Journal of Orthopaedic Research
13:570-577 The Journal of Bone and Joint Surgery, Inc.

Changes in Innervation of Long Bones after Insertion of an Implant: Immunocytochemical Study in Goats with Antibodies to Calcitonin Gene-Related Peptide and B-50/GAP-43

P. Buma, L. Elmans, and *A. B. Oestreicher

*Institute of Orthopaedics, Laboratory for Experimental Orthopaedics, Section of Histomorphology, University Hospital Nijmegen, Nijmegen, and *Rudolf Magnus Institute, Utrecht, The Netherlands*

Summary: In this study, we describe the distribution of fibres that contain calcitonin gene-related peptide in normal bones and in bones that are remodeling after insertion of an implant. With routine histology and antibodies to calcitonin gene-related peptide, small neural and free-running fibres staining positively for calcitonin gene-related peptide were found in the periosteum, endosteum, and cortical bone of the tibia in the goat. In many cases, the free-running fibres were associated with blood vessels that entered the bone through Volkmann's canals. The endosteal blood supply was destroyed as a result of insertion of the implant. The necrotic bone was no longer innervated, as shown by the lack of staining for the antibodies. At 6 weeks, a repair phase started with revascularization and remodeling of the necrotic endosteal bone. During this repair phase, there was increased innervation with fibres containing calcitonin gene-related peptide in the remodeling cavities at the interface between living and necrotic bone. These fibres ended blindly, with many large varicosities, and could be demonstrated by immunostaining with monoclonal antibodies to B-50/growth associated protein-43, an antibody to outgrowing neuronal fibres. The correlative occurrence between extensive sprouting of fibres containing calcitonin gene-related peptide and the remodeling of necrotic endosteal bone suggests that sensory fibres with calcitonin gene-related peptide have a regulatory role in the control of angiogenesis or of bone remodeling associated with the insertion of an implant, or with both processes.

It has been well known for several decades that bones and the surrounding soft tissues are innervated (31,32). Immunocytochemistry has shown that different chemically defined types of fibres are involved in the innervation of the skeleton (2,3,7,12,13,19,22,31). It is assumed that the fibres have more than one function, on the basis of experimental evidence, identification of the various neuroactive substances, and knowledge of the sympathetic and sensory origin of the fibres (5,15,17,20,42,54). Innervation may be involved in trophic effects on tissues, regulation of vascularization or bone remodeling, and pain and proprioception (2,3,19,26).

The present study focussed on the changes in the innervation pattern of sensory fibres associated with bone remodeling after insertion of an implant. In bone, the main function of sensory fibres that contain calcitonin gene-related peptide (CGRP) and substance P may be perception of pain (15,16,18,19,24,31,33,40,43). However, it also seems very plausible that CGRP plays a role in bone remodeling, on the basis of the pattern of innervation of molar teeth (45), the strong inhibitory influence of CGRP on osteoclastic bone resorption (8,57,58), the localization of peripheral CGRP binding sites (34), the effects of chemical sensory denervation, sympathectomy (19), and the proliferation of fibres containing CGRP around fracture sites (27).

The antibody to the B-50/growth associated protein (GAP)-43, which is a neuron-specific protein (29,30,46), may be of special interest in innervation studies. This antibody has been applied to detect outgrowing nerve fibres (48,52,53). A high concentration of B-50/GAP-43 has been found in axonal growth cones during embryonic (37,50) and postnatal development (10,11,39). The expression of B-50/GAP-43 is reinforced following nerve injury (46). Enhanced immunoreactivity to B-50/GAP-43 has been demonstrated in regenerating axons (48,52); thus, the antibody may be of use to demonstrate axonal sprouting (11,49,53).

The purpose of this study was to describe the changes in the pattern of sensory innervation in bone after insertion of an implant. As an experimental model, we used the tibia of the goat. It has been shown that part of the endosteal blood circulation is de-

Received March 14, 1994; accepted October 3, 1994.
Address correspondence and reprint requests to P. Buma at Institute of Orthopaedics, University Hospital Nijmegen, P.O. Box 9101, 6500 HB, Nijmegen, The Netherlands.

