The neuronal regulation of fracture healing

Effects of sciatic nerve resection in rat tibia

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The effect of sciatic nerve resection on tibial fracture healing was studied in rats 25 days post-trauma. To prevent differences in loading between sham-operated and nerve-resected animals the fractured limbs were cast-immobilized. On radiograms 8 of 11 fractures in the sham-operated animals showed very little callus formation in contrast to only 1 of 8 fractures in the group with nerve resection. Measured by single-photon absorptiometry, animals with sciatic nerve resection had a higher bone mineral content than the sham-operated animals. However, the mechanical strength in three-point cantilever bending was not better in the nerve-resected rats, implying a defective organization of the large callus. These results suggest neural regulation plays a role in the type of fracture healing, primary or secondary, and in the amount and quality of the callus.

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An almost total absence of innervation was recently observed in nonunited human diaphyseal fractures, raising the possibility that the lack of neural control may have led to delayed healing (Santavirta et al. 1992). Aro et al. (1981) studied tibial fracture healing after sciatic resection or spinal cord transsection, and observed increased tensile strength in the nerve-resected rats during the early stages of healing, and changes in callus appearance at later stages. After 28 days of healing Aro (1985) found that callus size was smaller in nerve-resected than in innervated tibiae. Removal of mechanoreceptors in the fibula led to nonunion (Aro et al. 1985). Frymoyer and Pope (1977) found more rapid fracture healing as assessed by both histology and mechanical testing in sciatically nerve-resected fibular fractures compared to controls. More recent studies have demonstrated in the rat that bone, callus and cartilage are innervated with neuropeptide-containing nerves of autonomic and sensory origin, both in normal (Kruger et al. 1989, Hukkanen et al. 1991, Hukkanen et al. 1992a,b) and in pathological conditions (Konttinen et al. 1990, 1992, Hukkanen et al. 1993). During normal fracture healing in the rat tibia, Hukkanen et al. (1993) found that neuropeptide-immunoreactive nerves undergo changes in an ordered and sequential manner from 7 to 21 days of healing, and they postulated that the nervous system was involved in bone growth and remodeling.

We studied the influence of nerve resection on the healing of a tibial fracture in the rat. In contrast to previous studies, the nerve-resected legs and sham-operated controls were both immobilized by casting to prevent differences in mechanical loading.

Animals and methods

19 male Wistar/Han/Mol SPF rats (Møllegård, Copenhagen, Denmark), 8 with resected sciatic nerves and 11 sham-operated rats, were randomly allocated from an original series of 45 rats (14 healing tibiae were used for immunohistochemistry for neuropeptides, and 12 animals were lost: 3 animals died during recovery from anesthesia after fracture of the tibia, while 9 animals were killed prior to 25 days because of ischemic skin injuries distal to the cast). Mean weight of the animals was 257 (226–270) g. The rats were kept single in wire-top plastic cages. They were anesthetized with a combination of fluanisone-fentanyl citrate and midazolam given subcutaneously for surgery and cast changes. The experi-

ment conformed to the Norwegian Council of Animal Research Code for the Care and Use of Animals for Experimental Purposes.

Tibial fracture

Surgery was performed under sterile conditions. The right tibiae of anesthetized animals were percutaneously fixed with a modular intramedullary nail by a modification of the technique previously described (Nordsletten et al. 1992). A stab incision was made over the patellar ligament, and an 18 G cannula inserted through the anterior tibial plateau into the medullary canal. A 21 G cannula, and a 25 G mandrin were inserted into the largest cannula, and all 3 were advanced as far distal as possible by combined axial pressure and rotation. The 2 outer cannulae were then withdrawn to the proximal part of the tibia, and with the mandrin inside, the tibia was fractured in the mediolateral direction with a fracture forceps (Ekeland et al. 1981). The 2 cannulae were then advanced distally past the fracture line (Figure 1). The nail was cut flush with the patellar ligament by retracting the skin, and the incision closed with 1 stitch. All fractures were stable at the end of the operation, and rotation was checked by comparing the alignment of the foot and the thigh. All animals were preoperatively given 1 intraperitoneal injection of 75 mg streptomycin (Streptocillin vet, NOVO, Copenhagen, Denmark).

Nerve resection

After the fracturing the right sciatic nerve was localized by dissection of the intermuscular septum through a 1 cm incision over the lateral upper part of the thigh. Half of the rats had been randomized to nerve resection, while in the other half the nerve was only identified. The wounds were closed in one layer.

