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Substance P- and calcitonin gene-related peptide-immunofluorescent nerves in the repair of experimental bone defects

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Summary. *Healing of an experimental bony defect in the rat's tibia was studied with an immunofluorescent technique to clarify when and where substance P (SP) and calcitonin gene-related peptide (CGRP) would develop. The normal tibia showed a few SP- and CGRP-immunofluorescent nerve fibres. In the experimental tibia, the number of these fibres increased on the 6th day after operation, reached a peak of proliferation on the 15th day and reverted to normal after the 24th day. The changes were associated with the development and decay of callus tissue suggesting that harmful stimuli from the injured site in a bone could be mediated by sensory nerves throughout the repair period. Most of the SP- and CGRP-immunofluorescence was seen near the vessels, frequently in the same nerve fibres. The SP- and CGRP-immunofluorescent nerves seemed to take part jointly in callus formation through the enhancement of local blood flow.*

Résumé. *Le procesus de guérison d'une perte de substance osseuse expérimentale a été étudié sur le tibia du rat par immunofluorescence afin de déterminer quand et où la substance P (SP) et la calcitonine peptide d'origine génique (CGRP) se développeraient. Le tibia normal ne montre qu'un petit nombre de fibres nerveuses immunofluorescentes SP et CGRP. Dans le tibia d'expérimentation, les fibres nerveuses immunofluorescentes SP et CGRP augmentent en nombre à partir du 6ème jour, atteignent leur maximum de prolifération le 15ème jour et reviennent à l'état normal après le 24ème jour post-opératoire. Ces modifications sont étroitement associées au développement et à la disparition du cal, suggérant ainsi que les stimuli nocifs provenant de la lésion osseuse pourraient être inactivés par les nerfs sensitifs durant la période de réparation. En outre, la plus grande partie de l'immunofluorescence SP et CGRP a été observée à proximité des vaisseaux, souvent dans les mêmes fibres nerveuses. Il semble que les nerfs immunofluorescents SP et CGRP participent conjointement à la formation du cal en augmentant la vascularisation locale.*

Introduction

Regulation of bone physiology by the nervous system is a subject of recent interest. Bone and periosteum have been shown to have many nerve fibres containing neuroactive peptides such as calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), substance P (SP) and neuropeptide Y (NPY) [3, 4, 11, 13, 14, 15]. In addition, CGRP has been reported to inhibit bone resorption [9, 31, 32] or to promote osteogenesis [2], while VIP is known to accelerate bone resorption [16].

The association between the nervous system and bone repair has also been documented. The rate of ossification is rapid in paraplegic limbs [1], and the healing of fractures seems to be accelerated in peripheral nerve injuries [10]. Nerve fibres containing substance P increase in number in healing fractures [26]. However, the role of

