Time-dependent sensory nerve ingrowth into a bone conduction chamber

Jan Erik Madsen[1,2], Mika Hukkanen[3], Per Aspenberg[4], Julia Polak[3] and Lars Nordsletten[1]

[1]Institute for Surgical Research, Rikshospitalet The National Hospital, University of Oslo, [2]Martina Hansens Hospital, Bærum, Norway, Departments of [3]Histochemistry, Imperial College School of Medicine, Hammersmith Campus, London, U.K., [4]Orthopedics, Lund University Hospital, Sweden. Correspondence: Jan Erik Madsen, Orthopaedic Department, Ullevål University Hospital, NO-0407 Oslo, Norway. Tel +47 22 11 95–00. Fax –58.
E-mail: j.e.madsen@klinmed.uio.no
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ABSTRACT – We studied time-dependent ingrowth of sensory nerve fibers into a bone defect in a rat bone conduction chamber model. In 10 male Sprague Dawley rats, a titanium chamber was implanted bilaterally in the proximal tibiae, representing an experimental bone defect. To mimic a clinical situation, the chambers were filled with a fresh blood clot. After 1, 2, 4, 6 and 8 weeks, 2 rats were fixed in vivo at each time before removal of specimens, and histological and immunohistochemical analyses. We used antisera against protein gene product 9.5, neural growth-associated protein 43/B-50, calcitonin gene-related peptide, and substance P, to locate regenerating sensory nerve fibers in the chamber. During bone defect healing, hematoxylin/eosin sections showed that new bone grew in through the ingrowth openings in the chamber, gradually filling it and replacing the blood clot. At 1 and 2 weeks after implantation, no nerve fibers could be detected. At 4, 6 and 8 weeks, however, small numbers of nerve fibers were seen in 8 of 11 specimens. The nerve fibers were located mainly in the dense fibrous tissue in close proximity to the new bone, and in some cases within the new forming bone. In this chamber model, the periosteum is not in contact with the bone ingrowth openings, and all ingrowing nerve fibers thus originated from the cortical bone, endosteum or bone marrow. We speculated that these late ingrowing sensory nerve fibers may actively participate in bone repair.

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Innervation of bone may represent a sophisticated regulatory element able to act both as a sensor of local demands, and as an effector on bone metabolism via release of locally acting neuropeptides (Bjurholm 1991, Hill et al. 1991, Hukkanen et al. 1995, Konttinen et al. 1996). During bone healing, sensory nerves grow into a fracture site in an ordered manner (Hukkanen et al. 1993), and may be important for the normal healing process, since no sensory nerves are found in pseudarthroses (Aro et al. 1985, Santavirta et al. 1992). The amount of fracture callus is affected by a peripheral nerve injury, because sciatic nerve resection promoted secondary fracture healing with large amounts of callus in a stable fracture model in the rat (Nordsletten et al. 1994), and bone metabolism in the posttraumatic state also depends on intact peripheral innervation (Madsen et al. 1996a). Further, sensory nerve fibers have been observed growing into bone grafts during incorporation (Madsen et al. 1996b), but the specific role of these ingrowing nerve fibers remains unclear.

In this study, we aimed to clarify the pattern of sensory nerve ingrowth into a bone defect at different times, and chose the bone conduction chamber as a model.

Material and methods

11 male Wistar rats were used, 10 for bone chamber implantations and 1 for making an allogenic blood clot fit into each chamber. The rats were 3 months old, and weighed 348 (339–358) g. They were kept alone in wire-topped plastic cages, in a 12-hour light and 12-hour dark cycle with free

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access to tap water and standard laboratory rodent pellet chow. The experiment conformed to the Norwegian Council of Animal Research Code for the Care and Use of Animals for Experimental Purposes.