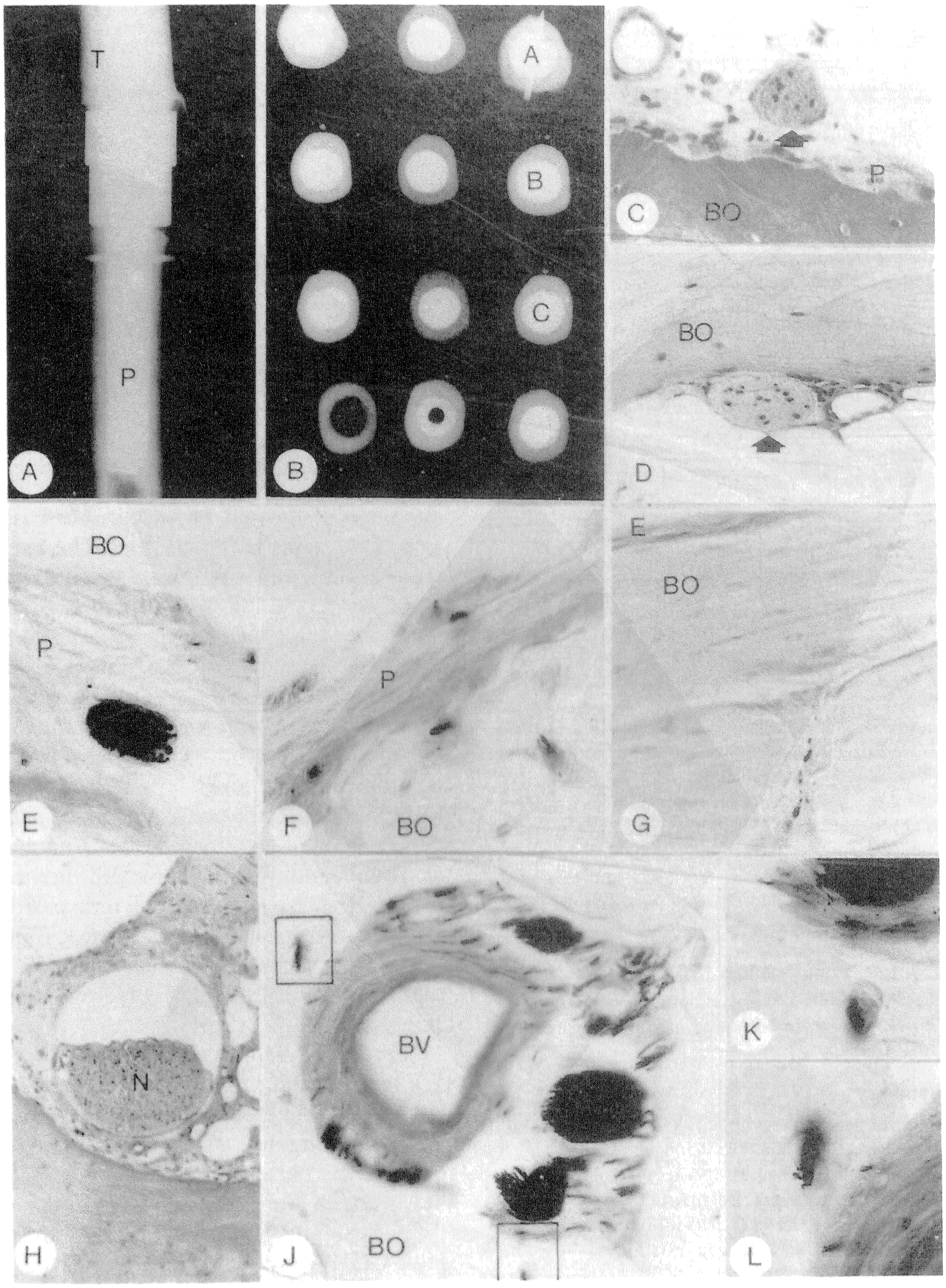

FIG. 1. The tapered part of the implant (P), coated with hydroxyapatite, was press fit into the distal part of the tibia (T) of the goat, and the proximal part was fixed with bone cement (**A,** ×1.1). Thick sections were cut at proximal (A), mid-shaft (B), and distal levels (C) along the implant 1 week (**B,** ×1.1), 6 weeks, and 12 weeks after insertion. In sections of control bones, staining with haematoxylin and eosin (**C** and **D,** ×240) showed small nerves (arrows) in the periosteum (P) and endosteum of the bone (BO), and immunostaining with antibodies to calcitonin gene-related peptide showed a small nerve (**E,** ×240), small bundles of nerve fibres partly in close association with the bone (**F,** ×300), and a free-running fibre in Volkmann's canal at an endosteal location (E) (**G,** ×270). Low magnification of a section from a control bone stained with haematoxylin and eosin (**H,** ×60) showed a rather thick nerve (N) in association with the nutrient vessels, which penetrates the bone. Immunostaining with antibodies to B-50/growth associated protein-43 in a section from a control bone (**J,** ×7) demonstrated its localization in nerves and free-running fibres in the nutrient canal. Large blood vessels (BV) are penetrating through the nutrient canal into the medullary cavity. In the upper and lower boxed areas (enlarged [×200] in **K** and **L**, respectively), small branches seem to penetrate into the cortical bone.

stroyed after insertion of an implant into the tibia (6); in time, the resulting necrotic bone was partly replaced by new bone as a result of bone remodeling of the necrotic endosteal areas. We do not know whether the remodeled bone becomes reinnervated, how rapidly reinnervation takes place, or what fibres are in-

volved. In this study, we focussed on fibres containing CGRP and used the antibody to B-50/GAP-43.

MATERIAL AND METHODS

Partly noncemented implants were placed in the midshaft of the tibia of the Dutch milk goat (*Capra hircus sana*). The straight tapered distal part of the implant was coated with a layer of approximately 60 μm plasma-sprayed hydroxyapatite, with a crystalline phase of about 70% (CAM Implant Service, Leiden, The Netherlands). After segmental resection, the proximal part of the implant was fixed with bone cement and the hydroxyapatite-coated distal end was press fit into the distal part of the bone, and they were subsequently coupled with each other (Fig. 1A). During the first 2 days after the operation, the goats were kept in a hammock to prevent early load-bearing. Qualitative evaluation of bone remodeling was performed using sequential fluorochrome labelling with oxytetracycline (for 7 days, beginning shortly after the operation), xylenol orange (for 7 days in the middle of the period), and calcein green (for 7 days directly before death). At 1, 6, and 12 weeks, four animals each were given a lethal dose of pentobarbitone. Immediately afterwards, perfusion was carried out with 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4. Each contralateral tibia was used as a control. All of the bones were disarticulated carefully, radiographs were taken, and the bones were fixed for at least another 7 days. A water-cooled saw (WOCO 50P/10; Conrad, Clausthal-Zellerfeld, Germany) was used to cut transverse slices every 3 mm. Radiographs of the slices were taken.

