Sensory nerve ingrowth during bone graft incorporation in the rat

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We studied nerve ingrowth into a cancellous bone graft in a bone conduction chamber model in the rat. Before implantation of the chamber bilaterally in the proximal tibiae of 8 Sprague-Dawley rats, a defatted cancellous bone graft from separate donor rats was fitted snugly into each chamber. After 6 weeks, the animals were perfused with Zamboni's fixative and the chambers were harvested. Immunohistochemical detection of nerve fibers was performed in cryostat sections, using antisera to protein gene product 9.5 (PGP 9.5), neural growth-associated protein GAP-43/B-50, calcitonin gene-related peptide (CGRP), substance P and C- flanking peptide of neuropeptide Y (CPON). Nerve fibers were found in 10 out of 16 samples in the newly formed bone, and also in the fibrous tissue which had penetrated deeper into the graft. The nerve fibers were mainly of sensory origin, as they showed immunoreactivity for CGRP and GAP-43/B-50. We speculate that the nerve fibers may act as transmitters of nociceptive impulses from the graft, and as transport pathways for neuropeptides that are actively involved in angiogenesis and in the recruitment and activity of osteogenic cell populations from the graft recipient.

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Submitted 95-08-02. Accepted 96-01-21

Nerve fibers have previously been detected in fracture callus during the early healing period (Hukkanen et al. 1993), and callus formation is probably increased in the paraplegic patient (Glenn et al. 1973). Thus, the peripheral nervous system may play a substantial role in fracture healing and bone remodeling (Nordsletten et al. 1994, Hukkanen et al. 1995). The role of the peripheral nervous system in the incorporation of bone grafts is unknown, and neural ingrowth into bone grafts has not previously been investigated. Therefore, we assessed nerve ingrowth into a cancellous bone graft during incorporation.

Animals and methods

8 male Sprague-Dawley rats (Møllegård, Copenhagen, Denmark) weighing 388 (376–396) g were used. The animals were kept alone in wire-top plastic cages at 22 °C with free access to tap water and standard laboratory rodent chow in a 12-hour light/12-hour dark cycle. All operations were performed under sterile conditions, the animals anesthetized with a combination of diazepam and pentobarbital injected intraperitoneally.

Bone conduction chamber

The bone conduction chamber has been described by Aspenberg and Wang (1994). A chamber was implanted in the proximal tibia on both sides in all animals. A longitudinal skin incision was made on the anteromedial side of the tibial metaphysis, and the periosteum was incised and raised just anterior to the insertion of the medial collateral ligament. The tibia was drilled through with a 1 mm burr and the hole in the medial cortex was enlarged to 2.7 mm. The bone graft was then inserted in the chamber, the proximal part of the graft having the densest bone was placed closest to the ingrowth openings. The chamber was screwed into position so that the pointed end of the chamber entered the opposite cortex and the bone ingrowth openings were at the level of the medial cortex. The implant was located approximately 1 mm below the growth plate of the proximal tibia. The wound was closed in two layers. All rats resumed full weight bearing within the first 3 days. No tibial fractures occurred and there were no signs of infection in any of the animals.

Bone grafts

All chambers were filled with a snugly fitted cancel-

lous bone graft before the implantation. The grafts were harvested as 6 mm long bone plugs from the proximal tibiae of separate donor rats, using a 2 mm cylindrical bone biopsy cutter. The bone rods were resected in the axial direction from the knee joint, each rat yielding 2 grafts. The epiphysis was excised from the grafts before they were frozen at –70 °C, and later defatted in chloroform:methanol 1:1, rinsed with methanol and water, and air-dried. The 2 grafts from each donor were kept as pairs, and each pair was implanted in the same recipient rat.

Tissue processing

After 6 weeks, the rats were anesthetized and perfused with fixative in vivo via cannulation of the ascending aorta through the left ventricle. A peristaltic pump was used, and 200 mL of phosphate buffered saline (PBS) was used to flush all blood out of the circulatory system before the rats were perfused with 300 mL of Zamboni's fixative (Hukkanen et al. 1995). The titanium chambers were rapidly removed, and the chamber content was immersed in Zamboni's fixative for 4 hours. The specimens were then repeatedly rinsed in PBS-sucrose and stored at 4 °C until further processing.