The bone conduction chamber

Properties and implantation technique of the titanium bone conduction chamber (BCC) have been described in detail (Aspenberg and Wang 1994). The chamber consists of a three-part titanium cylinder with two bone ingrowth openings at the cylinder base. The ingrowth openings come to lie embedded in bone after implantation, so that the recruitment of the ingrowing tissue in the BCC is from the metaphyseal bone alone. All operations were performed under aseptic conditions. The rats were anesthetized with a combination of Hypnorm (fluanisone 5 mg/mL, fentanyl citrate 0.16 mg/mL) and Dormicum (midazolam 2.5 mg/mL) injected subcutaneously, 0.2 mL per 100 g animal weight. The BCC was implanted bilaterally in the medial proximal tibia of the rats, located approximately 1 mm below the growth plate. Before implantation, the chambers were filled with a blood clot made from another animal immediately before starting the operations. Temgesic (buprenorphine 0.3 mg/mL) was given subcutaneously as analgesia for the first 2 days postoperatively.

Tissue processing

At 1, 2, 4, 6 and 8 weeks after BCC implantation, 2 rats were anesthetized, and fixed in vivo with Zamboni's fixative (85 mL 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and 15 mL saturated picric acid per 100 mL) via thoracotomy and cannulation of the ascending aorta (Hukkanen et al. 1995). Phosphate-buffered saline (PBS) was used to flush the circulatory system. The BCC was then rapidly removed, and the contents immediately immersed in Zamboni's fixative. After 4 hours, the specimens were rinsed repeatedly in PBS with 15% sucrose and 0.1% sodium azide, and the sucrose solution was then changed repeatedly over the first 2 days, while storing at 4 °C. After decalcification with EDTA, 15 µm cryostat sections were cut parallel to the specimen's longitudinal axis.

Evaluation

Hematoxylin and eosin (HE) staining was used to evaluate bone and fibrous tissue ingrowth and helped to localize the nerve fibers when compared with the immunostained sections. Bone and total tissue ingrowth into the chambers were assessed, using a computerized video digital table system (Videoplan, Zeiss) at a magnification of ×40. The area of new bone and total tissue ingrowth were measured, and by dividing this measure with the width of the specimen, the average distance which the new tissues had penetrated into the chamber was calculated (Aspenberg and Wang 1994).

Immunohistochemistry

Immunostaining with antisera against protein gene product 9.5 (PGP 9.5), neural growth-associated protein GAP 43/B-50 (GAP 43/B50), calcitonin gene-related peptide (CGRP), and substance P (SP) were used to locate and characterize nerve fibers. Since PGP 9.5 is a cytoplasmic ubiquitin C-terminal hydrolase present in all classes of nerves, and GAP 43/B-50 is a protein kinase C substrate, highly expressed in regenerating nerves, they represented general markers of nerve growth. SP and CGRP are neuropeptides mainly expressed by small and medium-sized sensory neurons (Hukkanen et al. 1995). More detailed characteristics of the antisera are given in Table 1. Decalcified cryostat sections were mounted onto APES-coated slides and rinsed in PBS. Endogenous peroxidase activity was exhausted by incubation in methanol with 0.3% hydrogen peroxide for 20 min. Sections were incubated with normal goat serum (dilution 1/30) for 30 min before overnight incubation at 4 °C with polyclonal antibodies (Table 1). They were then incubated with biotinylated goat anti-rabbit antibodies (dilution 1/100) for 60 min, and with a peroxidase-labeled avidin-biotin-peroxidase complex (ABC, dilution 1/200) for 60 min more (ABC Elite kit, Vector Laboratories, Peterborough, U.K.). The immunoreactivity was visualized by incubation for 5 min in a chromogen solution containing glucose oxidase-3,3'diaminobenzidine-nickel ammonium sulfate (Sigma) to amplify the reaction product (Hukkanen et al. 1993). Antibody specificity tests included absorption of the antibody with corresponding peptide (CGRP and substance P) and replacement of the

