Immunohistochemical localization of nerve growth factor in fractured and unfractured rat bone

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We detected nerve growth factor (NGF) by immunohistochemical localization in both fractured and unfractured rat rib. In unfractured bone, periosteal mesenchymal osteoprogenitor cells appeared to be the only skeletal cells which stained for NGF. Adjacent skeletal muscle fibers exhibited NGF staining both in fractured and unfractured bone. Fracture callus periosteal osteoprogenitor cells, marrow stromal cells, osteoblasts, young osteocytes and endothelial cells of new capillaries had moderate to heavy staining for NGF at 1 and 3 weeks after fracture. Deeply positioned osteocytes and osteoclasts showed no NGF staining. Most chondrocytes of fracture calluses stained for NGF, however, some chondrocytes did not stain which may indicate that NGF is produced at particular stages of chondrocytic differentiation. In calluses, periosteal matrix stained heavily for NGF when juxtaposed to cartilage and less obviously when associated with new bone at both 1 and 3 weeks post-fracture. However, other fibrous, cartilaginous and osseous matrices did not stain for NGF at any time. At 6 weeks post-fracture, NGF staining was largely confined to periosteal osteoprogenitor cells.

The detection of NGF in periosteal osteoprogenitor cells of unfractured rib points to these cells having a role in nerve maintenance in intact bone. Furthermore, the localization of NGF in osteoprogenitor cells, marrow stromal cells, osteoblasts, certain chondrocytes, endothelial cells, periosteal matrix of the fracture callus and skeletal muscle may mean that these entities participate in fracture innervation. The presence of NGF in the callus may also indicate a direct, as yet undefined action of this neurotrophin on skeletal cell metabolism.

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Innervation of bone appears to have trophic effects on both normal bone metabolism as well as fracture repair mechanisms. Sympathetic and sensory nerves are prominent in the periosteum of mammalian bones and are present amongst blood vessels and osteogenic cells in these areas (Hohmann et al. 1986, Bjurholm et al. 1988, Hill and Elde 1991). Bone deprived of its sympathetic innervation shows reduced bone deposition and mineralization as well as increased bone resorption (Sandhu et al. 1987, Hill et al. 1991). Furthermore, osteoporosis has been induced in non-weight bearing mandibular bones in animals which have been sympathectomized (Resende et al. 1991). Sensory nerves also appear to be important in normal bone metabolism and in bone fracture repair (Hukkanen et al. 1993).

The neurotrophin, nerve growth factor (NGF), is important for the appropriate development and maintenance of peripheral sensory and post-ganglionic sympathetic nerves (Levi-Montalcini and Angeletti 1968). Topical application of NGF to fractured rat ribs has been shown to increase the stiffness and breaking strains of fractures (Schuijers et al. 1995, Grills et al. 1997). However, the mechanisms by which the administration of NGF improved fracture healing in this model are unclear.

Increased sensory (Hukkanen et al. 1993, Aoki et al. 1994) and sympathetic innervation (Schuijers et al. 1995, Grills et al. 1997) of fracture calluses has been reported in animal experiments, with innervation maximal at approximately 3 weeks after fracture. NGF formation by cellular components of the callus has been put forward as a possible factor which increases this innervation (Nordsletten et al. 1994, Grills et al. 1997).

To date, no studies have been done to identify NGF in intact and/or fractured mammalian bone. In this report, we undertook immunohistochemical studies to investigate whether NGF could be identified in fractured and unfractured adult rat bone.

Animals and methods

Male Sprague-Dawley rats weighing between 200 and 250 g were used for the experiment. For examina-

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tion of fractured bone, rats were anesthetized by an intraperitoneal injection of 1 M ketamine, Rompun and acepromazine (4 mL/kg) and an incision was made on the lateral aspect of the trunk parallel to the ribs (approximately between ribs 5 and 7). The sixth rib was fractured by snipping it with a fine scissors, 2 cm from the vertebral column (Grills et al. 1997). Animals were then allowed to recover. Groups of 6 rats were killed at 1, 3 and 6 weeks post-fracture. For unfractured bone, 6 animals were sham-operated and allowed to recover. Pairs of rats were killed and one sixth rib was excised from each rat on the same days as in the fractured bone group.