The slices then were treated in the following ways. All prosthetic material was removed from the slices by push-out procedures. For routine histology, the slices were decalcified in 25% EDTA under radiographic control, embedded in polymethylmethacrylate, cut into thin sections (7 μm), and stained with haematoxylin and eosin. For fluorescence microscopy, unstained undecalcified slices, 3 mm thick, were embedded in polymethylmethacrylate and sectioned (20 μm) on a rotating water-cooled diamond saw (model 1600; Leitz, Wetzlar, Germany).

For immunocytochemistry, decalcified slices were transferred to 30% sucrose, deep frozen, and sectioned (80 μm) with a freeze microtome. Sections were collected in Tris buffered saline (0.05 *M* Tris, 0.9% NaCl, pH 7.4) for free-floating immunocytochemical processing.

Immunocytochemistry

Immunostaining was performed with polyclonal rabbit antibodies to CGRP (1:6,000) (RAS 6009-N; Peninsula Laboratories, Belmont, CA, U.S.A.), monoclonal antibodies to B-50/GAP-43 (1:4,000) (NM4; Innogenetics, Ghent, Belgium) (38), and polyclonal antibodies to B-50/GAP-43. The specificity of the antibodies to CGRP (5) and B-50 (10,11,39) has been tested previously. The specificity of the antisera was determined with use of the conventional method of substituting normal rabbit preimmune or mouse serum for the primary antiserum and omitting the primary antiserum step. The specificity of the CGRP antiserum was tested further by preadsorption of 5 ml of the diluted antiserum with 0.1 mg of CGRP (6009; Peninsula Laboratories).

The sections were stained overnight with the antiserum, stained for 2 hours with peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins or with peroxidase-conjugated rabbit immunoglobulins to mouse immunoglobulins (1:75) (Dakopatts, Glostrup, Denmark), and stained for 10 minutes with 0.04% 3,3-diaminobenzidine-4-hydrochloride (Merck, Darmstadt, Germany), 0.1% nickel ammonium sulphate, and 0.003% H_2O_2 in 0.05 *M* Tris buffer (pH 7.6). All dilutions of antibodies were made in Tris buffered saline to which 0.5% bovine serum albumin, 0.5% Triton X-100, and 1% normal goat serum were added. Before, between, and after each incubation step, the sections were washed three times for 20 minutes with fresh Tris buffered saline. All incubations were at room temperature and continuous gentle agitation was used. After the staining procedure, the sections were dehydrated and were mounted with Eukitt (Clichy, France) on gelatin-coated slides and protected with coverslips.

All of the sections were investigated and photographed by photomicroscopy (Zeiss II; Zeiss Instruments, Oberköchen, Germany). At each time period, remodeling cavities were quantitated in three equidistant cross sections (proximal, midshaft, and distal) of each tibia by direct light microscopic examination, at a magnification of ×50. Neuronal varicosities were quantitated in four random areas in the remodeling area of the cortical bone with a surface area of 1 mm^2, at a magnification of ×100.

RESULTS

The sections did not show any artifacts due to the freezing procedure. The fibres labeled with peroxidase-antiperoxidase could be recognized easily and were stained throughout the sections. The background immunostaining was very low or absent. The controls for specificity of the method (omission of the first incubation step and incubation with preimmune serum) were completely negative. After preadsorption of the CGRP antiserum with the peptide, specific immunostaining was completely absent. The decalcification procedure did not have an effect on the intensity of the immunostaining (5).

The immunocytochemical staining pattern and the sections stained with haematoxylin and eosin enabled the visualization of the gross and fine innervation of the tibia. In all specimens at all time periods, two types of fibres were observed: fairly thick fibres in nerves, showing no varicose structures (Fig. 1E and J), and free-running fibres, which generally had a varicose appearance (Figs. 1G and 2G).

Fibres were stained with CGRP in all nerves (Fig. 1E). Free-running fibres were located in the periosteum of the bone (Fig. 1F). Irrespective of the cross section investigated, many small nerves always were found in the periosteum of the tibia (Fig. 1C); often, they were in the soft tissues in direct contact with the periosteum. Small branches of these nerves, or single CGRP-positive fibres, entered the cortical bone and usually were associated with blood vessels located in Volkmann's canals (Fig. 1F). A fairly thick nerve or thick branch of nerves entered the medullary space through the nutrient canal (Fig. 1H-L). Small branches of the nutrient nerve entered the cortical bone (Fig. 1J-L). The rather thick nerve associated with the nutrient blood vessels branched into smaller nerves as soon as it entered the medullary canal. Small nerves were located in an endosteal position (Fig. 1D), from where many single fibres or small groups of fibres entered the cortical bone (Fig. 1G). In the control bones, no remodeling cavities were present. The mean (±SD) number of nerve varicosi-