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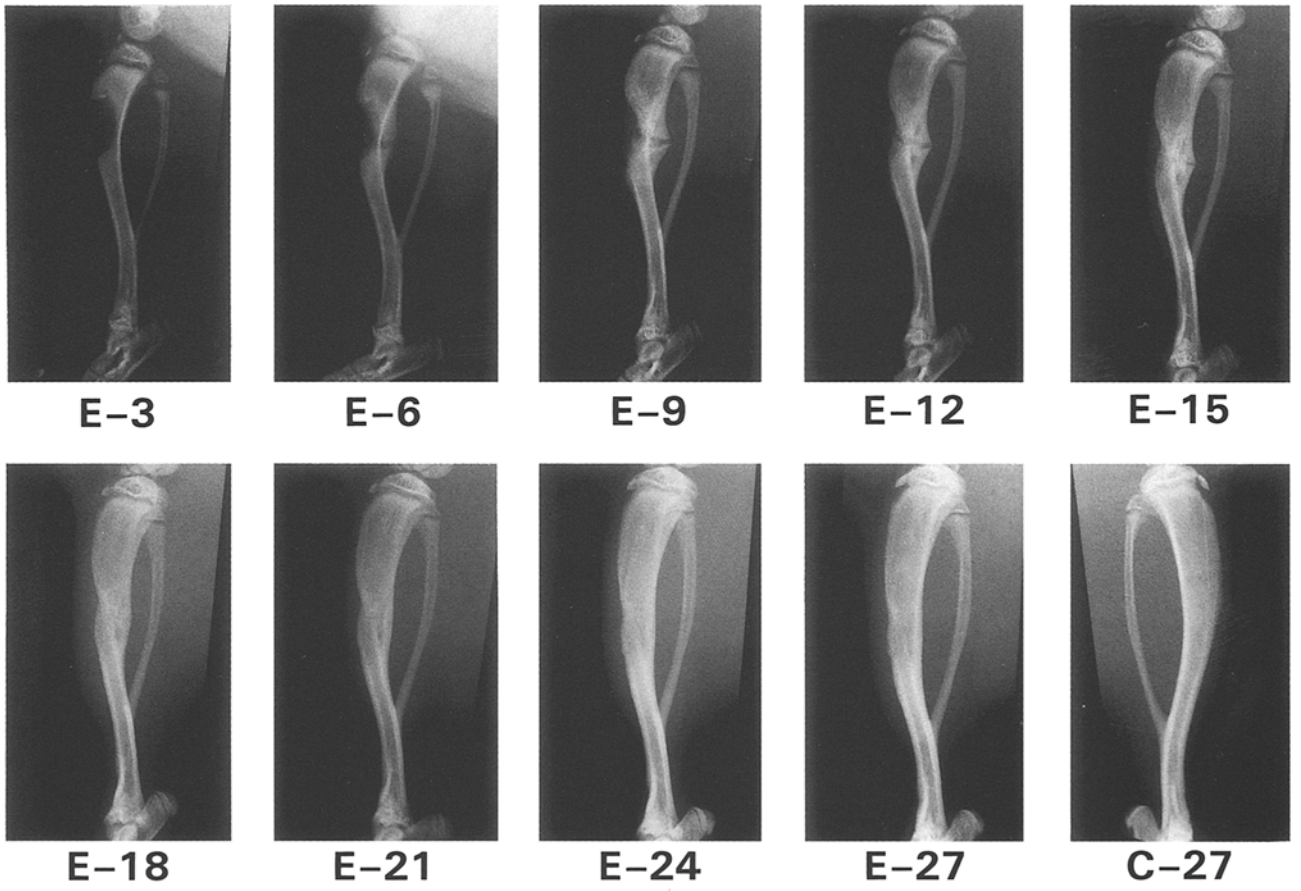

Fig. 1. Serial radiographs showing the repair of the experimental bony defect. On the 3rd day after operation, the defect was clearly seen (*E*-3). On the 6th day, some new bone was present around the defect and outside the posterior cortex of the tibia (*E*-6). Between the 9th and 12th day, callus increased in amount and density (*E*-9, *E*-12). On the 15th to 18th day, the defect was filled with new bone, and the new bone along the posterior tibial cortex tended to diminish (*E*-15, *E*-18). On the 21st day, the anterior tibial cortex had regained continuity and became dense; the posterior cortex also returned to normal (*E*-21). After the 24th day, the site of the defect was uniformly radiopaque (*E*-24). The length of the tibia on the experimental side (*E*-27) was equal to that of the control (*C*-27) indicating that no growth disturbance, or excessive growth, had occurred

neuropeptides in the repair of bony injuries has not been fully investigated.

The present study was undertaken to assess how sensory nerves could be involved during the repair of a bony defect. We investigated SP- and CGRP-containing fibres in relation to the radiological and histological sequences. We did not use a fracture model because the instability caused by a fracture might vary between individuals and this could affect the response of the nervous tissue.

Material and methods

We used 59 male Wistar rats aged 5 weeks at the start of each experiment. After intraperitoneal anaesthesia with sodium pentobarbital (50 mg/kg body weight), an oval bony defect, 5 mm × 3 mm, was made in the anterior cortex of the right tibia. The defect reached the endosteum of the posterior tibial cortex. The opposite tibia was also exposed but no defect produced, thus serving as a control. All the rats were allowed to walk freely in their cage throughout the experiment.

The animals were anaesthetised again to obtain bony specimens on days 3, 6, 9, 12, 15, 18, 21, 24 and 27 after operation. Ultrasoft radiographs of both legs were taken using Softex CMB-3. Rats with a complete fracture or a visible deformity were excluded.

All the specimens were prepared after in vivo perfusion and in vitro demineralisation by Bjurholm's method [5]: through a 19 gauge catheter inserted into the heart towards the aorta, 100 ml of Tyrode's buffer at 16–18°C, 100 ml of the same solution at 4°C and 250 ml of Zamboni's buffer were instilled in this order. We excluded the rats if their eyeballs, tail or feet did not become yellowish. Both tibiae were excised and kept in Zamboni's buffer at 4°C for 2 days. They were then immersed in ethylenediaminetetraacetic acid (EDTA)-cacodylate solution (EDTA 40 g, sodium cacodylate 24.2 g, sodium hydrate 15–16 g/1000 ml, pH 7.3) for a further 10 to 14 days for demineralisation.