15 mm thick cryostat sections were cut parallel to the longitudinal axis of each specimen, the central section being in the center of the specimen. Hematoxylin and eosin (HE) were used to evaluate bone ingrowth. The distribution of nerve fibers and their peptide contents were studied, using antisera to protein gene product 9.5 (PGP 9.5), neural growth-associated protein GAP-43/B-50, calcitonin gene-related peptide (CGRP), substance P and C-flanking peptide of neuropeptide Y (CPON). PGP 9.5 is a cytoplasmic ubiquitin C-terminal hydrolase present in all classes of nerves. GAP-43/B-50 is a protein kinase C-substrate and highly expressed in regenerating nerves, while CGRP and substance P are neuropeptides mainly expressed by small and medium-sized sensory neurones. CPON is found in postganglionic sympathetic nerve fibers (Hukkanen et al. 1995).

Evaluation

The neural ingrowth was determined in each specimen. From each tissue block, 4 non-consecutive sections, cut 75 µm apart, were immunostained for each antiserum. Tissue morphology was assessed with HE sections, and immunoreactive structures were related to the general morphology. The nerve fiber types were registered according to the immunostaining patterns, and the total density of nerve fibers in each specimen was quantified with a scale from 0–3, based on the evaluation of the microscopic findings in the immunostained sections: 0) no nerve fibers present, 1) few fibers present, 2) moderate density of nerve fibers, and 3) dense innervation of the new bone and ingrowth into the deeper areas of the chamber.

Table 1. New bone ingrowth distance into the bone conduction chamber 6 weeks after implantation, and amount of nerves that are immunoreactive to protein gene product 9.5, neural growth-associated protein, calcitonin gene-related peptide and substance P, scored on a scale from 0 (no nerves) to 3 (dense innervation)

Rat No.	Side	Bone ingrowth distance (mm)	Amount of sensory nerves
1	l	0.93	2
	r	2.73	0
2	l	2.40	1
	r	1.86	2
3	l	1.84	1
	r	1.65	3
4	l	3.54	0
	r	1.09	0
5	l	1.53	0
	r	2.25	0
6	l	0.78	2
	r	1.90	3
7	l	2.81	0
	r	0.46	1
8	l	3.09	1
	r	0.45	1

Bone ingrowth into the chambers was assessed in the HE sections using a computerized video digital table system (Videoplan™, Zeiss) at magnification 40×. The area of new bone ingrowth was measured, and by dividing this measure by the width of the specimen, the average distance which the new bone had penetrated into the graft was calculated (Aspenberg and Wang 1994).

Results

Histological examination revealed new cancellous bone in all 16 chambers. The mean distance that the new bone had penetrated into the bone graft was 1.83 (0.45–3.54) mm (Table 1). The grafts were resorbed proximally in the chamber and the new bone growing in through the bone ingrowth openings formed an ossicle with bone marrow. Distal to this new bone, the graft was unresorbed, but loose areolar fibrous tissue containing some lymphocytes and polymorphonuclear leucocytes penetrated the graft all the way into the distal end of the chamber. Between the graft and the new bone there was a dense layer of fibrous tissue, with the fiber orientation parallel to the surface of the ingrowing bone.

In 2 animals, no nerves were detected in the chamber on either side. In 2 other animals, nerves were

present unilaterally, and in the remaining 4 animals bilaterally (Table 1). The nerve fibers were mainly located in the dense fibrous tissue between the new bone and the graft, often orientated perpendicularly to the surface of the ingrowing bone (Figure 1). Nerve fibers were also seen deep in the loose areolar tissue penetrating the bone graft, but usually only a few nerve fibers extended deeply into the bone grafts. GAP-43/B-50 immunoreactivity was strong in all of the positive specimens, indicating extensive nerve growth. The regenerating nerves showed numerous varicocities along the fibers (Figure 1), and very few fibers related to vascular structures. All regenerating nerves were of sensory origin, as CGRP immunoreactivity was strong. Substance P immunoreactivity was sparse, while CPON immunoreactive nerve fibers could not be seen in any of the specimens studied. PGP 9.5 immunoreactivity was strong in all nerve fibers, but also in many invading soft tissue cells, making the interpretation of the PGP 9.5 results uncertain.

Regression analysis revealed no correlation between the amount of new bone penetrating the graft and the density of ingrowing nerve fibers.

