

Immunologic studies of nonunited fractures

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We studied tissue samples of noninfected delayed union or nonunion of diaphyseal bones in 10 patients immunopathologically and neuroimmunologically 4 to 25 months after the primary injury. Samples mostly consisted of vascularized connective tissue of varying density with the proline-4-hydroxylase-containing fibroblast as the major cell type. Most inflammatory cells were CD4 T-lymphocytes and their number was always twice that of the CD8 positive cells. Staining for CD11b positive monocyte/macrophages showed in all samples positive cells scattered in the connective tissue stroma with perivascular enrichments. Mast cells were absent or very rare.

Our findings suggest that delayed union and nonunion tissue consists of vascularized connective tissue, which mostly contains 5B5 fibroblasts, CD11b macrophages and vascular endothelial cells with only few immigrant recently recruited monocytes or lymphoid cells. Almost all resident cells seem to be involved in tissue remodeling as suggested by their content of fibroblast-type MMP-1 and its proteolytic activator MMP-3 or stromelysin. The most striking finding was the paucity or total lack of peripheral innervation, which may have to do with the nonunion of the fracture.

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Nonunion of fractures has been studied in material obtained at operations (Urist et al. 1954, Judet et al. 1958, Bohr 1971), and several animal models have also been advocated (Neto et al. 1984, Aro et al. 1985). The roles of disruption of vascularization (Trueta 1965), enhanced bone resorption (Trueta 1965, Vaes 1988) and neural factors (Aro et al. 1985) have been pointed out. However, with conventional study methods several aspects of the pathobiology of the process leading to fracture nonunion could not be studied.

We analyzed samples of human delayed unions and nonunions for the presence of inflammatory cells, matrix metalloproteinases, and neural tissue with the use of immunohistochemical techniques.

months after the initial trauma, while 2 patients were operated on for established nonunion at 16 and 25 months.

Seven of the patients were men and three were women, with an average age of 48 (17–64) years. Two of the tibial fractures (Cases 2 and 5) were sustained in

Table 1. Clinical data on 10 patients treated with Phemister operation for noninfected delayed union or nonunion after diaphyseal fractures

Case	Age	Sex	Location of fracture	Type of fracture	Months from injury to operation
1	60	F	lower tibia	open, gr I	7
2	44	F	lower tibia	open, gr I	4
3	44	M	lower tibia	closed	5
4	64	M	middle tibia	open, gr I	5
5	27	M	middle tibia	open, gr I	7
6	42	M	upper humerus	closed	25
7	45	M	middle tibia	closed	6
8	45	M	middle tibia	open, gr II	16
9	60	F	upper humerus	closed	4
10	52	M	middle tibia	closed	4

Material and methods (Table 1)

We studied the data of 10 patients who had surgery to treat delayed union or nonunion of tibial fractures (8 patients) or humerus fractures (2 patients). In 8 cases the surgery was performed to treat delayed union 4–7

motor cycle accidents and one tibial fracture (Case 7) was sustained in an explosion; all the other fractures were due to falls on level ground (usually on icy roads).

None of the fractures was infected either primarily or at the time of surgery; in each case the ESR and C-reactive protein values were normal, and the bacterial cultures obtained at operations were negative.

At surgery, three parallel representative samples, each about 4 x 4 mm in size, were collected from delayed union/nonunion tissue from the area between the diaphyseal cortices below the pseudocapsule (Urist et al. 1954) and were then processed for inflammatory-cell analysis, analysis of matrix metalloproteinases and for neuroimmunological studies.

Staining of inflammatory cells

For immunostaining of the inflammatory cell population (Hsu et al. 1981, Kontinen et al. 1983, Kontinen et al. 1989a, Santavirta et al. 1990a), we used mouse monoclonal antibodies against CD2 (for total T-cells), CD4 (inducer/helper T-cells), CD8 (suppressor/cytotoxic T-cells), 2H4 (suppressor inducer T-cells), 4B4 (helper inducer T-cells), CD19 (resting B-lymphocytes), CD11b (monocyte/macrophages), GpIb and GpIIb-IIIa (platelets), Ia (MHC locus II antigen), CD25 (interleukin-2 receptor) and PCA-1 (plasma-blasts and plasma cells). All the monoclonal antibodies were obtained from Ortho Diagnostic System, Raritan, New Jersey, except the Pan B (anti-CD19) which was from Dakopatts, Copenhagen, Denmark. The method (including avidin-biotin-peroxidase complex method, counterstaining, staining controls and counting of the cells) was recently reported by us in detail elsewhere (Santavirta et al. 1990a). Mast cells were stained using toluidine blue (Kontinen et al. 1990c, Santavirta et al. 1990a).