Using a cryostat cooled at –20°C, the demineralised tibiae were cut into sections, 10 µm thick, perpendicular to the long axis of the bone. Sixteen sections were obtained from the experimental side by cutting 4 consecutive slices at 4 different sites along the tibia. These were mounted directly on object glasses at room temperature. Eight of the 16 specimens were

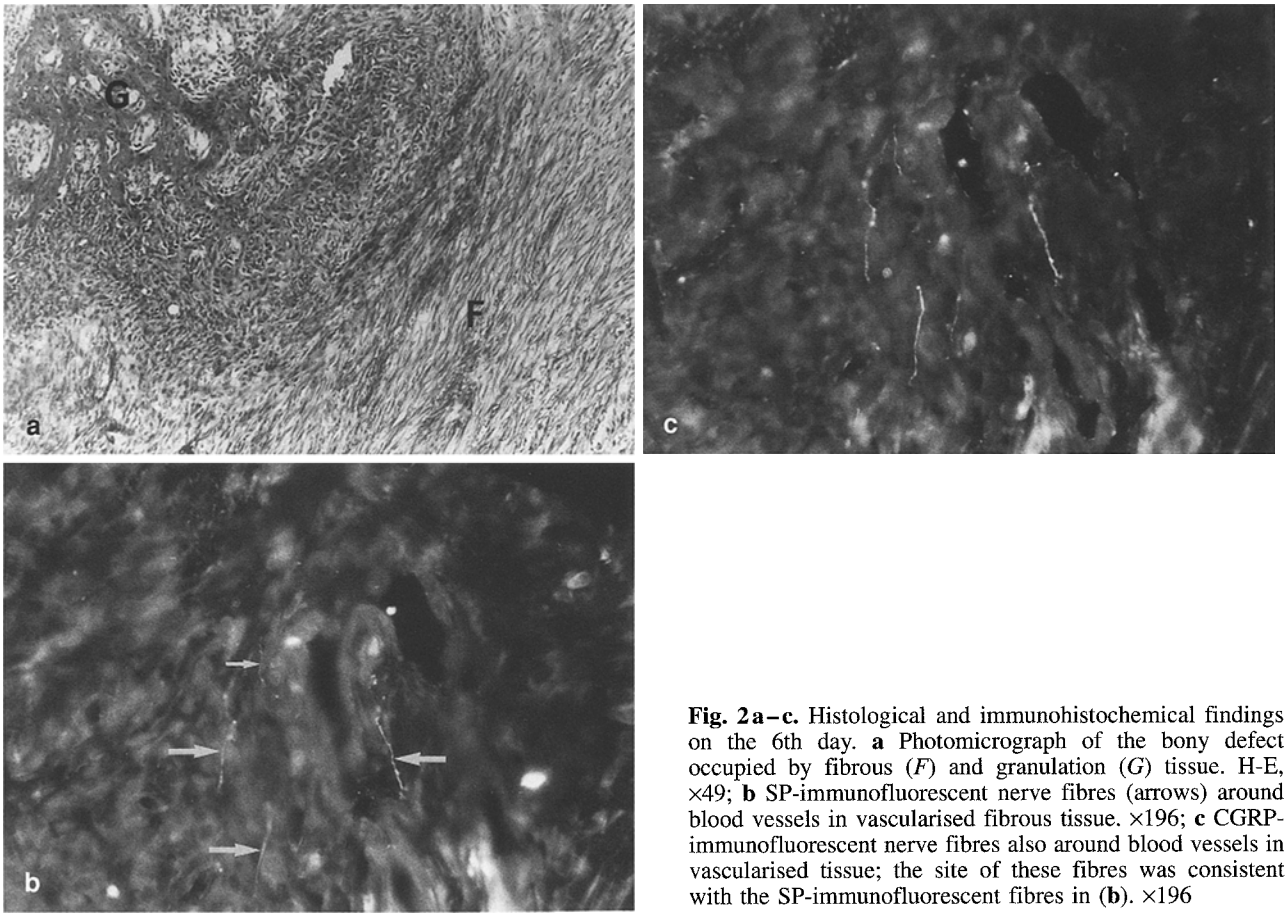

Fig. 2a–c. Histological and immunohistochemical findings on the 6th day. **a** Photomicrograph of the bony defect occupied by fibrous (*F*) and granulation (*G*) tissue. H-E, ×49; **b** SP-immunofluorescent nerve fibres (arrows) around blood vessels in vascularised fibrous tissue. ×196; **c** CGRP-immunofluorescent nerve fibres also around blood vessels in vascularised tissue; the site of these fibres was consistent with the SP-immunofluorescent fibres in (**b**). ×196