The rat submandibular gland, a tissue known to synthesize large amounts of NGF (Levi-Montalcini and Angeletti 1968), was excised from an anesthetized rat and processed similarly to fracture calluses. This tissue acted as an immunohistochemical reference for NGF staining.

Fracture calluses and the submandibular gland were fixed in 4% paraformaldehyde, 0.25% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 24 h at 4 °C, then processed to LR gold acrylic resin (London Resin Company, U.K.) using previous methods (Grills et al. 1990). Fracture calluses were not decalcified. One micron thick sections were cut, using an ultramicrotome equipped with a diamond knife. Sections were subsequently placed on Vectabond reagent-coated (Vector Laboratories, USA) glass histologic slides.

Immunohistochemistry

LR gold-embedded sections were rehydrated in distilled water, then washed in 10 mM phosphate buffered saline (PBS), pH 7.2, for 10 min and to inhibit nonspecific binding were subsequently immersed in 5% normal goat serum (Amersham, U.K.) for 30 min.

After this, sections were incubated in a 1/60 dilution of rabbit anti-2.5S rat NGF antiserum (Sigma Chemical Co, St Louis, USA) overnight in a humidifier and subsequently washed in three changes of PBS for 10 min each. Sections were then incubated in a 1/200 dilution of biotinylated goat-anti-rabbit antiserum (Amersham, U.K.) for 75 min and subsequently washed in three changes of PBS for 5 min each. Sections were next exposed to a 1/40 dilution of Auro Probe™ LM streptavidin-gold (Amersham, U.K.) for 60 min followed by a wash in distilled water for 5 min and then flooded with IntenSE™ M (Amersham, U.K.) silver enhancement mixture for 6–18 min, depending on ambient room temperature. Sections were washed for 5 min in distilled water. Slides were subsequently dried on a hotplate and sections were coverslipped using DePeX.

All procedures were undertaken at room temperature. Goat serum, antibodies and streptavidin-gold were diluted in a solution containing PBS, 0.1% bovine serum albumin, 0.1% tween and 0.05% sodium azide.

Nonspecific stainings included omission of the primary antibody or the secondary antibody from either of these steps.

After immunostaining, slides were examined and photographed uncounterstained under a light microscope at 100 and 400 times magnification with a blue filter. Brown-black deposits, which were distinct from nonspecific background stainings, indicated NGF positive staining.

Results

In unfractured ribs, light immunostaining for NGF was seen only in skeletal muscle fibers, periosteal osteoprogenitor cells and superficial osteocytes in cortical bone. However, deep cortical osteocytes as well as both periosteal and osseous matrices did not stain for NGF in unfractured ribs (Figure 1).

At 1 week post-fracture, calluses consisted predominantly of osteoprogenitor cells producing fibrous tissue, which were associated with small amounts of cartilage between the fracture ends (external callus). Direct trabecular bone formation by osteoblasts occurred in collars on periostea adjacent to fracture sites (primary callus). At 3 weeks after fracture, primary callus formation in periostea had ceased and in external callus, fibrous tissue persisted. At this stage, external callus cartilage was more evident than at day 7; indicating that endochondral ossification was then more advanced. Immunohistochemistry of fractures revealed that NGF staining was prominent at

Figure 1. Section of outer edge of unfractured rat rib showing NGF staining of periosteal osteoprogenitor cells (OP), a superficial osteocyte (small arrow) and skeletal muscle fibers (M). Bone matrix (B) and deep osteocytes (large arrows) do not stain for NGF (uncounterstained), ×400.