Cast immobilization

The fractured limb in all rats was immobilized in a plaster of Paris with extension of the foot, and with 90° flexion of the knee (Tuukkanen et al. 1992). The cast was sprayed with pyroleum animale crudum to prevent destruction by gnawing (Lindboe and Presthus 1985). The casts were checked every second day and changed when necessary.

Evaluation

After 25 days of healing, the animal's both tibiae and femora were resected, with care taken to leave the callus of the right tibiae intact. The lengths of the left tibiae were measured with sliding calipers (accuracy of ±0.05 mm), and both tibiae were weighed. Alignment of the fractures was assessed macroscopically, and the intramedullary nails were removed carefully. The bones were frozen wet at –20°C.

There were no clinical or radiographic signs of infection in the healing tibiae. The healing fractures in the right tibiae were located 15.6 *1.3* (SD) mm and 15.4 *1.7* mm from the malleolar plane in the nerve-resected and sham-operated animals, respectively, and the lengths of the left tibiae were 39.0 and 39.2 mm. Weight gain was 52.6 *16.2* g in the nerve-resected animals, and 42.8 *12.7* g in the sham-operated ones ($P < 0.05$). Inspection showed severe atrophy of the muscles in the thigh, but particularly in the lower leg on the fractured side. All resected sciatic nerves were inspected at the time of termination of the experiment and they were still severed.

Radiographic examination

Radiographs were taken of the healing tibiae and scored blindly and independently for the presence of callus by 2 radiologists with long experience in orthopedics. Each radiogram was rated from 0 to 3, and the average of the frontal and side views was calculated. The scoring was: 0) no callus, 1) just visible callus, 2) normal amount of callus, and 3) excessive callus. A score of less than 1 was taken as "no callus formation".

Single-photon absorptiometry

The bone mineral content (BMC) and the width of the callus were later assessed by single-photon absorptiometry (SPA) on a Gambro Densitometer (Gambro AB, Lund, Sweden), applying a ^{241}Americium radiation source equipped with software for small bones (Nordsletten et al. 1994). The collimator width was 3.2 mm, time interval 0.5 s, and the scan speed 0.25 mm/s. Each healing tibia was immersed in water and measured 3 times, and the means for BMC and callus width were recorded.

Mechanical testing

For mechanical testing the tibiae were thawed in Ringer solution, and then loaded until fracture in three-point ventral cantilever bending at a rate of 0.095 rad/s (5.43°/s) with the fulcrum placed over the callus. The position of the fracture was measured from the malleolar plane with sliding calipers. The corresponding point was marked on the left tibia, which was then fractured in the same way. The load in the test apparatus was measured with a load cell which was connected to a microcomputer via an amplifier. The load/deflection curve was recorded online in WorkBenchMac (Strawberry Tree Incorporated, Sunnyvale, CA, U.S.A.). Ultimate bending

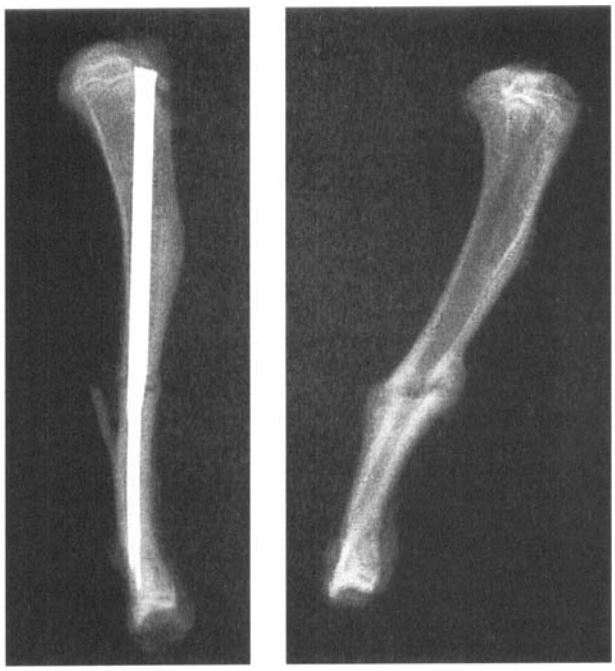

Figure 1. Tibial fracture stabilized by percutaneous intramedullary fixation with a modular nail consisting of three parts threaded into and past each other. The radiograph was taken after 25 days in a sham-operated animal showing the lack of callus formation, and the modular intramedullary nail (A). Abundant callus formation in tibial fracture after 25 days of healing in sciatic nerve-resected rat (B).

moment, ultimate energy absorption, bending stiffness and deflection were read directly or calculated from the computer recordings (Nordsletten and Ekeland 1993).