used for immunohistochemical study, while the remainder were stained with haematoxylin-eosin (H-E). The control side was treated in the same way, except that only immunohistochemical staining was carried out.

Immunohistochemical study. The sections cut on the cryostat were dried in the room atmosphere and exposed to anti-SP or anti-CGRP rabbit IgG (CRB Co Ltd, USA) for 24 h in a humid atmosphere. Following this first-step immunological reaction, the sections were rinsed in 0.01 M phosphate buffer solution (PBS) for 30 min. They were then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (anti-rabbit IgG goat IgG, BTI Co Ltd, USA) diluted to 1:300 for 30 min at 16–18°C, followed by rinsing with 0.01 M PBS for 30 min. The sections were finally mounted in glycerine-PBS and examined with a fluorescence microscope (Leiz Co. Ltd. Germany). The location of the SP- and CGRP-immunofluorescent nerve fibres was determined by comparing each immunohistochemical section with the adjacent slice stained by H-E.

Results

Ultrasoft radiographic images

Neither bone formation nor bone resorption was seen in the control tibiae. In the bony defect on the experimental side, callus formation was recognised on the 6th day, and was most marked between the 12th and 18th days. The defect was almost completely repaired after the 24th day (Fig. 1).

Histological findings

On the 3rd day after operation, blood clots, granulation and poorly vascularised fibrous tissue occupied the bony defect. On the 6th day, fibrous tissue with fairly good vascularity developed within the defect (Fig. 2a). Some cartilaginous tissues were also encountered and new bone was seen outside the posterior tibial cortex.

On the 9th day, new bone and fibrous tissue replaced the bony defect; the fibrous tissue was well vascularised near the periosteum around the defect (Fig. 3a). Proliferation of new bone and cartilaginous tissue was marked on the posterolateral aspect of the tibia.

On the 12th to the 15th day, the fibrous tissue inside the defect decreased in volume, while new bone increased markedly (Figs. 4a, 5a). Similarly, on the posterolateral aspect of the tibia, cartilaginous tissue decreased and, instead, the new bone