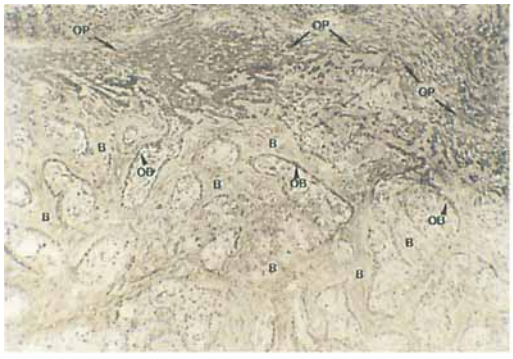

Figure. 2. Section of fractured rat rib 7 days post-fracture (fracture site out of picture, to right) showing prominent NGF staining of both periosteal osteoprogenitor cells (OP) and osteoblasts (OB) forming new trabecular bone (B) of the callus (uncounterstained), ×100.

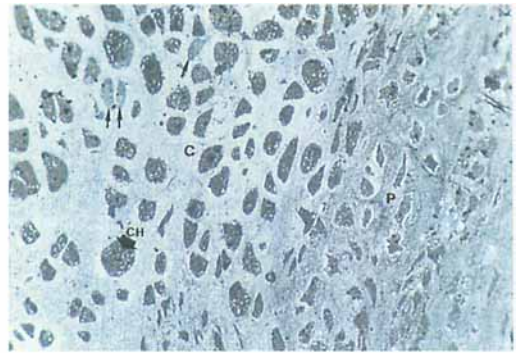

Figure. 3. This micrograph shows the interface between cartilage (C) and periosteal tissue (P) of the callus 7 days post-fracture. In periosteal tissue, osteoprogenitor cells and their surrounding matrix stain for NGF. Most chondrocytes (CH) of cartilage show staining for NGF, whilst some chondrocytes do not stain for NGF (small arrow). Cartilage matrix (C), like that of bone, however, does not stain for NGF (uncounterstained), ×400.

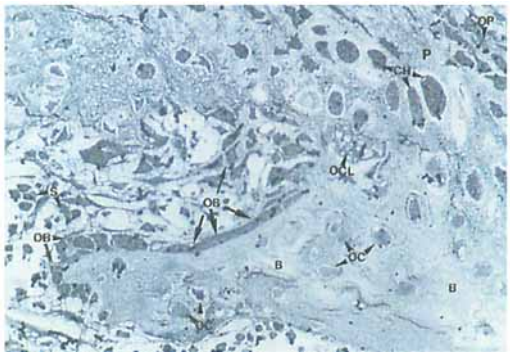

Figure 4. Section of fractured rat rib 21 days post-fracture. There is prominent NGF staining in marrow stromal cells (S) and osteoblasts (OB) which are forming new bone (B). Most chondrocytes (CH), osteoprogenitor cells (OP) and the matrix of the periosteal/chondral junction (P) show staining for NGF. Osteocytes (OC), an osteoclast (OCL) process and bone matrix (B), however, do not stain for NGF (uncounterstained), ×400.

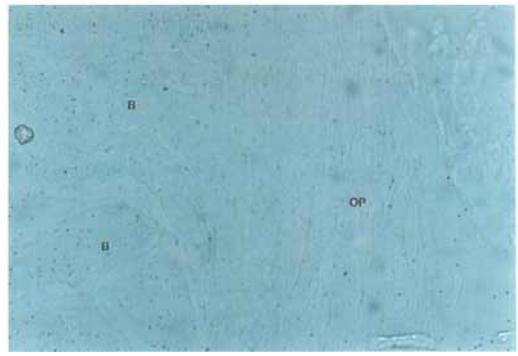

Figure 5. Representative nonspecific stained section of fractured rat rib (7 days post-fracture). Osteoprogenitor cells (OP) of a thickened periosteum on bone (B) show only background staining (uncounterstained), ×100.