Proline 4-hydroxylase and carboxy-terminal propeptide of type I collagen immunostaining were done with monoclonal antibodies (Kontinen et al. 1989c, 1990a).

Staining of matrix metalloproteinases

For immunohistochemical analysis of MMP-1 (matrix-metalloproteinase-1; mesenchymal fibroblast-type collagenase), MMP-3 (matrix-metalloproteinase-3; stromelysin), and MMP-8 (granulocyte collagenase), we used 6-µm-thick cryostat sections which were air-dried and fixed in acetone at +4 °C for 5 min (Cooper et al. 1983, Sorsa 1987, Sorsa et al. 1988). Endogenous peroxidase activity was blocked with 0.3% H₂O₂ for 20 min. Nonspecific binding sites were blocked by incubation in 1:20 normal horse serum for

20 min, after which the MMP-positive cells were demonstrated with the avidin-biotin-peroxidase complex (ABC) immunostaining method (Hsu et al. 1981). Details of staining of matrix metalloproteinases have recently been published by Sorsa (1987), Sorsa et al. (1988), Suomalainen et al. (1991), and Santavirta et al. (1992). The slides were also stained for endogenous peroxidase using the 3,3'-diaminobenzidine reaction only. Evaluation of staining was done by routine light microscopy from immunostained and counterstained coded specimens.

Staining of neuronal markers

The method for neuroimmunohistochemical analysis has been described in detail by Bjurholm et al. (1988a), Bjurholm et al. (1988b), Kontinen et al. (1989b), Kontinen et al. (1990b), Hukkanen et al. (1992a), and Santavirta et al. (1990b). The tissue specimens were immediately fixed in Zamboni's fluid in the operating room (fixation time 4 to 6 hours at +4 °C), rinsed overnight at +4 °C in phosphate-buffered-saline (0.1-M PBS, pH 7.4) containing 15% sucrose and then transported to the immunohistochemistry laboratory the following morning.

Neural markers used were: PGP 9.5 (ubiquitine C-terminal hydrolase; antiserum was obtained from Ultracelone, Cambridge, U.K.), polyclonal neurofilament (intermediate neuronal filament; antiserum was kindly provided by Dr. Doris Dahl, Veterans Administration, Boston, MA); substance P, CGRP, synaptophysin, vasoactive intestinal peptide (VIP), C-flanking peptide of neuropeptide Y (CPON) and tyrosine hydroxylase (all antisera kindly provided by Dr. Julia M. Polak, Hammersmith Hospital, Royal Postgraduate Medical School, London, England). As positive sample controls in the neuroimmunohistological study, we used 8 human coracoacromial ligaments which were obtained at shoulder surgery and were processed and stained at the same time.

Results

The general morphology of the samples in all the cases was quite similar and apparently not dependent on the interval from the fracture causing injury to the Phemister operation. Each sample exhibited strong variations in tissue morphology between different microscopic fields and the variations were similar in all samples. All samples contained connective tissue of varying density, in which tissue fibroblast-like mononuclear cells seemed to predominate in routine hematoxylin

Table 2. Schematic summary of main results of immunopathological and neuroimmunological findings in 10 samples of delayed union and nonunion tissue

Case	Inflammation total T-cells, CD2	Collagenase MMP-1 and MMP-3	Perivascular nerve endings PGP 9.5	Pain fibers subst. P
1	—	+++	+/-	—
2	+	+++	—	—
3	+	+++	—	+/-
4	+	+++	—	+/-
5	+	+++	+/-	—
6	—	+++	—	—
7	+	+++	—	—
8	+	+++	—	—
9	+	+++	—	—
10	++	+++	—	—

— no positively stained cells;
+/- rare, occasional;
+ occasional;
++ occasional with local enrichments;
+++ high density of positive cells.

staining. The cellularity varied inside each sample from poorly cellular, tight connective tissue areas to highly cellular strangs with occasional cartilage or bony islets. All samples were relatively vascular.

Staining for CD2 reflecting the total number of local T-cells revealed in most samples occasional positive cells in the stroma and sometimes a higher density of positive cells perivascularly (Figure 1 and Table 2). In two samples (Cases 1 and 6) there were no CD2 positive cells at all. Most of the local T-cells were of the CD4 inducer/helper T-cell subset, which was about twice as frequent as the CD8 suppressor/killer T-cell subset. CD19-positive resting B-lymphocytes were only found in four samples (Cases 5, 6, 8 and 9).