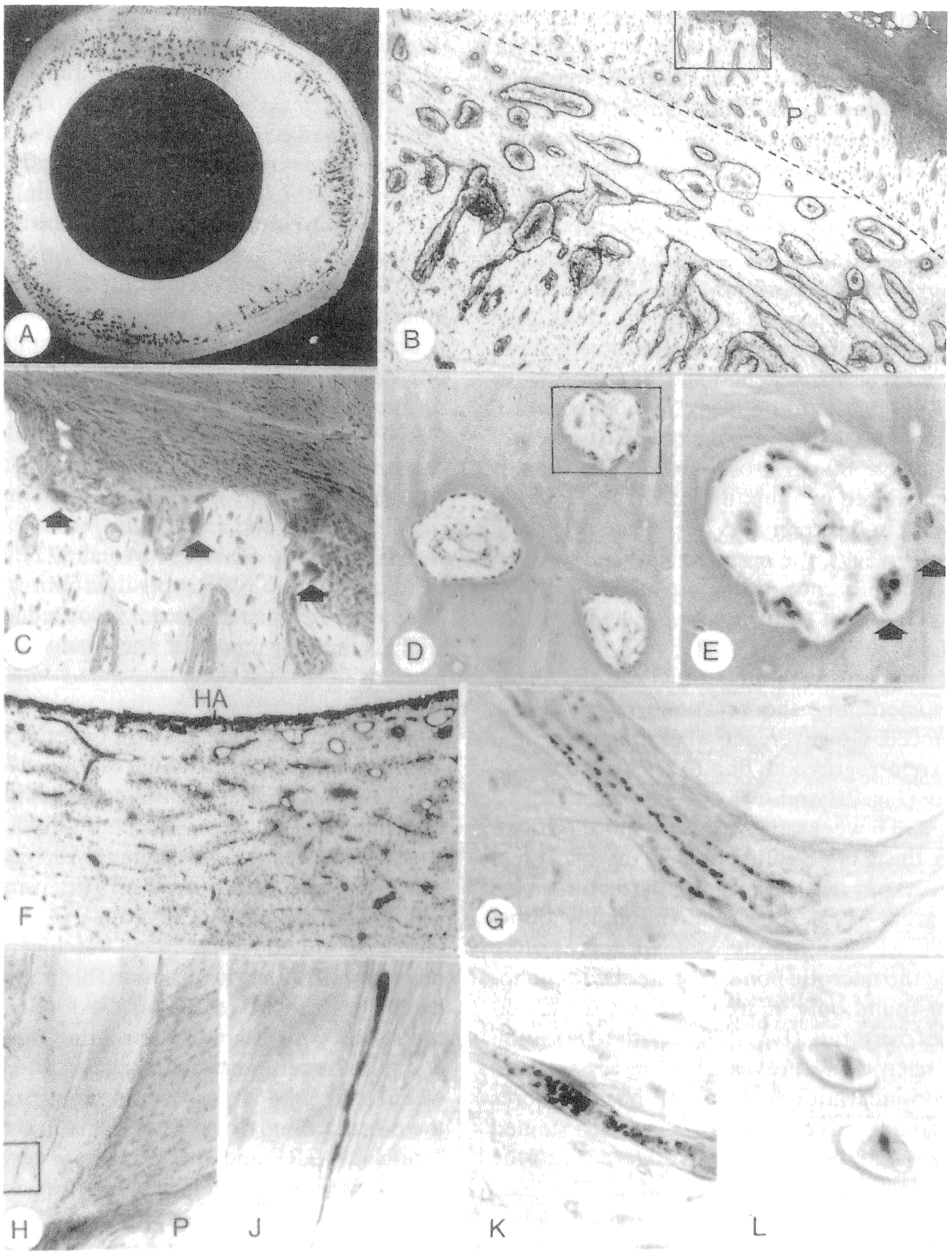

FIG. 2. A: Radiograph (×2) of a 100 μm thick section, after removal of the prosthetic material, taken 6 weeks after the operation, showing porosis of the cortical bone. **B:** Section stained with basic fuchsin (×30), 6 weeks after the operation, showing the periosteal reaction boundary (broken line) between the pre-existing cortical bone (lower portion) and the periosteal reaction (upper portion). Note the porosis of the cortical bone. **C:** Enlargement (×170) of the boxed area in **B,** showing many osteoclasts that are resorbing the periosteal reaction (arrows). **D:** Section stained with haematoxylin and eosin (×80), 6 weeks after the operation, showing remodeling cavities at the interface between living and necrotic bone. **E:** Enlargement (×220) of boxed area in **D,** showing osteoclasts (arrows). **F:** By 12 weeks, creeping substitution has reached the hydroxyapatite (HA) layer around the implant (×40). **G:** A section (×300) at 6 weeks shows many fibres staining positively for calcitonin gene-related peptide (CGRP) in the remodeling cavity. **H:** Low magnification (×50) of a section 6 weeks after the operation, showing a blood vessel penetrating the cortical bone. **J:** Enlargement (×250) of encircled area of **H**, showing blindly ending nerve fibre that stained positively for B-50/growth associated protein-43. The fibre is directed toward the endosteal side of the bone. **K:** At 6 weeks, a large number of varicose fibres that stained positively for CGRP are in the remodeling cavity (×300). **L:** At 12 weeks after the operation, a normal pattern of innervation has been restored. There are sparse CGRP-positive fibres in the cortical bone close to the implant (×240).

ties was 18.4 ± 8.2 per sampling area, all located in Volkmann's canals.