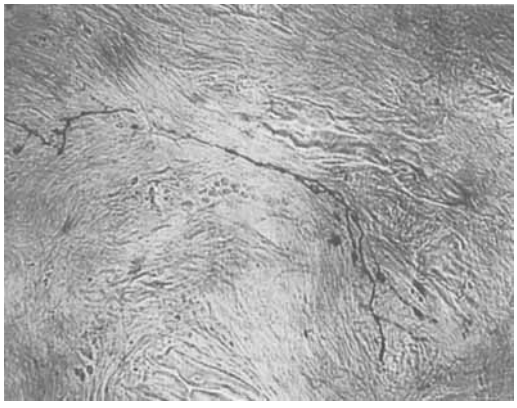

Figure 1. GAP-43/B-50 immunostaining nerve fibers (arrow) in dense fibrous tissue between new bone and cancellous bone graft (×40).

Discussion

Our study is the first to report ingrowth of nerves in an osseous graft. The density of nerve fibers in the chamber was not related to the amount of ingrowing bone. Using a bone conduction chamber for this study, one ensures that all living tissue in the chamber originated from the ingrowth openings. The morphological pattern of the nerve fibers indicated that the nerves invaded the chamber and implanted allograft in front of the ingrowing bone, as the nerves were mostly found embedded in fibrous tissue, in some instances penetrating the whole allograft.

Nerve fibers immunoreactive for GAP-43/B-50, a nonspecific marker for neural growth, was the most prominent finding in the specimens. However, all fibers also stained strongly for CGRP, which is synthesized mainly in small- and medium-sized sensory neurones in the dorsal root ganglia (Amara et al. 1982). The detected nerve fibers were thus of sensory origin. Few nerve fibers were in close relation to vascular structures, and postganglionic sympathetic fibers, identified by CPON immunoreactivity, were absent. This pattern of nerve ingrowth parallels that recently described in fracture callus in the early fracture-healing process (Hukkanen et al. 1993, 1995), where time-dependent sensory nerve fiber proliferation into the fracture site was detected from 7 to 25 days after fracture. In our study, nerve fibers were present in 10 out of 16 samples only; whether this represents region-specific variations in the specimens or a true lack of innervation remains to be seen. In this model, the ingrowing nerve fibers must pass through the bone ingrowth openings in the chamber, and the nerves probably originate from the cortical bone adjacent to the chamber. This may represent a significant difference in nerve growth conditions, compared to the rat tibial fracture model in which we found large amounts of regenerating nerve fibers deep in the fracture callus after 25 days of fracture healing (Hukkanen et al. 1995). A significant part of the regenerating nerve fibers in this fracture model may originate from the surrounding periost.

One can only speculate about the role of the nerve fibers in the incorporation of bone grafts. Normal graft incorporation involves several important steps, where the initial necrosis of all cells in the graft is followed by an inflammatory response, and a subsequent fibrovascular response. Revascularization is an essential step towards subsequent bone regeneration via recruitment of osteogenic cells from the recipient. During this process, the peripheral nerves may act as transport pathways for neuropeptides actively involved in angiogenesis and vascular control, as previously suggested in fracture repair (Hukkanen et al. 1993). CGRP may have a direct influence on several steps in the graft incorporation process, as it is one of the most potent vasodilator agents identified (Brain et al. 1985). Further, CGRP has been shown to stimulate bone formation in vitro (Bernard and Shih 1990), and to enhance bone mineralization in vivo (Ancill et al. 1991). Additionally, neuropeptides are known to exert direct effects both on osteoblasts and osteoclasts (Bjurholm et al. 1992), and the peripheral nervous system may also influence the long-term remodeling

of the bone graft by transmitting nociceptive or postural signals centrally, thus regulating the degree of loadbearing. This parallels the process of fracture healing, where the amount and structure of the callus forming after fractures may be regulated by the peripheral nervous system (Nordsletten et al. 1994). If the role of the detected nerve fibers is primarily nociceptive, this may explain why there was no correlation between the amount of bone and nerve fibers, as no loading occurs in the bone conduction chamber, and therefore no adequate signals controlling the amount of bone production could be found.

Immunohistochemical staining enabled us to separate sensory nerve fibers from autonomic fibers, but a more exact distinction between different sensory fiber types would be valuable in understanding the function of the nervous system in the incorporation of bone grafts.

Acknowledgements

We are grateful to Dr. A B Oestreicher, Rudolf Magnus Institute for Neurosciences, The Netherlands, for antibodies for GAP-43/B-50, and to the Grand Charity at Freemasons for their financial support.

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