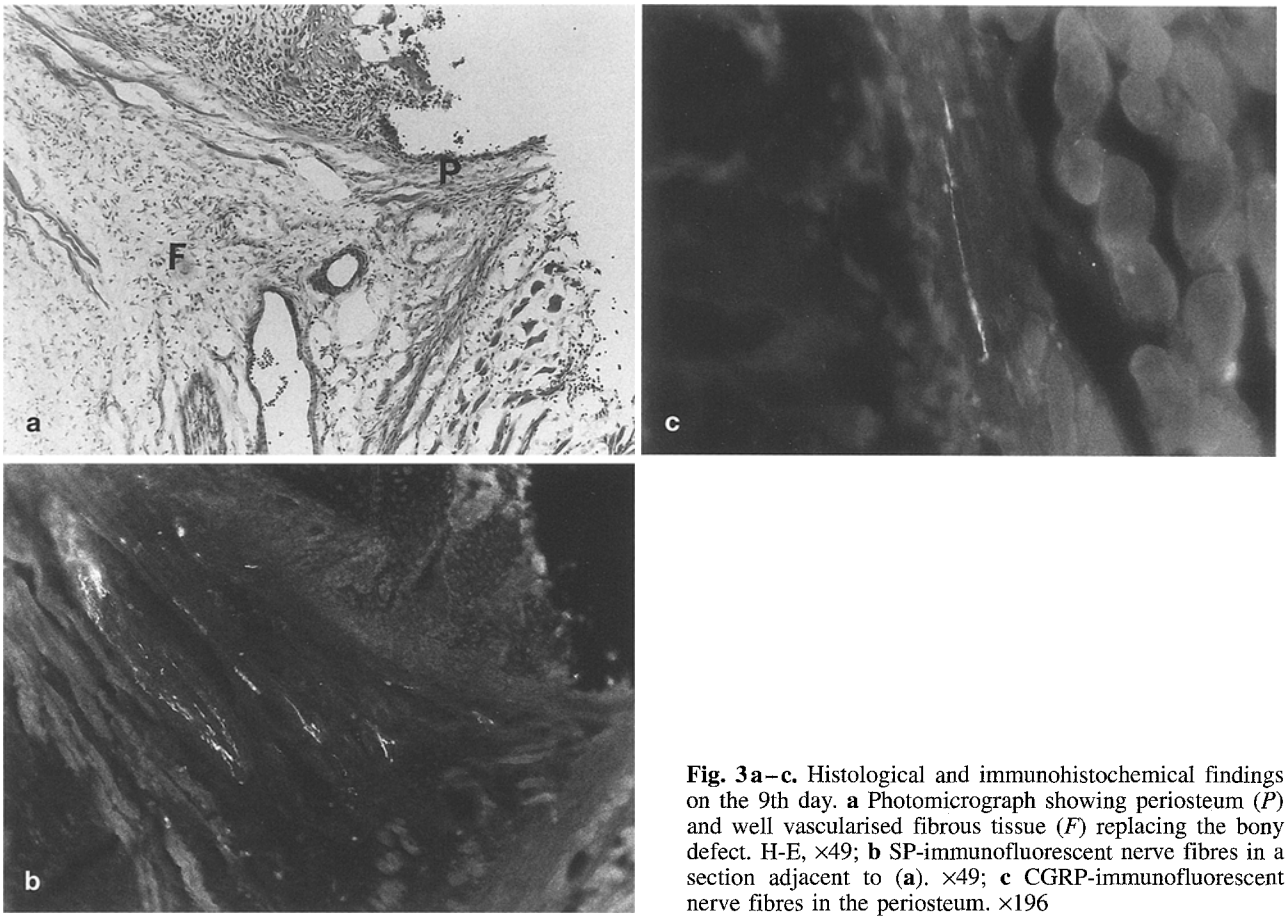

Fig. 3a–c. Histological and immunohistochemical findings on the 9th day. **a** Photomicrograph showing periosteum (*P*) and well vascularised fibrous tissue (*F*) replacing the bony defect. H-E, ×49; **b** SP-immunofluorescent nerve fibres in a section adjacent to (**a**). ×49; **c** CGRP-immunofluorescent nerve fibres in the periosteum. ×196

was larger. On the 15th day, the periosteum had restored the continuity of the surface of the bony defect.

On the 18th day, osteogenesis developed further in the defect and on the posterior tibial surface; the anterior tibial cortex appeared almost continuous. On the 21st day, the amount of new bone was significantly decreased both inside and outside the tibia, and the vascularity of the bone marrow was also reduced. By the 24th to the 27th day the periosteum, cortex and bone marrow had returned to their normal appearance.

Immunohistochemical findings

In the control tibiae, SP- and CGRP-immunofluorescent nerve fibres were only observed in the periosteum. The number of these fibres ranged from 0 to 2 (average 0.82) per section throughout the 59 animals examined. This justified the proposition that a section with more than 3 immunofluorescent fibres showed nerve fibre proliferation. Accordingly we regarded a limb as showing nerve fibre proliferation if the average number of immunofluorescent fibres in 8 sections exceeded 3. Then we calculated the rate of nerve fibre proliferation by dividing the number of limbs with nerve fibre proliferation by the number of limbs examined in individual postoperative periods, separately for SP- and CGRP-immunofluorescence, to give the time-related changes of these neuropeptides. This showed that both the SP- and CGRP-immunofluorescent fibres increased with time after operation, reaching a peak of proliferation on the 12th to 15 days, and then returned to normal with no proliferation after the 24th day (Table 1).

The findings in the experimental tibiae are detailed as follows. On the 3rd day, there was no proliferation of SP- and CGRP-immunofluorescent fibres; on the 6th day (n = 8), SP- (Fig. 2b) and CGRP-immunofluorescent fibres (Fig. 2c) were frequently encountered in the periosteum and in the well vascularised tissue within the defect. The coexistence of SP- and CGRP-immunofluorescence in the same nerve fibres was frequently seen (Fig. 2b, c).

