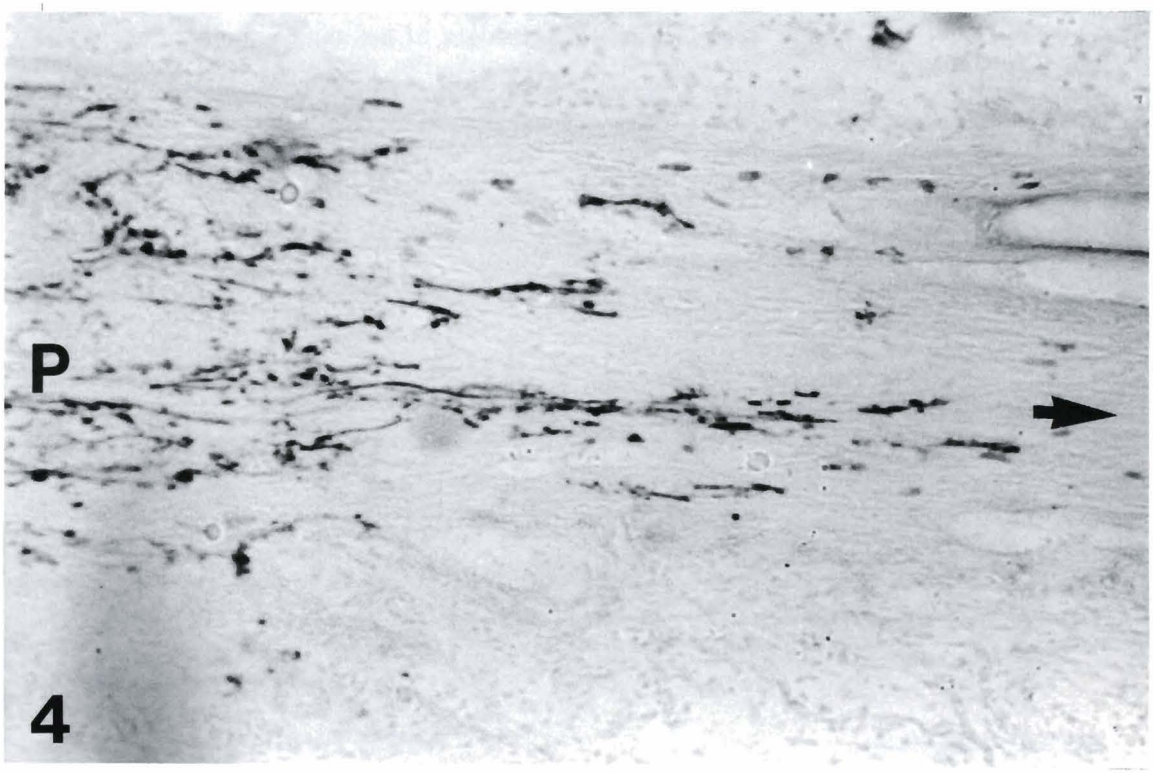

Fig. 4. Regeneration of CGRP-immunoreactive fibres though a muscle graft. The fibres penetrates into the graft from the proximal nerve stump (P), slowly advancing into the graft (direction indicated by the arrow). With time, the regenerating fibres reach the distal stump and reinnervate the target organ. ABC-peroxidase method.

