IS NERVE IN-GROWTH REQUIRED FOR NORMAL FRACTURE HEALING?

+*Li, J; *Ahmad, T; *Ahmed, M (A-AP Research Commission); *Spetea, M; *Kreicbergs, A (A-Swedish Medical Research Council, AO Research Commission)
+Karolinska Institutet, Stockholm, SWEDEN. Orthopedic Research Laboratory, Research Center M3:02, Karolinska Hospital, S-171 76 Stockholm, SWEDEN,
+46851772699, Fax: +46851776103, tony@ort.ks.se

INTRODUCTION Patients with head injuries show brisk and excessive callus formation in fractured limbs. Similarly, abnormal callus formation is seen in hemiplegic patients with fracture on the paralysed side. The fracture callus of the neurologically deficient limb, despite being large in size, has been shown to be poorly mineralized and mechanically weak. These observations as well as experimental findings suggest that an intact nervous system is required for normal fracture healing.

Several in vitro studies have shown that neuronal mediators, so-called neuropeptides, are involved in local bone formation and resorption. In embryonic limb development, it has been shown that the occurrence of nerve fibers in diaphyseal bone coincides with mineralization. These findings imply that the nervous system is an important participant in the ossification process. In fracture, it may prove that regeneration of the injured nerves is a prerequisite for normal healing.

In recent years, neuronal proteins specific for nerve regeneration (growth associated protein – GAP-43) and nerve maturity (protein gene product – PGP 9.5) have been identified and isolated. Using antibodies against these proteins it is possible to demonstrate regeneration and end-differentiation of nerves by immunohistochemistry.

In the present study, based on the hypothesis that nerve in-growth is a critical component of fracture healing, we analysed the occurrence of regenerating and mature nerve fibres over time in fracture callus.

METHODS Twelve male Sprague Dawley rats, weighing 230-290 g were utilised. The right tibiae were fractured under Hypnorm® anaesthesia and fixed with a 17 G cannula needle in the medullary canal. The left un-fractured tibia served as an internal control. Progress of fracture healing was monitored by X-ray. Experiments were approved by the local animal ethics committee.

Three rats were killed at 3 days, 1, 2 and 3 weeks post-fracture and perfused with Zamboni's buffered paraformaldehyde solution for in vivo fixation. Subsequently, right and left tibiae were dissected and demineralized in a solution containing 7% AICl3, 5% formic acid and 8.5% HCl. The specimen was divided into two halves sagitally and then sectioned at a thickness of 15μm using a Leitz Cryostat.

The indirect immunofluorescence method was used for staining with GAP-43 and PGP 9.5. In brief, the sections were incubated with antiserum to GAP-43 (1:2000) and then with biotinylated antibodies. Cy2-conjugated avidin was used for the fluorescent staining. For double-staining, after the completion of staining with first antibody, the sections were incubated with avidin blocking solution followed by biotin blocking solution. Incubation with the second antiserum to PGP 9.5 (1:10,000) was performed in the same manner as for the first peptide. For fluorescent staining of PGP 9.5, the sections were incubated with Cy3-conjugated avidin. A Nikon epifluorescence microscope was used for photography.

RESULTS To assess the normal occurrence of regenerating and mature nerve fibres in fibrocartilage as early as 1 week post-fracture. Arrows indicate GAP-43-positive nerves. V=new vessel; P=hyperplastic periosteum; FC=fibrocartilage callus; CA=cartilage callus.

DISCUSSION Our study suggests that there is an expression of GAP-43 even under normal circumstances, as seen in the un-fractured tibia. This expression probably reflects longitudinal nerve growth in growing rats rather than nerve regeneration. This is supported by the absence of signs of nerve sprouting.

In the fractured tibia, however, there was intense nerve regeneration in the early phase of fracture healing. Thus, a prominent expression of GAP-43 was seen in the hyperplastic periosteum and the callus fibrocartilage as early as 1 week post-fracture. This expression remained high in the fractures up to 3 weeks, when healing essentially was completed. Possibly, this persisting occurrence of GAP-43 is necessary for the ensuing ossification and bone remodeling. PGP 9.5 expression was markedly low at one week, but became pronounced at 3 weeks, probably reflecting functional maturation of the regenerated nerves in the healing fracture. It may prove that the strong regenerative capability of nerves seen in the fractures is a prerequisite for normal fracture healing.

Our results point to the possibility that regenerating nerves provide the delivery system for GAP-43 and neuronal mediators required for normal callus formation and/or neovascularization.