Staining for CD11b showed monocyte/macrophages scattered in the connective tissue stroma with perivascular enrichments (Figure 2), and CD11b-positive cells were in all samples more frequent than CD2-positive T-cells. Staining for endogenous peroxidase was negative (Figure 3).

Interestingly, staining for 2H4 positive suppressor inducer T-cells was usually negative, and only two patients' samples (Cases 2 and 8) showed occasional positive cells, whereas the capillary endothelial and postcapillary venule endothelial cells stained in all samples positive for 4B4 (which is often advocated as a marker for helper inducer T-cells) and also the majority of all cells in the connective tissue stroma were positive for 4B4 (Figure 4).

Staining for Ia (MHC locus II antigen) showed that in all samples most capillary and postcapillary venule

endothelial cells stained positive, while the number of positive cells in the connective tissue varied and most samples showed local enrichments of positive cells perivascularly and/or at areas where there was a higher density of inflammatory cells. However, staining for CD25 (interleukin-2 receptor) was negative in all samples except in Case 6, where there were occasional positive cells perivascularly.

Toluidine blue staining for mast cells showed rarity or absence of positive cells in the connective tissue (Figure 5) with the exception of Case 3 where 2-5 percent of the cells in the stroma were positive.

Staining for MMP-1 (matrix metalloproteinase-1; fibroblast collagenase) showed that in each sample all the capillary and postcapillary venule endothelial cells as well as about 90 percent of the cells in the connective tissue stroma stained positive (Figure 6). In cartilaginous areas several chondrocytes were also found to be positive. The cellular staining profile for MMP-3 (Stromelysin) was quite similar (Figure 7).

In the staining for MMP-8 (for granulocyte collagenase) only occasional intravascular positive granulocytes were found while in one sample (Case 8) also rare positive tissue neutrophils were seen. Staining for Gp Ib and IIb/IIIa was negative in all patient samples.

In the staining with 5B5 for proline 4-hydroxylase the capillary and postcapillary venule endothelial cells were negative in all but two samples (Cases 7 and 8) where some endothelial cells were slightly positive. Most of the cells (85-90%) in the connective tissue stroma stained positive.

From the neuroimmunohistochemical point of view, the most prominent finding was the lack of peripheral nerves in the delayed union/nonunion tissue. Staining for the general neuronal marker PGP 9.5 did not show any free or perivascular nerve endings in eight patients (Figure 8), while a few nerves were seen in two samples (Cases 1 and 5). Control samples of human coracoacromial ligaments, similarly processed and stained showed in the periligamentous sheath rich perivascular innervation with PGP-9.5-containing nerves (Figure 8). Similarly, the neurofilament staining showed rare solitary nerve fibers in three samples (Cases 3-5), and staining for synaptophysine showed only occasional perivascular fibers in two samples (Cases 4 and 8). Staining for VIP showed rare positive solitary perivascular fibers in three samples (Cases 2-4). Staining for substance P was negative in all except two samples (Cases 3 and 4), where there were a few solitary and very small positively stained perivascular fibers. Similarly in CGRP staining, occasional positive fibers were found in two samples (Cases 3 and 4). Very rare CPON positive fibers were found in four samples (Cases 3-5 and 8) and tyrosine hydroxylase staining showed a similar finding.

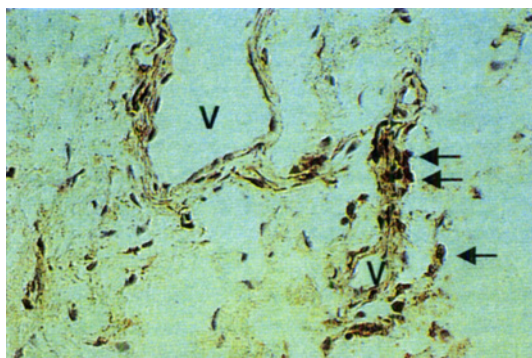


Figure 1. Case 2. Delayed union after open fracture of the lower tibial diaphysis in a 44-year-old woman. Sample of the delayed union tissue was obtained at Plemister operation 4 months after injury. CD2-positive T-lymphocytes (some have been marked with arrows) have been stained with monoclonal antibody applied in avidin-biotin-peroxidase staining with hematoxylin counterstain. Delayed-union tissue was noninflammatory with very few immigrant lymphocytes, here surrounding a small venula (V), x 480.