Only a brief description is given of the effects of the insertion of the implant on bone, as the details of histological reactions have been published elsewhere (6). Clinically, the goats functioned well. At 3 weeks,

the limb with the implant could be loaded normally. Immediately after insertion, there were no significant gaps between the implant and the cortical bone (Fig. 1A and B). At 3 weeks, the first signs of creeping substitution of the necrotic cortical bone were found. At 6 weeks, radiographs and histology showed sequential osteoclastic porosis and new bone formation in the necrotic bone (Fig. 2A and B). This process of creeping substitution (Fig. 2D and E) started at the transition between living peripheral and endosteal necrotic bone and moved toward the implant. Where the endosteal layer of necrotic bone was relatively thin, the first bone ingrowth reached the hydroxyapatite layer around the implant at 6 weeks (6). At 6 weeks, the mean number (±SD) of remodeling cavities in the tibia was 459 ± 89 per cross section. The remodeling process resulted in a mixture of vital remodeled bone embedded in the remnants of the necrotic bone (Fig. 1F). At 12 weeks, the creeping substitution had reached the circumference of the implant surface and new bone was in direct contact with the hydroxyapatite layer (Fig. 1F).

Periosteal reactions were moderate (Fig. 2B). Proximal to the osteotomy, the reactions were slightly more pronounced. However, at 6 weeks the periosteal reactions were in regression, due to periosteal osteoclastic activity (Fig. 2B and C).

At 1 week and 6 weeks, there was no more positive staining with the CGRP antiserum in the endosteal necrotic bone. Apparently, the neural structures also had disappeared, as there was no immunostaining for the B-50/GAP-43 antibody. Stained nerves were found outside the necrotic bone. At 6 weeks, immunoreactivity was found only in areas where vascularization had been restored (Fig. 2G). At the transition between the necrotic and revascularizing areas, there was increased innervation with CGRP-positive fibres (Fig. 2K). In contrast to control bone, in which stained fibres were found only occasionally, staining with the CGRP antibodies in bones with an implant revealed many varicose fibres in almost all of the remodeling cavities. In the sampling areas, 23.4 ± 5.2 remodeling cavities were found, and they contained 483 ± 79.5 neural varicosities (a mean of 21 varicosities per cavity). Fibres immunostained for the B-50/GAP-43 antibody were at the same location and had the same morphology. Occasionally, bludgeon-like structures were associated with blood vessels that entered the bone (Fig. 2H and J). The blindly ending thick parts of the structures always were directed toward the implant. They strongly resembled growth cones that have been found during embryonal development of the pyramidal tract (11) and were present only at 6 weeks. At 12 weeks, the proliferated CGRP-positive fibres had disappeared and the preoperative situation had been restored. The bone around the implant had become reinnervated with sparse CGRP-stained fibres (Fig. 1L), and there were no longer any growth cones.

DISCUSSION

Insertion of an implant into the tibia of the goat had distinct effects on bone remodeling and the pattern of sensory innervation. We observed initial degeneration of endosteal bone innervation, followed by proliferation of CGRP-positive fibres during creeping substitution of necrotic bone. To date, changes in the innervation pattern of bone after pathological disorders have been described in the human femoral head after arthrosis (41) and in the innervation of the arthrotic knee with fibres containing substance P and CGRP in humans (56) and in mice (5). In addition, it has been demonstrated in humans (13,36) and rats (25) that, in the presence of arthritis, the local levels of substance P and CGRP in the fibres may be depleted. Recently Hukkanen et al. (27) described the proliferation of CGRP-positive fibres periosteally around fracture sites. To our knowledge, the current study is the first report on the changes in innervation patterns in cortical bone associated with the insertion of an implant.

On the basis of the present observations, it seems very plausible that the innervation of long bones is associated strongly with this vascularity. This means that endosteal necrosis induced by endosteal devascularization always is associated with necrosis of the accompanying nerve supply. The repair phase, in which necrotic bone is revascularized by peripheral blood vessels, also is a phase in which peripheral fibres reinnervate the cortical bone. This idea is supported by the presence of the B-50/GAP-43-positive structures that were found at the interface between the living and necrotic bone, particularly after 6 weeks, when revascularization and creeping substitution of the necrotic bone took place. The immunostaining patterns for CGRP and B-50 that were observed after 6 weeks were morphologically similar to those for the growth cones of the regenerating sciatic nerve (52) and those reported in the developing pyramidal tract (10,11,28). Although the exact nature of these structures cannot be judged by use of light microscopy alone, it seems likely that axonal sprouts, which reinnervate necrotic bone, are formed during the repair phase.

Studies on the distribution of CGRP immunoreactivity in many species (2,14,23,51) have strongly suggested that, in a large proportion of sensory fibres, CGRP is co-localized with substance P. Fibres containing CGRP and substance P may be the most important ones to transduct nociceptive impulses to the spinal cord (15,24,43,56), but other types of fibre also may be involved (17). However, the function of CGRP-positive fibres in the tibia of the goat should be in-

terpreted with great care, because it is not known whether all fibres demonstrated in the present study originated from primary sensory neurons. Both physiological and anatomical tracer studies with horseradish peroxidase have shown that part of the innervation of the lower extremity is of sympathetic origin (17,54). Moreover, careful comparison of the number of articular afferents that display a nociceptive function, as determined by electrophysiological studies, and the proportion of fibres containing substance P or CGRP in the medial articular nerve indicates that, at most, only 30% of the nociceptive-specific articular afferents contain substance P or CGRP, or both. Thus, if the CGRP-positive fibres in the present study were of sensory origin, other undetected fibres also may be involved in the perception of pain. Therefore, detailed tracer studies are needed to evaluate the exact nature of the fibres demonstrated in the present study. Bearing this in mind, it can be speculated that the pain in the middle of the thigh that sometimes occurs after the insertion of a noncemented prosthesis (55) is mediated by fibres containing CGRP.