Table 1. Characteristics of antisera used in the study

Antigen	Dilution	Absorption	Source	Reference
PGP 9.5	1/10000	N/A	Ultraclone, Cambridge, UK.	Gulbenkian et al. 1987
GAP-43/B-50	1/4000	N/A	Dr. Oestreicher, The Netherlands	Oestreicher et al. 1983
α-CGRP	1/4000	0.1 nM/mL	Hammersmith Hospital, UK	Merighi et al. 1988
SP	1/4000	1.0 nM/mL	Hammersmith Hospital, UK	Merighi et al. 1988

Absorption = antiserum absorbed with corresponding peptide, lowest concentration where significant reduction in immunoreactivity was noted, N/A = not applicable, α-CGRP = antiserum raised against a synthetic rat peptide, antiserum cross-reacts with α-CGRP, substance P = antiserum raised against synthetic rat peptide, antiserum cross-reacts with other tachykinins.

primary antiserum with normal serum. Method specificity tests included omission of the secondary or ABC complex.

Nerve fiber types were characterized by the immunostaining patterns, and the number of fibers were semi-quantified, using a subjective scale from 0 to 3, based on the microscopic findings: 0) no nerve fibers present, 1) few fibers present, 2) moderate density of nerve fibers, 3) dense innervation (Madsen et al. 1996b).

Results

At 8 weeks, the left leg chamber in one of the two rats was loose, and its contents were discarded. Otherwise, there were 4 samples for each time point.

At 1 week, HE sections showed that polymorphonuclear leukocytes had invaded the fibrin clot a distance of 1–2 mm from the ingrowth end. Only occasional spindle-shaped cells were seen. At 2 weeks, fibrous tissue had penetrated the clot about 2–3 mm. This tissue was rich in spindle-shaped cells with moderate amounts of fibrous matrix. There were numerous sinusoids and capillaries and evidence of fresh bleeding at the edge towards the remainder of the fibrous clot deeper into the chamber. At the ingrowth end, islands of membranous ossification were seen. The bone or osteoid had a woven character. At 4 weeks, the callus- like fibrous tissue had reached 2.5–4 mm into the chamber. A front of membranous ossification was seen at a distance of 2–2.5 mm from the ingrowth end. Behind this front, the newly formed bone was resorbed to make place for a large number of marrow cavities filling the ingrowth end of the chamber with a hematogenous marrow. At 6 weeks, the morphology was similar to that at 4 weeks. Ingrowth had proceeded, the fibrous callus reaching 3–4.5 mm and the bone 2–3 mm into the chamber. The many different marrow cavities had now joined to form one big marrow space in the proximal end of the chamber, also containing fat cells. No remnants of the fibrin clot were seen. At 8 weeks, ingrowth of fibrous tissue and bone had proceeded to 6–7 and 5–6 mm, respectively (Figure 1). There were no other differences compared to 6 weeks, but in 2 cases, lumps of macrophages were seen with phagocytosed material. The bone ingrowth distance was greater than in previous studies using the chamber (Aspenberg and Wang 1994, Madsen et al. 1996b).

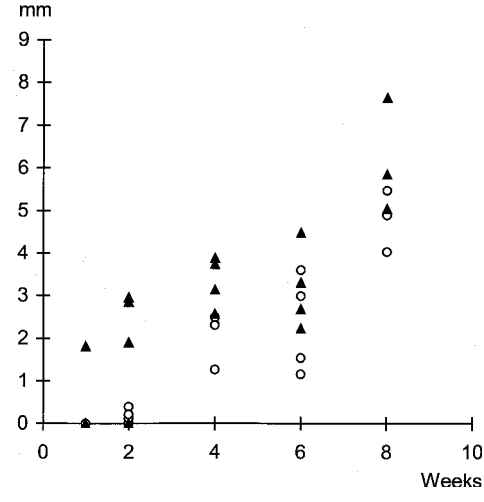

Figure 1. Bone ingrowth distance (BD ●) and total tissue ingrowth distance (TD ▲), in millimeters (mm), into the titanium bone conduction chamber 1, 2, 4, 6 and 8 weeks after implantation in the proximal tibia.