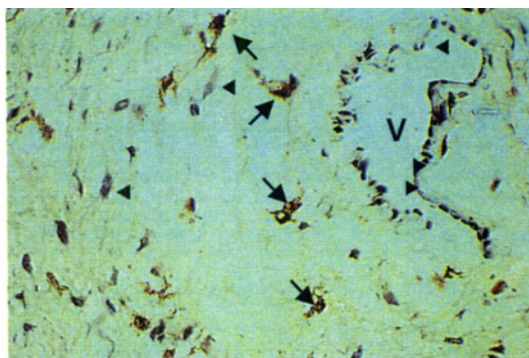


Figure 2. Case 1. Delayed union in the lower tibial diaphysis 7 months after open fracture in a 60-year-old woman. A typical case of delayed union was characterized by mesenchymal response and absence of perivascular lymphocytes, as demonstrated in this CD11b staining for monocytes/macrophages (arrows). Vascular endothelial (v venula) cells and some fibroblast-like stromal cells are not peroxidase-labeled (arrow heads) and can be discerned by hematoxylin counterstain, x 480.

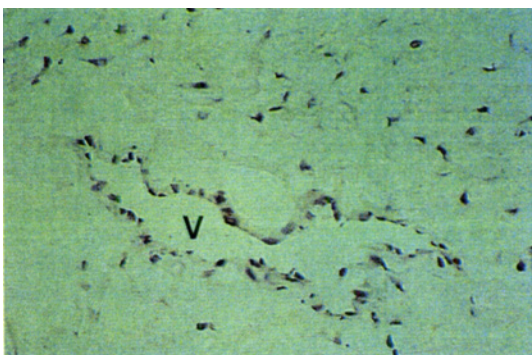


Figure 3. Case 1. The sample was here histochemically stained for endogenous peroxidase (Px). Endogenous peroxidase is expressed by peripheral blood monocytes recently recruited to tissues and such cells cannot be seen at all in situ. The staining shows that almost all cells of the monocyte/macrophage lineage are mature tissue macrophages (endogenous peroxidase negative, CD11b positive) and only occasionally recently recruited endogenous peroxidase positive monocytes are seen in situ, x 480.

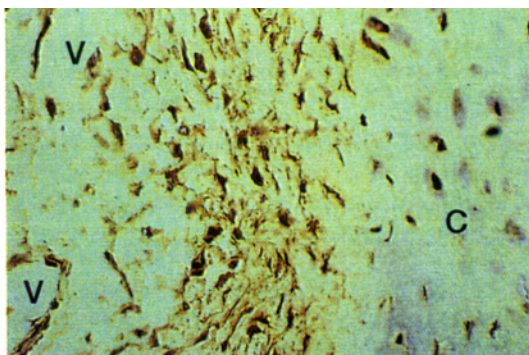


Figure 4. Case 1. Cartilage (C) is seen to the right. There is accumulation of 4B4 positive mononuclear cells on the border of the cartilage at the center of the field. Also occasional chondrocyte-like cells in the cartilage are 4B4 positive (arrows), x 480.

Discussion

The biology of fracture healing in long bones has been extensively investigated with morphologic and biochemic methods. Only few studies report on pathobiological investigations on delayed union and nonunion of fractures of long bones (Urist et al. 1954, Trueta 1965). In addition, experimental delayed union/ non-union models have been difficult to develop.

Urist et al. (1954) suggested that fibrinoid degeneration of connective tissue in the interior of the callus is the main cause of delayed union and that this process resembles chronic adventitious bursitis. Further, their

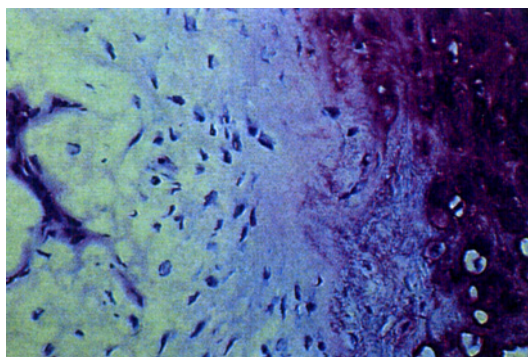


Figure 5. Case 1. The same area as in Figure 4 in routine toluidine blue staining shows metachromatic staining of the newly formed ectopic cartilage. Note also the rarity or, in this case, total absence of toluidine blue positive mast cells in the connective tissue of the delayed union, a common feature in our series, x 480.