^{85}Strontium incorporation

3 days before the rats were killed they were given an intraperitoneal injection of one μC/100 g body weight of $^{85}SrCl_2$ (Kirkeby and Berg-Larsen 1991). ^{85}Sr activity was counted in healing and intact tibiae and femora by placing the sample in the center of a gamma counter and expressed as count ^{85}Sr per mg wet weight of bone. Due to intestinal injection in 3 animals, ^{85}Sr incorporation could be calculated in only 5 healing tibiae in the animals with nerve resection.

Statistics

Results are given as mean *SD* for the nerve-resected and the sham-operated animals. Fracture healing was expressed as the ratio of the mechanical value for the healing tibia over the value for the contralateral left tibia. The groups were compared by Mann-Whitney U, with the level of significance set at $P < 0.05$. Group differences in the presence or absence of callus on radiograms was analyzed by the Chi-square test. Linear regression analysis was applied for comparison between callus weight and BMC.

Results

Wet weights of the fractured tibiae were 0.92 *0.09* g in the nerve-resected animals, and 0.79 *0.08* g in the sham-operated group ($P < 0.01$). The callus in the healing tibiae was larger in the group with nerve resection (Figure 1): There was no callus formation in 8 of the 11 innervated fractures, as opposed to only 1 out of 8 in the nerve-resected ones. Calculation of the weight of the callus as the difference in wet weight between the healing and the contralateral intact tibia showed a callus weight of 0.16 *0.08* mg in the nerve-resected animals compared to 0.04 *0.08* mg in the sham-operated ones ($P < 0.001$). The bone mineral content (BMC-g/cm) of the callus evaluated by SPA was 53 percent higher in the nerve-resected animals (Table 1, $P < 0.02$), and the callus

Table 1. Bone mineral content (BMC) and callus width measured by single-photon absorptiometry in 25-day-old healing tibial fractures in sciatic nerve-resected (SNR) and sham-operated (Sham) rats. Mean *SD*

	BMC (mg/cm)	Callus width (mm)
SNR (n 8)	72 *20*[a]	5.45 *0.84*[a]
Sham (n 8)	47 *10*	4.54 *0.26*

[a] $P < 0.02$, SNR versus Sham.

was 20 percent wider. The linear regression between BMC and callus weight was r 0.81 ($P < 0.001$), and between BMC and radiological scoring it was r 0.76 ($P < 0.001$).

Alignment was good in all fractures, and for this reason they could be used for mechanical evaluation. Fractures were short transverse or oblique, with an intermediary fragment in only 1 case. In 3 fractures in the sham-operated group, the 2 parts of the tibia fell apart during removal of the intramedullary nail. These fractures were given zero value for all mechanical parameters. The mechanical results were not significantly different for any parameters in the two groups studied.

The ^{85}Sr activity of the healing tibiae were 9 percent higher in the nerve-resected than in the sham-operated animals (not significant).

Discussion

This study shows that innervation has a regulatory role in the healing process of diaphyseal fractures. Resection of the sciatic nerve led to a larger callus formation with a higher bone mineral content as compared to animals with intact sciatic nerves. However, the mechanical results of the healing tibiae were not significantly different after 25 days in nerve-resected and sham-operated rats suggesting that both the quantity and the quality of callus formation are influenced by the nervous system.

In our study the hind limbs of both the nerve-resected and the sham-operated animals were cast-immobilized to prevent uncontrolled and unequal loading of the fractured tibiae. Aro et al. (1981) studied the effect of nerve resection on healing tibial fractures, and assumed that the legs were loaded equally. However, sciatic nerve resection clearly changes the loading pattern and it does not normalize with time (Chen et al. 1992). In the present study, weight bearing was prevented by casting the limbs, although it is not possible to rule out that muscle contraction inside the cast may have influenced the healing process. The importance of immobilization of the innervated fractures is clearly evidenced by a comparison of Aro's study with the present one: Aro (1985) found a larger callus formation in the control animals than in the nerve-resected ones after 28 days of healing, while in the present study callus was larger in the nerve-resected limbs. In fact, weight bearing has been shown to increase the callus size substantially (Sarmiento et al. 1977).