Although it is not known if CGRP can be released from the varicosities into the surrounding tissues, a local efferent function of fibres containing CGRP also seems plausible. CGRP may be involved in the regulation of vascular sprouting and the stimulation of angiogenesis or in the regulation of osteoclast and osteoblast activity, or it may be involved in all of these. Its involvement in the regulation of osteoclast and osteoblast activity seems to be the most plausible. Bone architecture is a result of continuous modeling and remodeling of the cortical and trabecular components of bone. It generally is assumed that the mechanical environment is a major determinant of the physiological behaviour of mammalian cancellous and cortical bone, in such a way that the bone structure is always optimal for the prevailing mechanical environment. However, mechanical stimuli are mediated by biological factors that stimulate or inhibit cells involved in the alteration of bone stock. Very little is known about the nature of the chemical factors involved in these regulatory processes of cellular activity during bone remodeling. Prostaglandins may be involved in bone formation, but the source is not yet known (1). Localization and *in vitro* studies have supported the view that the innervation of bone not only plays a sensory role but also may be involved in bone metabolism. Substance P can stimulate bone resorption by inducing the macrophage-mediated release of interleukin-1 (29) and tumor necrosis factor-α (34). In addition, it may mediate the release of oxygen derivatives, which also may stimulate bone resorption (9,44). With respect to CGRP, the pattern of innervation of molar teeth (45) strongly suggests that it has a role in mineralization. Furthermore, in *in vitro* experiments, it has been found that besides having a vasodilatory action (4,47), CGRP also exhibits a strong inhibitory influence on osteoclastic bone resorption (8,57,58). Mantyh et al. (35) found peripheral CGRP binding sites, which suggests the presence of CGRP receptors. It has been known for some time that vasoactive intestinal peptide has a very strong stimulatory action on bone resorption (21).

In conclusion, after insertion of an implant into the tibia of the goat, necrosis and subsequent dynamic neural sprouting and reinnervation associated with the process of creeping substitution of the necrotic bone were observed. The exact functional relationship between innervation of bone and bone remodeling remains to be clarified.

Acknowledgment: The authors would like to express their gratitude to Mrs. H. Versleyen for expert technical assistance, P. van Loon for performing the operations on the goats, and Dr. Marc Mereken and Innogenetics for providing the B-50 monoclonal antibody.

REFERENCES

1. Binderman I, Zor U, Kaye AM, Shimshoni Z, Harell A, Somjen D: The transduction of mechanical force into biochemical events in bone cells may involve activation of phospholipase A_2. *Calcif Tissue Int* 42:261-266, 1988
2. Bjurholm A, Kreicbergs A, Brodin E, Schultzberg M: Substance P- and CGRP immunoreactive nerves in bone. *Peptides* 9:165-171, 1988
3. Bjurholm A, Kreicbergs A, Terenius L, Goldstein M, Schultzberg M: Neuropeptide Y-, tyrosine hydroxylase- and vasoactive intestinal polypeptide-immunoreactive nerves in bone and surrounding tissues. *J Auton Nerv Syst* 25:119-125, 1988
4. Brain SD, Williams TJ, Tippins JR, Morris HR, MacIntyre I: Calcitonin gene-related peptide is a potent vasodilator. *Nature* 313:54-56, 1985
5. Buma P, Verschuren C, Versleyen D, Van der Kraan P, Oestreicher AB: Calcitonin gene-related peptide, substance P and GAP-43/B50 immunoreactivity in the normal and arthrotic knee joint of the mouse. *Histochemistry* 98:327-339, 1992
6. Buma P, Slooff TJJH, Versleyen D: A simple, rapid and reliable procedure for the visualization of bone with implants. *Clin Mater* 15:93-99, 1994
7. Cavanaugh JM, El-Bohy A, Hardy WN, Getchell TV, Getchell ML, King AI: Sensory innervation of soft tissues of the lumbar spine in the rat. *J Orthop Res* 7:378-388, 1989
8. D'Souza SM, MacIntyre I, Girgis SI, Mundy GR: Human synthetic calcitonin gene-related peptide inhibits bone resorption in vitro. *Endocrinology* 119:58-61, 1986
9. Garret IR, Boyce BF, Oreffe ROC, Bonewald L, Poser J, Mundy GR: Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. *J Clin Invest* 85:632-639, 1990
10. Gorgels TGMF, Oestreicher AB, de Kort EJM, Gispen WH: Immunocytochemical distribution of the protein kinase C substrate B-50 (GAP43) in developing rat pyramidal tract. *Neurosci Lett* 83:59-64, 1987
11. Gorgels TGMF, Van Lookeren Campagne M, Oestreicher AB, Gribnau AA, Gispen WH: B-50/GAP43 is localized at the cytoplasmic side of the plasma membrane in developing and adult rat pyramidal tract. *J Neurosci* 9:3861-3869, 1989
12. Grönblad M, Liesi P, Korkala O, Karaharju E, Polak J: Innervation of human bone periosteum by peptidergic nerves. *Anat Rec* 209:297-299, 1984
13. Grönblad M, Konttinen YT, Korkala O, Liesi P, Hukkanen M, Polak JM: Neuropeptides in synovium of patients with rheumatoid arthritis and osteoarthritis. *J Rheumatol* 15:1807-1810, 1988