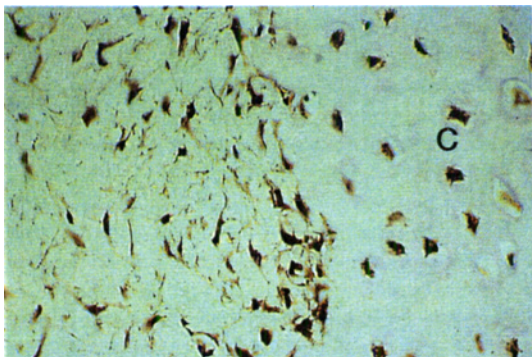


Figure 6. Case 1. Fibroblast-type collagenase or matrix-metalloproteinase-1 (MMP-1) was expressed in almost all stromal fibroblasts (dark stained cells) and also by most although not all cells in the cartilage (C), x 480.

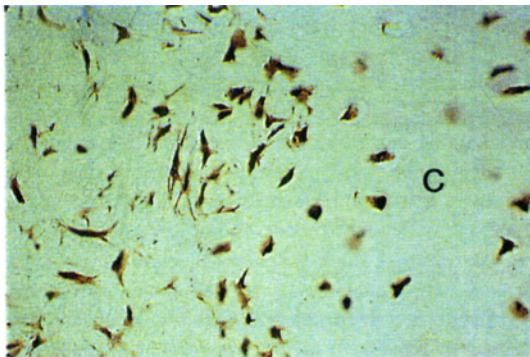
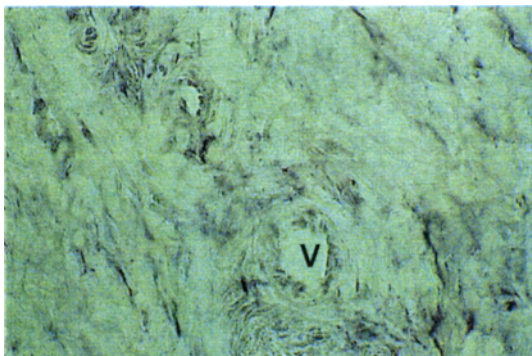


Figure 7. Case 1. Stromelysin or MMP-3 staining discloses an almost identical tissue localization as was seen for MMP-1 in the previous figure, where the sample was from the same patient and area, x 480.

Figure 8. From the neuroimmunohistological point of view, the most prominent finding in the delayed union tissue was an almost total lack of peripheral nerves.



Case 4. In this sample, staining of a general neuronal marker, PGP 9.5, does not show any perivascular or free nerve endings, x 480.



In contrast, similarly processed connective tissue in this sample from coracoacromial ligament obtained in Neer's acromioplasty operation shows rich perivascular innervation by PGP 9.5 containing nerves in the periligamentous sheath (arrows), x 480.

report suggested that the qualitative changes in the delayed union/nonunion tissue during the first two years are relatively small, this being in accordance with our results; the gross routine histology was relatively uniform in all our ten cases and was not dependent on the interval from the primary injury. It is not known why a small proportion of diaphyseal fractures do not unite in due time. Our results showed uniform gross histomorphology, immune-histopathology and neuroimmunology in samples obtained 4 to 25 months from injury. This suggests that the reason for development of nonunion lies in the early phase of fracture healing. Our samples, obtained in each case below the pseudocapsule (Urist et al. 1954) from the intercortical space, consisted of fibrous connective tissue of varying density, containing occasional cartilagenous and bony islets and the predominant cell was morphologically fibroblast-like. Also, all samples were vascular-

ized with capillaries and occasional arterioles and scattered postcapillary venules. This does not support Trueta's (1965) theory of vascular disruption and avascularity as a reason for the development of nonunion.