The fixation of the tibial fractures was done percutaneously with modular intramedullary nails, which have about the same stiffness and strength as intact tibiae of rats used in the present experiment (Nordsletten et al. 1992). The fractures were made with a fracture forceps, which causes minimal soft tissue damage. Despite the small trauma resulting from surgical fixation and the low energy fracture, the healing tibiae regained only about 10 percent of the bending strength of the intact contralateral tibiae. One explanation of this could be the combination of intramedullary nailing and casting which prevented loading and gave a very stable fixation. 8 out of 11 fractures in the sham-operated animals showed no callus formation. By defining primary fracture union as absence of callus on radiograms (Chao et al. 1989), these 8 fractures apparently had started to heal by primary union. In contrast, all except one nerve-resected fracture had developed callus with a relatively high BMC. This difference in healing type may imply that in fractures with a nerve supply the sensory innervation recognizes movement of the fracture and that, with stable fixation and minimal movement, nerves may mediate signals that lead to primary bone healing. Sensory nerves have been detected inside callus 7 days after fracture (Hukkanen et al. 1993). Fractures probably unite through a bridging callus, which without innervation will occur regardless of the degree of stability. In humans this phenomena is probably reflected by excessive callus formation in paraplegic patients (Eichenholtz 1963). Primary union with end-to-end consolidation in rat tibial fractures treated with a rigid nail has been reported by Aro et al. (1982). The combination of nailing and casting in the present study probably provided an even more stable fixation.

To be able to normalize the healing strength of the intact tibiae a three-point cantilever bending test was used. The healing tibiae had only regained 7 percent and 11 percent, respectively, of the strength of the intact tibiae in the sham- and nerve-resected animals. BMC of the callus was 53 percent higher in the nerve-resected animals as measured by SPA. This group difference was in reality larger, since the 3 fractures in the sham-operated group which fell apart during removal of the nail could not be measured by SPA. However, some of the difference may have been due to a lesser degree of bone resorption at the fracture site in the nerve-resected tibiae, since some of the original bone on each side of the fracture was also measured. Interestingly, the larger callus with a higher degree of mineralization in the animals with nerve resection was not significantly stronger than the small callus in the sham-operated animals. A defective organization of the callus due to the lack of appropriate neural influence may explain this.

A previous study of healing, rat tibiae has shown an increased density of calcitonin gene-related peptide (CGRP)—and protein gene product 9.5 (PGP 9.5)—immunoreactive nerves in the periosteum of normally innervated callus, with a more than fourfold increase, 21 compared to 7, days after fracture (Hukkanen et al. 1993). At 7 days post-fracture immunoreactive nerves were also seen in the middle of the callus. The main role of the CGRP-containing peptidergic sensory system has been suggested to be involvement in tissue maintenance and renewal during normal function and following injury (Kruger et al. 1989, Hukkanen et al. 1993). In the degenerating segment of a cut nerve trunk, Schwann cells proliferate. This coincides with an increased expression of growth factor receptors and growth-associated proteins both distal to the nerve section (Bosch et al. 1989) and in the dorsal root ganglia (Sommervaille et al. 1991). This could be a source of neurotrophic factors (nerve growth factor-NGF and others) (Varon and Bunge 1978), which could influence fracture healing. During embryogenesis, the peripheral tissues produce NGF, thereby attracting the ingrowth of nerves (Varon and Bunge 1978, Frenkel et al. 1990b). In fracture repair, a process similar to embryonic bone formation (Frenkel et al. 1990a), the production of NGF is probably necessary for the ingrowth of nerves. It is possible that in nerve-resected animals there was a sustained high concentration of NGF in the fracture which may have stimulated the callus formation. NGF-receptors have been observed both in cartilage and bone cells in chicken (Frenkel et al. 1990b).

Vascularization is of definite importance for fracture healing (Wilson 1991). Takahashi et al. (1990) found that sciatic nerve resection did not increase tibial bone blood flow in the rat after 6 hours, 4 or 12 weeks, whereas spinal hemi-transsection did. Changes in bone and callus blood flow in nerve-resected compared to normal tibiae have not been reported. Since CGRP-containing sensory innervation may be important in controlling the vascular supply of a fracture and in angiogenesis (Hukkanen et al. 1993), differences in blood supply may have been of significance for the results of the present study.

Nonunited human fractures show a paucity of nerve endings, which may indicate that lack of innervation plays a causative role (Santavirta et al. 1992). The present study evaluated fracture healing 25 days after fracture. In both groups little strength had been regained, in the innervated tibiae probably because of small callus formation with primary healing in several cases. The combination of internal fixation and casting probably delayed the healing process. There was more callus in the nerve-resected tibiae which, however, was weak. This is a further sign of neural regulation of fracture healing.

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