14. Gulbenkian S, Merighi A, Wharton J, Varndell IM, Polak JM: Ultrastructural evidence for the coexistence of calcitonin gene-related peptide and substance P in secretory vesicles of peripheral nerves in the guinea pig. *J Neurocytol* 15:535-542, 1986
15. Hanesch U, Heppelmann B, Schmidt RF: Substance P- and calcitonin gene-related peptide immunoreactivity in primary afferent neurons of the cat's knee joint. *Neuroscience* 45:185-193, 1991
16. Henry JL: Effects of substance-P on functionally identified units in cat spinal cord. *Brain Res* 114:439-451, 1976
17. Heppelmann B, Schaible H-G: Origin of sympathetic innervation of the knee joint in the cat: a retrograde tracing study with horseradish peroxidase. *Neurosci Lett* 108:71-75, 1990
18. Hill EL, Elde R: Calcitonin gene-related peptide-immunoreactive nerve fibers in mandibular periosteum of rat: evidence for primary afferent origin. *Neurosci Lett* 85:12-178, 1988
19. Hill EL, Elde R: Distribution of CGRP-, VIP-, DβH-, SP-, and NPY-immunoreactive nerves in the periosteum of the rat. *Cell Tissue Res* 264:469-480, 1991
20. Hill EL, Turner R, Elde R: Effects of neonatal sympathectomy and capsaicin treatment on bone remodeling in rats. *Neuroscience* 44:747-755, 1991
21. Hohmann EL, Levine L, Tashjian AH Jr: Vasoactive intestinal peptide stimulates bone resorption via a cyclic adenosine 3′5′-monophosphate-dependent mechanism. *Endocrinology* 112:1233-1239, 1983
22. Hohmann EL, Elde RP, Rysavy JA, Einzig S, Gebhard RL: Innervation of periosteum and bone by sympathetic vasoactive intestinal peptide-containing nerve fibers. *Science* 232:868-871, 1986
23. Holzer P: Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience* 24:739-768, 1988
24. Hope PJ, Jarrott B, Schaible H-G, Clarke RW, Duggan AW: Release and spread of immunoreactive neurokinin A in the cat spinal cord in a model of acute arthritis. *Brain Res* 533:292-299, 1990
25. Hukkanen M, Grönblad M, Rees R, Konttinen YT, Gibson SJ, Hietanen J, Polak J, Brewerton DA: Regional distribution of mast cells and peptide containing nerves in normal and adjuvant arthritic rat synovium. *J Rheumatol* 18:177-183, 1991
26. Hukkanen M, Konttinen YT, Rees RG, Gibson SJ, Santavirta S, Polak JM: Innervation of bone from healthy and arthritic rats by substance P and calcitonin gene-related peptide containing sensory fibers. *J Rheumatol* 19:1252-1259, 1992
27. Hukkanen M, Konttinen YT, Santavirta S, Paavolainen P, Gu X-H, Terenghi G, Polak JM: Rapid proliferation of calcitonin gene-related peptide-immunoreactive nerves during healing of rat tibial fracture suggests neural involvement in bone growth and remodelling. *Neuroscience* 54:969-979, 1993
28. Joosten EA, Gribnau AAM, Dederen PJWC: Postnatal development of the cortical spinal tract in the rat: an ultrastructural anterograde tracer HRP study. *Anat Embryol* 179:449-456, 1989
29. Kimball ES, Persico FJ, Vaught JL: Substance P, neurokinin A, and neurokinin B induce generation of IL-1-like activity in P388D1 cells: possible relevance to arthritic disease. *J Immunol* 141:3564-3569, 1988
30. Kristjansson GI, Zwiers H, Oestreicher AB, Gispen WH: Evidence that the synaptic phosphoprotein B-50 is localized exclusively in nerve tissue. *J Neurochem* 39:371-378, 1982
31. Kruger L, Silverman JD, Mantyh PW, Sternini C, Brecha NC: Peripheral patterns of calcitonin-gene-related peptide general somatic sensory innervation: cutaneous and deep terminations. *J Comp Neurol* 280:291-302, 1989
32. Kuntz A, Richings CA: Innervation of the bone marrow. *J Comp Neurol* 83:213-222, 1945
33. Kurashi Y, Hirota N, Sugimoto M, Satoh M, Takagi H: Effect of morphine on noxious stimuli-induced release of substance P from rabbit dorsal horn in vivo. *Life Sci* 33(Suppl 1):693-696, 1983
34. Lotz M, Vaughan JH, Carson DA: Effect of neuropeptides on production of inflammatory cytokines by human monocytes. *Science* 241:1218-1221, 1988
35. Mantyh PW, Mantyh CR, Brecha NC, Kruger L, Sternini C: Autoradiographic localization of calcitonin gene-related peptide binding sites in the rat brain, guinea pig periphery and human spinal cord. Presented at the annual meeting of the Society for Neuroscience, Sheffield, England, September, 1985
36. Mapp PI, Kidd BL, Gibson SJ, Terry JM, Revell PA, Ibrahim NBN, Blake DR, Polak JM: Substance P-, calcitonin gene-related peptide- and C-flanking peptide of neuropeptide Y-immunoreactive fibres are present in normal synovium but depleted in patients with rheumatoid arthritis. *Neuroscience* 37:143-153, 1990