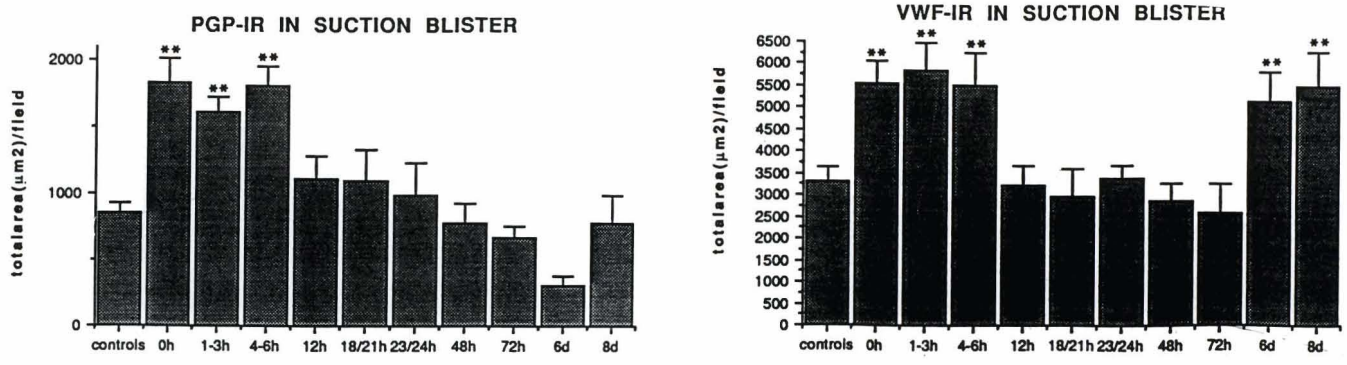

Fig. 5. Diagrams showing the changes of PGP and vWf immunoreactivities measured at different time points after suction blister. The values between 0 hour and 72 hours are from the blister area, while the values of controls and at day 6 and 8 are from the whole sub-epidermal layer of the biopsies, as there is no blister present. At these two late time points, the repair changes of vWf are already evident, as identified by significant increases. However, this process is delayed for PGP-immunoreactive nerves, which do not show any regeneration increase until 8 days. **: $p<0.01$ vs control.

due to nerve injury. Soon after the start of keratinocyte regeneration, nerve proliferation was observed first in the sub-epidermal layer at the edge of the blister, then spreading gradually towards the centre of the blister closely following the vascularisation and re-epidermalisation processes. Image analysis quantification highlighted the pattern of these changes, and showed that the initial increases were statistically significant (Fig. 5) (Gu et al., 1994). It is interesting to note that sensory fibres are the first to be recognised during the regeneration process, a fact which may be linked with the temporary itching often reported in clinical practice during the re-epidermalisation of wound healing. CGRP and SP are co-localised in sensory fibres and both have a vasoactive role (Brain and Williams, 1985). SP has also been linked to mast cell degranulation (Lembeck and Gamse, 1982; Stead et al., 1989) and to histamine release and hitch reaction (Hagermark et al., 1978; Lembeck and Gamse, 1982), indicating an added involvement of these peptides during wound healing and nerve regeneration.

Cultured keratinocytes have been used for some time for grafting of burn injuries (Teepe et al., 1990; Nanchahal and Ward, 1992). However, direct grafting of cultured keratinocytes is not always successful. An improvement has been found when the cultured epidermal layer is grafted onto a dermis, as these composite kerato-dermal grafts showed histological features similar to those of normal skin (Kangesu et al., 1993a,b). The success of a skin graft depend of the revascularisation of the tissue and its reinnervation, in order to re-establish functional viability. Preliminary studies using conventional microscopy have shown the pattern of the vascular and nerve growth during skin graft healing (Manek et al., 1993b). However, more detailed information can be obtained using laser confocal microscopy, which has the ability to scan optically through thick tissue specimen (Shotton, 1989), offering considerable advantages compared to light microscopy (Murray, 1992; Gardner, 1993).

In order to study the effect of cultured keratinocytes on the healing process and to avoid the influence of surrounding epidermis, a graft chamber was used to isolate the wound (Kangesu et al., 1993a). In this model, the blood vessels were seen to penetrate the wound from the deep dermis, with the vessels extending perpendicularly towards the surface of the epidermis in a linear pattern. There have been some reports of similar vertical neo-capillaries in early stages of wound healing (Rigal et al., 1989), running in parallel with the