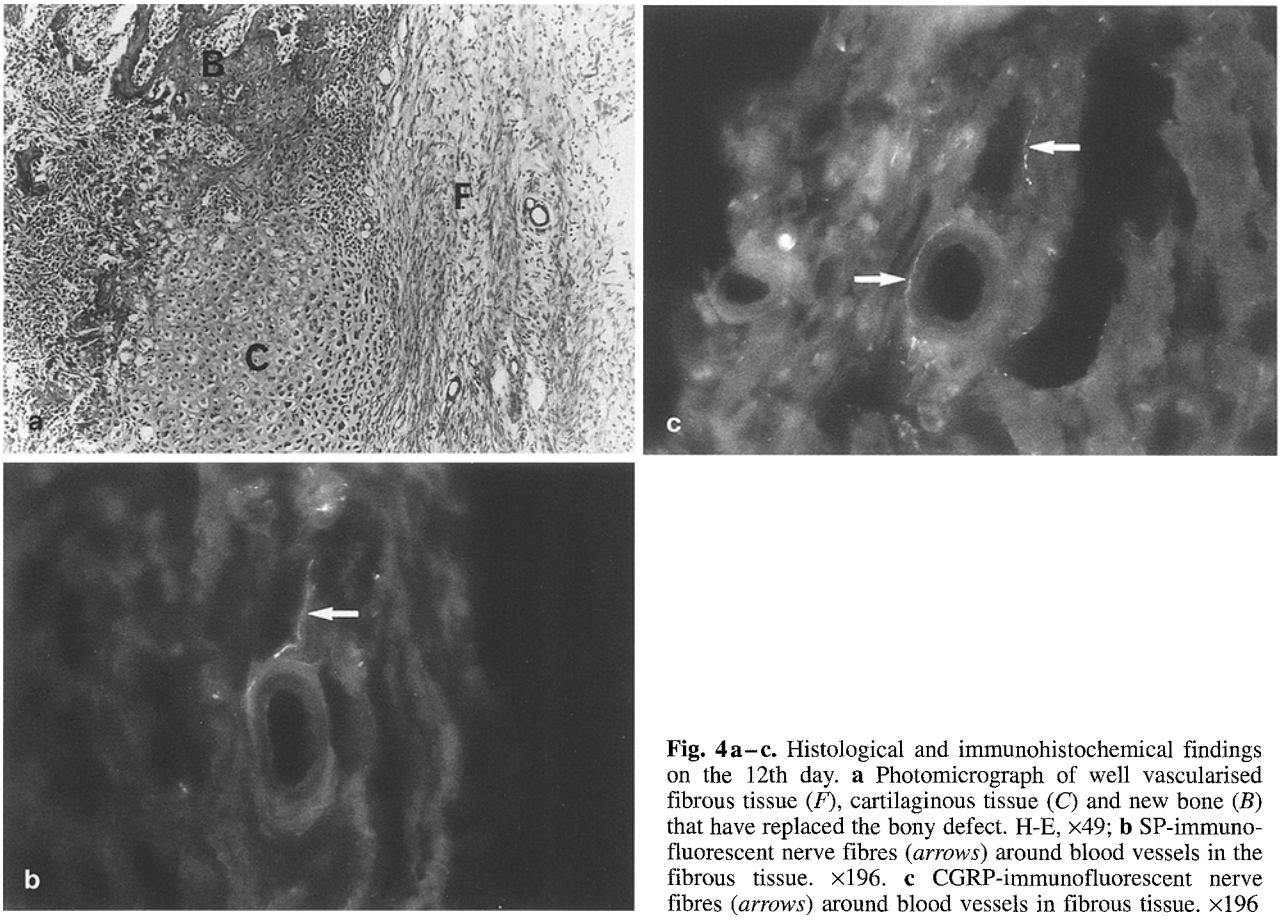

Fig. 4a–c. Histological and immunohistochemical findings on the 12th day. **a** Photomicrograph of well vascularised fibrous tissue (*F*), cartilaginous tissue (*C*) and new bone (*B*) that have replaced the bony defect. H-E, ×49; **b** SP-immunofluorescent nerve fibres (*arrows*) around blood vessels in the fibrous tissue. ×196. **c** CGRP-immunofluorescent nerve fibres (*arrows*) around blood vessels in fibrous tissue. ×196

On the 9th day (n = 8), SP- and CGRP-immunofluorescent fibres were abundant not only within the bony defect, but also in the periosteum on the posterolateral cortex. They were often seen around blood vessels in the fibrous tissue (Fig. 3b, c). No immunofluorescent fibres were found in the cartilaginous tissue which did not contain blood vessels.