The presence of inflammatory cells in the early phase of fracture healing is well known (Lindholm et al. 1969, McKibbin 1978), and especially during the first days polymorphonuclear leucocytes, macrophages and mast cells make their appearance and participate in the process of clearing up of the debris (Lindholm 1969). Little has been written about the role of inflammatory cells in delayed union and nonunion. In our series, the presence of T-lymphocytes was uniformly modest, and among them there were more inducer/helper T-cells than suppressor/killer T-cells while B-lymphocytes were almost nonexistent. CD11b-positive tissue macrophages were common in all samples, whereas recently recruited endogenous peroxidase-

positive monocytes were rare. Although macrophages are not able to resorb bone directly, osteoclasts are activated by the intense macrophage reaction and the surrounding bone undergoes progressive resorption and fibrous substitution (Vaes 1988). The rarity or absence of mast cells, which are commonly present in the early phase of fracture healing (Lindholm et al. 1969), was striking. In the healing process, monocytes/macrophages usually attempt to clear the involved area of exogenous as well as endogenous substances and debris, and this process is followed by invasion of the site by fibroblasts and by synthesis of extracellular matrix by activated fibroblasts (Konttinen et al. 1988, Konttinen et al. 1990a). Some of the monokines act as chemoattractants for fibroblasts; they induce proliferation of fibroblasts or synthesis of the extracellular matrix (Konttinen et al. 1988).

In all samples, nearly all the cells in the connective tissue stroma as well as the capillary and postcapillary venous endothelial cells stained positive for interstitial collagenase (MMP-1 and stromelysin or MMP-3), while MMP-8 containing polymorphonuclear neutrophilic leukocytes were absent or rare. This together with the presence of fibroblasts containing the carboxy-terminal propeptide of type I collagen (a fibroblast activation marker) suggests, that the fibroblasts in situ may be involved in a type of tissue remodeling involving both synthetic and degradative processes (Fessler and Fessler 1978, Gavrilovic et al. 1987, Konttinen et al. 1988). Macrophages have been shown to produce interstitial collagenase identical to fibroblast collagenase (Welgus et al. 1985, Wilhelm et al. 1986, Santavirta et al. 1992). This is suggestive of an attempt on behalf of the local phagocytic and secretory macrophages to respond to the stimulus initiating and perpetuating the process which leads to deficient fracture healing.

The finding that the capillary and postcapillary venulae endothelial cells stained positively for both MMP-1 and MMP-3 has not been previously reported in connection with fracture healing, but is in accordance with some earlier observations, in particular during actively ongoing wound healing and neovascularization associated with reparative connective tissue formation (Wilhelm et al. 1986, Banda et al. 1988, Mignotti et al. 1989, Santavirta et al. 1992). The absence of catalytically somewhat similar but immunologically distinct MMP-8 of the matrix metalloproteinase family speaks for the specificity of the matrix metalloproteinase stainings. Interleukin-1 is a major inflammatory cytokine with a multitude of biological activities and it is a potent inducer of collagenase mRNA and protein in connective tissue fibroblasts (Gavrilovic et al. 1987, Konttinen et al. 1992). Interestingly, most cells in the connective tissue stroma in

our samples stained positive for proline 4-hydroxylase while the capillary endothelial cells were negative (Konttinen et al. 1989c). Collagen, which is the major protein component in wound healing, is produced by fibroblasts and 4-hydroxyproline and is essential for the formation of the right-handed triple helix through hydrogen bonds.

From the neuroimmunohistological point of view the most prominent finding was the lack or rarity of peripheral nerves in delayed union/nonunion tissue, as was seen in the staining for the general neuronal markers PGP 9.5, neurofilaments and synaptophysin. Currently, little is known about the innervation in normal fracture healing. In rats, periosteum, bone marrow, and the growth plates were found to be relatively well innervated, whereas the innervation of the diaphyseal and metaphyseal bone was more scarce (Hukkanen et al. 1992a). In fractures of rat tibias, we found a rapid increase of PGP 9.5- and CGRP-immunoreactive nerve fibers during the first 3 weeks (Hukkanen et al. 1992b). Also, delayed union/nonunion is a painful condition, but staining for substance P which detects local pain fibers (Bjurholm et al. 1988a, Bjurholm 1989) was negative in all but two samples where there were only rarely occasional positive fibers. Interestingly, Aro et al. (1985) reported that in their experimental study removal of periosteal neural mechanoreceptors caused nonunion in the rat fibula. Currey (1968) hypothesized that neural elements monitor the magnitude of bone strain for adaptive bone remodeling. Also the lack of VIP positive nerves is noteworthy. Hohmann et al. (1983) demonstrated that VIP may induce bone resorption in vitro. However, neither the physiological role nor the source of VIP is established in bone. VIP is also synthesized by extraneural cells, e.g., mast cells and immunocompetent cells (Goetzl et al. 1990). As control samples for neuroimmunohistochemical studies we used coracoacromial ligaments from painful shoulders; in general fewer neuroimmunoreactive nerves are found in inflammatory tissue (Konttinen et al. 1990b).

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