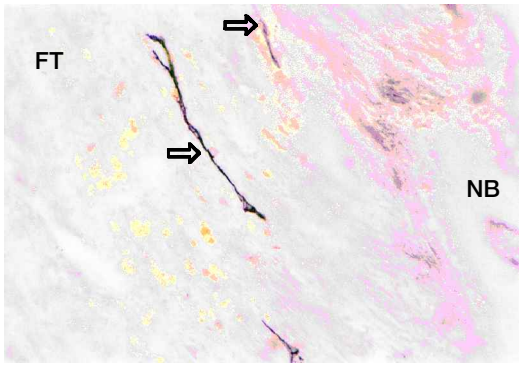

Figure 2. Nerve fibers immunoreactive to neural growth-associated protein GAP 43/B-50 (open arrows) in a bone conduction chamber 6 weeks after implantation. The nerve fibers are typically located in the transitional zone between newly formed bone (NB) and the dense fibrous tissue (FT) preceding this new bone (×125).

Table 2. Nerve fiber density in the right (R) and left (L) bone conduction chambers 1, 2, 4, 6, and 8 weeks after implantation, scored as amount of nerve fibers immunoreactive to substance P, calcitonin gene- related peptide, protein gene product 9.5, and neural growth-associated protein on a scale from 0 (no nerves) to 3 (dense innervation)

Weeks after implantation	Animal number	Side	Nerve density
1	1	R	0
		L	0
	2	R	0
		L	0
2	3	R	0
		L	0
	4	R	0
		L	0
4	5	R	1
		L	1
	6	R	1
		L	2
6	7	R	2
		L	2
	8	R	0
		L	0
8	9	R	0
		L	2
	10	R	1
		L	No specimen

Immunostainings revealed no nerve fibers in the BCC at 1 and 2 weeks (Table 2). At 4 weeks, nerve fibers immunoreactive for PGP 9.5, GAP-43/B-50, SP, and CGRP could be seen in all sections, but in small numbers. No samples staining positively for general nerve growth markers alone were found. Morphology and presence of SP and CGRP in the actively growing fibers indicated that they were of sensory origin and, in all sections, they were mainly localized in the fibrous tissue developing in front of the newly formed bone, close to the new bone (Figure 2). Some nerve fibers were seen deep in the distal end of the chambers, where a looser, areolar fibrous tissue replaced the blood clot, whereas very few nerve fibers were seen inside the new forming bone, and no fibers in the marrow cavities. At 6 weeks, nerve fibers were found in both BCC from one of the animals, while in the other rat, no nerves could be detected (Table 2). At 6 and 8 weeks, most of the nerve fibers were located in the dense fibrous tissue immediately adjacent to the new bone. The nerve fibers mainly ran parallel to the connective tissue fibers, and had no close contact with blood vessels, apart from what might be expected from random. At 8 weeks, bone and fibrous tissue almost filled the BCC, and very few nerve fibers were seen compared to the previous time points at 4 and 6 weeks. In one specimen at 8 weeks, nerve fibers could not be identified, whereas the contralateral specimen and the third 8-week specimen showed few sensory fibers immunoreactive for all epitopes studied. Most fibers still seemed to be localized in the bone/fibrous tissue interface in front of the new bone, but compared to the previous time points, they were in closer contact with the bone. A few intraosseous sensory nerve fibers were seen, as well as a few nerve fibers immunoreactive for PGP 9.5 and GAP-43 in the endosteal area.

Thus, nerve fibers immunostaining for PGP 9.5, GAP-43/B-50, SP, and CGRP were found at 4, 6, and 8 weeks, in bone and fibrous tissue in different samples, with no apparent differences in distribution pattern between the epitopes.