On the 12th day (n = 6) to the 15th day (n = 7), SP- and CGRP-immunofluorescent fibres slightly decreased in number inside the fibrous tissue, but were still abundant in the periosteum, being found most frequently close to the blood vessels (Fig. 4b, c). Immunofluorescent fibres were also seen around the relatively large blood vessels within the new trabeculae (Fig. 5b, c).

On the 18th day (n = 5), the distribution pattern of immunofluorescent nerve fibres was similar to that on the 15th day, but the number of fibres had decreased. On the 21st day (n = 7), the immunofluorescent fibres were less frequent. On the 24th day (n = 5) to the 27th day (n = 5), no specimens showed proliferation of either SP- and CGRP-immunofluorescent fibres.

Table 1. Rate of proliferation of SP- and CGRP-immunofluorescent nerve fibres

Days after operation	Control side	3	6	9	12	15	18	21	24	27
Number of limbs examined	59	8	8	8	6	7	5	7	5	5
SP-immunofluorescence[1]	0 (0)	0 (0)	4 (50)	6 (75)	5 (83)	6 (86)	3 (60)	3 (43)	0 (0)	0 (0)
CGRP-immunofluorescence[1]	0 (0)	0 (0)	6 (75)	8 (100)	6 (100)	7 (100)	5 (100)	4 (57)	0 (0)	0 (0)

[1] Number of limbs with nerve fibre proliferation (more than three immunofluorescent fibres in a section in average). Percentages are given in parentheses