both 1 and 3 weeks post-fracture in the following: skeletal muscle fibers, osteoprogenitor cells in thickened periostea of new calluses, osteoblasts forming new trabecular bone near fracture sites, marrow stromal cells, superficial osteocytes of new trabecular bone, most chondrocytes of calluses (Figures 2 and 4) and endothelial cells of new callus capillaries (results not shown). Some chondrocytes, as well as deep osteocytes in new trabecular bone or cortical bone and osteoclasts, did not stain for NGF. In calluses, periosteal matrix stained heavily for NGF when juxtaposed to cartilage and less obviously when associated with new bone at both 1 and 3 weeks post-fracture whilst other fibrous, cartilaginous and osseous matrices did not appear to stain for NGF at any time (Figures 3 and 4). By 6 weeks after fracture, all fibrous tissue and cartilage of calluses had been replaced by trabecular bone and light NGF staining was limited to periosteal osteoprogenitor cells and osteoblasts of the endosteum (results not shown).

Nonspecific immunohistochemistry showed minimal background staining of tissue components at all times (Figure 5).

Discussion

To our knowledge, this is the first study which has localized NGF in skeletal cells and matrix of both fractured and unfractured adult mammalian bone. NGF has been detected in chondrocytes and osteoblasts of embryonic chick skeletal tissue which sug-

gests that these cells may govern innervation in this embryo by synthesizing and secreting NGF (Frenkel et al. 1990). Fracture callus is initially composed of similar embryonal-like skeletal cells (Hulth 1989) and it was therefore expected that cells of the callus could similarly contain NGF. In the chick embryo, Frenkel et al. 1990 reported that all chondrocytes stained for NGF, whilst in the current experiments most chondrocytes of the callus stained for NGF. However, some did not stain for this neurotrophin. This finding may indicate that NGF appears at a particular stage during chondrocytic differentiation in callus development. We are currently performing in situ hybridization investigations to determine the time when various skeletal cells are induced to express NGF in fractures.

Sensory and sympathetic nerve fibers are mainly found in the periosteum (Hohmann et al. 1986). Therefore the presence of NGF in osteoprogenitor cells in this region may contribute to the normal maintenance of these nerve fibers, a role which has been attributed to NGF (Levi-Montalcini and Angeletti 1968).

Rat skeletal muscle has previously been shown to produce NGF (Amano et al. 1991) and the immunohistochemical identification of NGF in similar tissue in the current experiments supports this finding. NGF formation by this tissue could conceivably not only influence neural metabolism of skeletal muscle but also affect adjacent tissues, such as periosteum in intact bone or calluses in fracture repair.

The failure to detect NGF in osseous matrix and deeply embedded osteocytes may be due to absence of nerves in mineralized bone matrix (Hohmann et al. 1986, Bjurholm et al. 1988, Hill et al. 1991). On the other hand, superficial osteocytes in cortical bone of unfractured rib and in new trabecular bone of fracture calluses may affect neural metabolism, since NGF staining was evident in these cells.

Fibrous and cartilaginous matrices did not exhibit immunostaining for NGF, a finding similar to that in the embryonic chick study (Frenkel et al. 1990). The presence of NGF in periosteal matrices juxtaposed to cartilage and new bone, however, may indicate that reinnervation of the periosteum is important in fracture repair, a tissue which is highly innervated (Hohmann et al. 1986), as opposed to non-innervated cartilage or bone matrices. This observation may also indicate that NGF, like some other growth factors e.g., insulin-like growth factor I (Pfeilschifter et al. 1995), can be stored in certain matrices and thus be made available for physiologic responses by release from such tissue. Furthermore, the presence of NGF in these matrices suggests that NGF possesses its own associated binding protein in these matrices. Further experiments will be undertaken to determine whether such a binding protein exists.

Detection of NGF in skeletal cells and certain matrices of the unfractured bone and the fracture callus indicates that this growth factor is involved in nerve maintenance in intact bone as well as nerve fiber upkeep and proliferation in bone repair. Moreover, recent findings by Jehan et al. (1996) have shown that cultured ROS 17/2.8 osteoblast-like cells possess the low affinity NGF receptor and respond in a dose-dependent manner to the presence of $1,25(OH)_2D_3$ by an increase in NGF mRNA. These results therefore may indicate that NGF has direct, as yet undefined, actions on osseous cells.

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