37. Meiri KF, Pfenninger KH, Willard MB: Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones. *Proc Natl Acad Sci U S A* 83:3537-3541, 1986
38. Mercken M, Lubke U, Vandermeeren M, Gheuens J, Oestreicher AB: Immunocytochemical detection of the growth-associated protein B-50 by newly characterized monoclonal antibodies in human brain and muscle. *J Neurobiol* 23:309-321, 1992
39. Oestreicher AB, Gispen WH: Comparison of immunocytochemical distribution of the phosphoprotein B-50 in the cerebellum and hippocampus of immature and adult rat brain. *Brain Res* 375:267-279, 1986
40. Otsuka M, Yanagisawa M: Does substance P act as a pain transmitter? *Trends Pharm Sci* 8:506-510, 1987
41. Reimann I, Christensen SB: A histological demonstration of nerves in subchondral bone. *Acta Orthop Scand* 48:345-352, 1977
42. Salo PT, Tatton WG: Age-related loss of joint innervation. In: *Transactions of the Combined Meeting of the Orthopaedic Research Societies of USA, Japan, and Canada*, pp 81. Calgary, Canada, Committee of the Orthopaedic Research Societies, 1991
43. Schaible H-G, Jarrott B, Hope PJ, Duggan AW: Release of immunoreactive substance P in the spinal cord during development of acute arthritis in the knee joint of the cat: a study with antibody microprobes. *Brain Res* 529:214-223, 1990
44. Serra MC, Bazzoni F, Della Bianca V, Greskowiak M, Rossi F: Activation of human neutrophils by substance P: effect on oxidative metabolism, exocytosis, cytosolic Ca2+ concentration and inositol phosphate formation. *J Immunol* 141:2118-2124, 1988
45. Silverman JD, Kruger L: An interpretation of dental innervation based upon the pattern of calcitonin gene-related peptide (CGRP)-immunoreactive thin sensory axons. *Somatosens Res* 5:157-175, 1987
46. Skene JHP: Axonal growth-associated proteins. *Annu Rev Neurosci* 12:127-156, 1989
47. Struthers AD, Brown MJ, Macdonald DWR, Beacham JL, Stevenson JC, Morris HR, MacIntyre I: Human calcitonin gene related peptide: a potent endogenous vasodilator in man. *Clin Sci* 70:389-393, 1986
48. Tetzlaff W, Zwiers H, Lederis K, Cassar L, Bisby MA: Axonal transport and localization of B-50/GAP-43-like immunoreactivity in regenerating sciatic and facial nerves of the rat. *J Neurosci* 9:1303-1313, 1989
49. Van Lookeren Campagne M, Oestreicher AB, Van Bergen en Henegouwen PMP, Gispen WH: Ultrastructural double localization of B-50/GAP43 and synaptophysin (p38) in the neonatal and adult rat hippocampus. *J Neurocytol* 19:948-961, 1990
50. Van Lookeren Campagne M, Dotti CG, Jap Tjoen San ERA, Verkleij AJ, Gispen WH, Oestreicher AB: B-50/GAP43 localization in polarized hippocampal neurons in vitro: an ultrastructural quantitative study. *Neuroscience* 50:35-52, 1992
51. Venesio T, Mulatero B, Fasolo A: Coexistence of substance P and calcitonin gene-related peptide in the frog spinal cord. *Neurosci Lett* 80:242-250, 1987
52. Verhaagen J, van Hoof COM, Edwards PM, De Graan PNE, Oestreicher AB, Schotman P, Jennekens FGI, Gispen WH: The kinase C substrate protein B-50 and axonal regeneration. *Brain Res Bull* 17:737-741, 1986
53. Verhaagen J, Oestreicher AB, Edwards PM, Veldman H, Jennekens FGI, Gispen WH: Light- and electron-microscopical

study of phosphoprotein B-50 following denervation and reinnervation of the rat soleus muscle. *J Neurosci* 8:1759-1766, 1988
54. Widenfalk G, Wiberg M: Origin of sympathetic and sensory innervation of the knee joint: a retrograde axonal tracing study in the rat. *Anat Embryol* 180:317-323, 1989
55. Wixson RL, Stulberg D, Mehlhoff M: Total hip replacement with cemented, uncemented, and hybrid prostheses: a comparison of clinical and radiographic results at two to four years. *J Bone Joint Surg [Am]* 73:257-263, 1991
56. Wojtys EM, Beaman DN, Glover RA, Janda D: Innervation of the human knee joint by substance-P fibers. *Arthroscopy* 6:254-263, 1990
57. Zaidi M, Chambers TJ, GainesDas RE, Morris HE, MacIntyre I: A direct action of human calcitonin gene-related peptide on isolated osteoclasts. *J Endocrinol* 115:511-518, 1987
58. Zaidi M, Fuller K, Bevis PJR, GainesDas RE, Chambers TJ, MacIntyre I: Calcitonin gene-related peptide inhibits osteoclastic bone resorption: a comparative study. *Calcif Tissue Int* 40:149-154, 1987