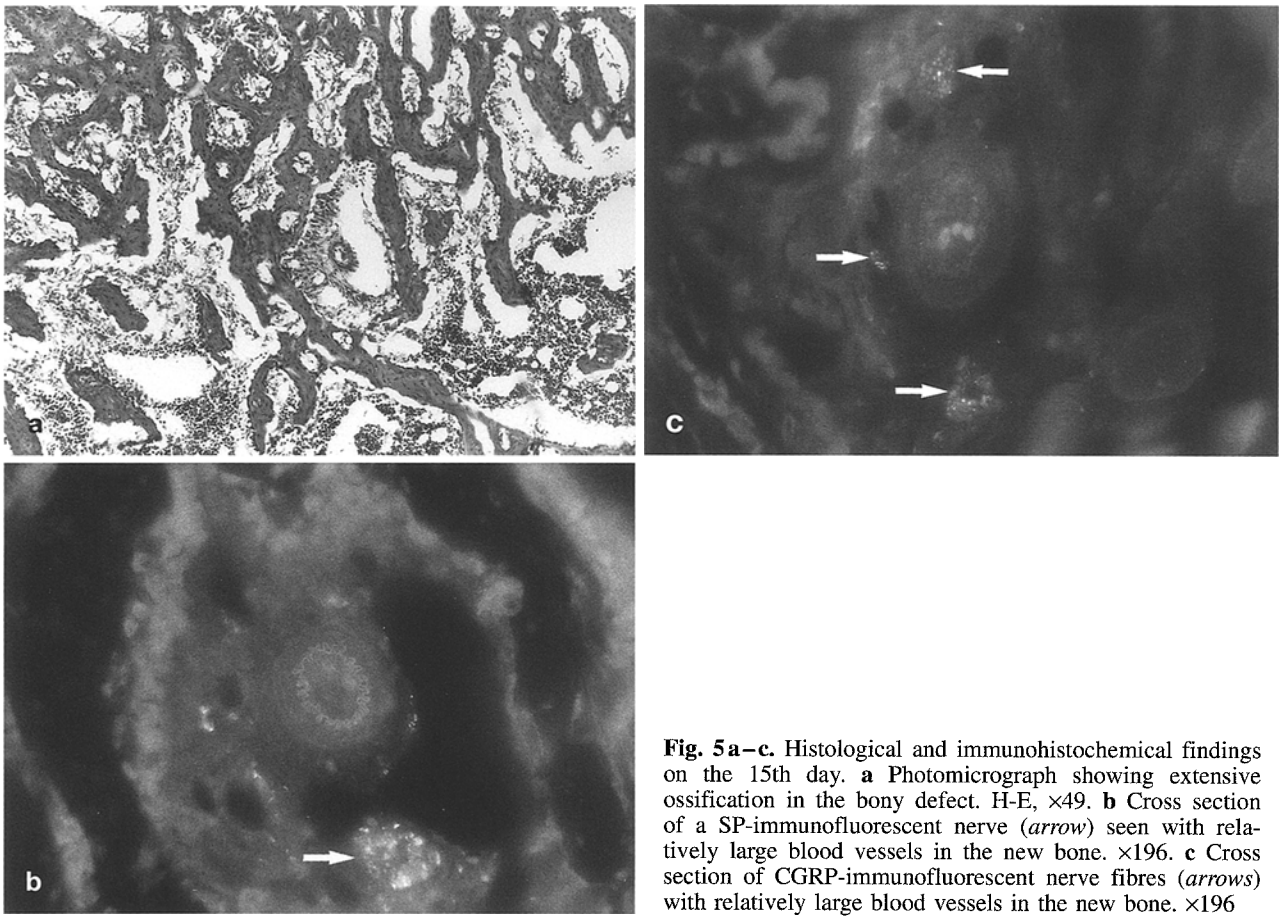

Fig. 5a–c. Histological and immunohistochemical findings on the 15th day. **a** Photomicrograph showing extensive ossification in the bony defect. H-E, ×49. **b** Cross section of a SP-immunofluorescent nerve (*arrow*) seen with relatively large blood vessels in the new bone. ×196. **c** Cross section of CGRP-immunofluorescent nerve fibres (*arrows*) with relatively large blood vessels in the new bone. ×196

Discussion

This study demonstrated that the development of SP- and CGRP-immunofluorescent nerve fibres was closely associated with the new bone formation seen in radiographs. Moreover, SP- and CGRP-immunofluorescent nerve fibres were abundant during the biologically active phase of bone repair.

Both SP and CGRP in the bone and periosteum have been shown to originate from the primary sensory nerves, while VIP and NPY come from sympathetic neurones [13, 14]. It has also been reported that SP and CGRP are produced in the dorsal root ganglion and then transported to the nerve endings [12]. Thus these neuropeptides can be a marker of primary sensory nerve terminals. The SP- and CGRP-immunofluorescent fibres observed in this study are most likely to originate from primary sensory nerves and have played a protective nociceptive function until the bone was well stabilised as suggested by Rusanen et al. [26]. This does not, however, imply that nociceptive sensation from the callus tissue is transmitted by the SP and CGRP per se. SP and CGRP are known to be released in the central branches of the primary sensory neurones by nociceptive stimuli to transmit pain sensation to the spinothalamic tract [18, 22, 27], whereas the sensory transmission through the peripheral branches is in the reverse direction to the transport of the neuropeptides.

The role of SP and CGRP in the nerve endings seems to be efferent in nature. Both are potent vasodilators [6, 7, 8, 30]. Our study has demonstrated that SP- and CGRP-immunofluorescent fibres were most often found close to the blood vessels in the fibrous callus, new trabeculae and periosteum. New vascularisation in the callus was most marked on the 9th to 18th day after operation, which was in keeping with the time when SP- and CGRP-immunofluorescent fibres accumulated. These findings suggest that SP and CGRP could have acted on blood vessels to enhance local blood flow during new bone formation. The importance of angiogenesis in osteogenesis shown in previous studies [24, 28] supports this possibility. Also, the direct osteogenic action of CGRP [9, 31, 32] might have affected the reparative process.

Our results can be safely applied to fracture healing which consists of inflammatory, repair and reconstructive phases [25]. In the histological findings we have described, the 3rd day corresponds to the inflammatory phase, the 6th to 21st day to the repair phase, and the 24th to the 27th day to the reconstructive phase. The immunohistochemical results can also be interpreted in terms of these three phases. The SP- and CGRP-immunofluorescent nerve fibres proliferated early in the repair phase (6th day), were most marked in the middle of this phase (12th to 18th day), and were less frequent at the end (21st day). These changes confirmed those reported by Rusanen et al. [26], although they did not demonstrate such changes in CGRP-immunoreactive nerves.

The proliferation rate of SP-immunofluorescent nerve fibres was constantly lower than that of CGRP-immunofluorescent fibres throughout the experiment, although the time-related changes were similar (Table 1). The distribution pattern of peptidergic nerves has been reported to vary in different organs [21, 23]. The significance of less abundant SP-immunofluorescent fibres in osteogenesis and the coexistence of SP- and CGRP-immunofluorescence within a nerve fibre (Fig. 2b, c) should be studied, since they may imply interaction between the two peptides as suggested in earlier studies [17, 19, 20, 29].

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