Discussion

We found no nerve fiber ingrowth into the BCC at 1 and 2 weeks after implantation. At 4, 6 and 8 weeks, however, a few sensory nerve fibers were seen within the chambers. By filling the BCC with a blood clot, we wanted to mimic a clinical bone

defect. In the in vivo fracture situation, the periosteum surrounding the fracture site plays an important role in early fracture healing (Aro et al. 1982, Aro et al. 1985, Hukkanen et al. 1993), and previous studies have mainly focused on periosteum-derived nerves. Immunoreactive sensory nerve fibers in experimental tibial bone defects and fractures have been observed even 1 week after operation, and periosteal nerve fibers were still proliferating actively 2 and 3 weeks after fracture (Hukkanen et al. 1993, Aoki et al. 1994). Proliferating nerve fibers have also been found in bone marrow spaces adjacent to a fracture after 25 days of healing (Hukkanen et al. 1995). In our BCC model, all ingrowing tissue originates from the cortical bone or bone marrow in the proximal tibia, since the periosteum is not in contact with the bone ingrowth openings (Aspenberg and Wang 1994, Madsen et al. 1996b). This model therefore mainly permits nerve growth from the endosteal compartment and bone marrow, and nerve fibers within the chamber at the early post-implantation stages could not be detected. Nerve fibers were observed in the bone defect 4, 6, and 8 weeks after implantation. This showed that the osseous tissue around the defect was able to supply sensory nerve fibers independent of the surrounding periosteum.

The BCC model differs from a clinical bone defect. It provides an unloaded environment for bone regeneration, which may affect the pattern of nerve ingrowth, compared to the loaded clinical situation. However, nerve fibers have been found in fracture callus also in other models that did not allow functional weightbearing (Aoki et al. 1994, Hukkanen et al. 1995, Madsen et al. 1998). Moreover, the presence of nerve fibers during late bone defect healing in a BCC is not strong evidence for nerve involvement in bone repair. We could not find nerve fibers in all chambers even at the later time points in the present study, as was also the case in a previous study involving bone graft incorporation (Madsen et al. 1996b). This may be the result of several factors: the number of nerve fibers in bone are normally few (Hukkanen et al. 1992b), and the innervation shows considerable regional variations in the tibia, since the tibial epiphysis is considerably richer in sensory innervation than the shaft (Hukkanen et al. 1992b, Ahmed et al. 1994, Hara-Irie et al. 1996). The number of nerve fibers in bone and surrounding tissues may also vary in different pathologic conditions (Hukkanen et al. 1992a), and the proliferation of local nerves may be rapid and transient (Hedberg et al. 1995), and therefore detectable only at certain short phases during bone repair. Thus, nerve fiber ingrowth during the early phase of fracture healing or bone formation may be dependent on an intact periosteum with normal innervation, and these nerve fibers may have specific modes of action determining the later fate of bone repair. Sensory nerve fibers may act as transport pathways for several biologically active neuropeptides, and especially CGRP has been implicated (Konttinen et al. 1996). As CGRP is a strong vasodilator, it may exert vascular control and influence angiogenesis during early fracture healing (Brain et al. 1985, Hukkanen et al. 1995). Further, it impairs bone resorption by inhibiting osteoclast activity and recruitment (D'Souza et al. 1986, Zaidi et al. 1987, Hara-Irie et al. 1996), and osteogenesis is stimulated by direct receptor effects on osteoblasts (Bernard and Shih 1990, Bjurholm et al. 1992). CGRP may also act as a sensory transmitter in Aδ and C type sensory nerve fibers, being actively involved in the protection of the fracture site against unwanted movement (Hukkanen et al. 1995).

We found that sensory nerve fibers, immunostaining for SP and CGRP, and originating from the endosteal or bone marrow spaces, appeared in the later stages of bone repair. This may be compatible with an active participation of the peripheral sensory nervous system during the later phases of bone repair and remodeling.

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