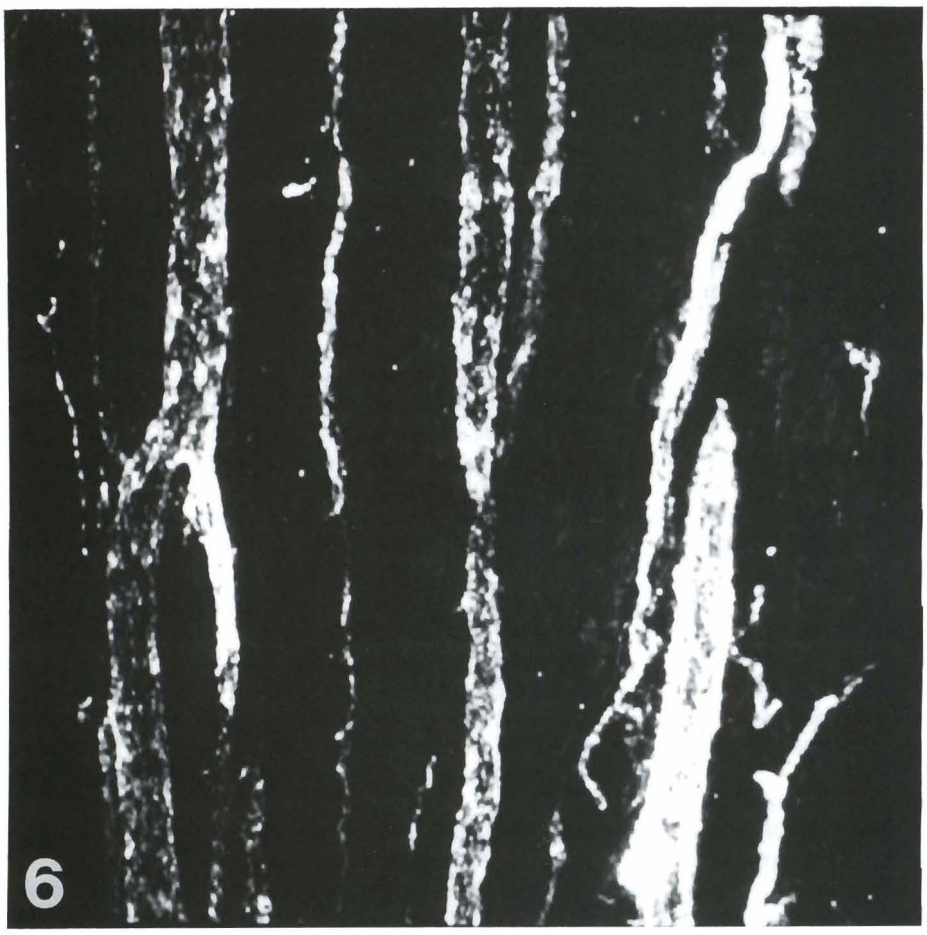

Fig. 6. Confocal image of blood vessels immunostained for vWf in the deep dermis during early wound healing. To note the linear arrangement of the vessels, perpendicular to the surface of the epidermis and with few anastomotic branches.

fibroblasts, which are arranged perpendicularly to the epidermis (Arnold and West, 1991). Within 2 weeks, these linear blood vessels reached the epidermis, and soon after anastomotic branches started to connect the vertical vessels (Fig. 6). The anastomosis appeared first in the deep dermis, spreading toward the superficial dermis, slowly restoring a normal vascular network (Gu et al., 1995). A similar pattern of regeneration was observed for the nerves, with fibres growing vertically from the base of the wound seemingly along the blood vessels pathways, as seen in burn wound healing (Kishimoto, 1984). However, the reinnervation was slower to proceed than vascularisation, reaching the epidermis at later time, and the network of fibres was not yet fully developed by the time the vascularisation showed a normal pattern of distribution (Gu et al., 1995).

Consistently with other studies of cutaneous wound healing in human (Gu et al., 1994) and rat (Manek et al., 1993a), neovascularisation precedes reinnervation, with nerve fibres following the pattern of blood vessel formation, as observed in other experiments (Varon and Williams, 1986; Turkof et al., 1993). An interesting finding was that the growth of blood vessels and nerves took place only in the area with epidermal cover. This was particularly evident as the cultured keratinocytes contracted towards the centre of the wound after grafting onto the dermis, thus exposing a thin peripheral rim of granulation tissue (Kangesu et al., 1993b). In these exposed dermal areas there was no sign of neovascularisation or reinnervation following the initial formation of granulation tissue (Gu et al., 1995). Thus, it is possible that keratinocytes might influence angiogenesis and reinnervation by exerting a stimulatory effect, as also noted during the repair process in suction blister.

In conclusion, the results of these studies would indicate that during wound healing and nerve regeneration there is a close relationship between epidermis, blood vessels and nerve fibres, which is more complex than previously thought and might involve numerous neural and cellular factors.

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