Noncollagenous proteins in heterotopic ossification

Immunohistochemical analysis in 15 paraplegies

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We used immunohistochemical techniques to investigate the distribution pattern of osteonectin, osteocalcin, bone sialoprotein II and the small proteoglycans decorin and PG 100 during different stages of heterotopic ossification (HO) in pressure sores of paraplegic patients. All these noncollagenous proteins (NCPs) accumulated in fibroblasts and preosteoblasts, predominantly in the activity centers of early osteogenetic areas. Mature types of HO showed a more discrete expression pattern for this protein group, with weaker reactions in the narrow osteoblastic rims. Decorin was detected predominantly in the stroma of HO.

Our results indicate that the NCPs are important components during the pathogenesis of HO and that fibroblasts may serve as osteoprogenitor cells.

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Recent data suggest that noncollagenous proteins (NCPs) influence local mechanisms of calcification (Fisher et al. 1987). The main components of the NCPs are osteonectin (Termine et al. 1981), osteocalcin (Hauschka et al. 1975), bone sialoprotein I (osteopontin), sialoprotein II, and the small proteoglycans biglycan (PG I), decorin (PG II), and PG 100 (Fisher et al. 1987, Schwarz et al. 1990, Bosse et al. 1993). These proteins are not totally bone-specific, and they are expressed and distributed in a substantially divergent fashion (Bosse et al. 1990). However, they show a high affinity to the mineralization of bone matrix (Bolander et al. 1988).

We report the presence of NCPs in heterotopic ossification (HO), in pressure sores in paraplegia.

Patients and methods

Our probands, aged 23–74 years, comprised 15 paraplegic patients with traumatic transverse lesions of the cord. All suffered from recurring pressure sores with a mean diameter of 8 cm; they occurred for the first time on average 3 months after the accident. Surgical extirpation of the pressure sores was preceded by local anti-inflammatory treatment; no systemic treatment was given, for example, no bisphophonates were administered. The heterotopic ossifications in the pressure sores measured between 0.3 and 3 cm and were mostly nodular.

Tissue preparation and immunohistochemistry

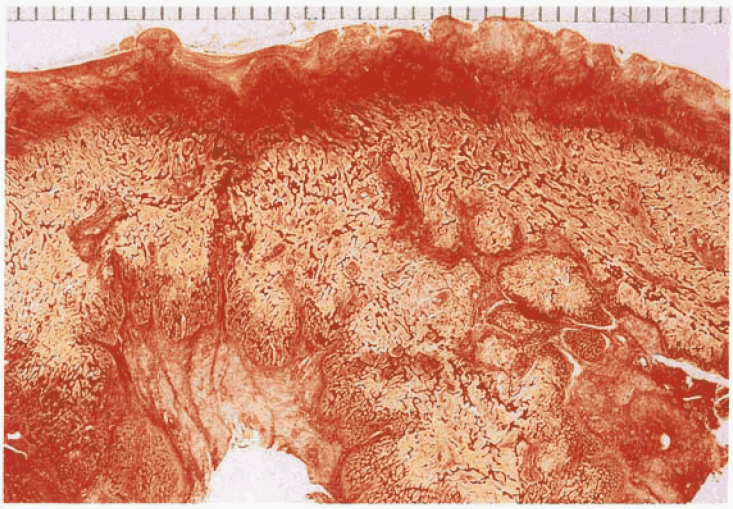
Standard tissue preparations embedded in paraffin were obtained from the pressure sores, if necessary, decalcified in 10% EDTA. 4 µm sections were placed on lysine-coated slides, dried over night at 37 °C, and deparaffined with xylol. Immunohistochemistry was performed using either the ABC method (Hsu et al. 1981) or the APAAP technique (Cordell et al. 1984); identical results were obtained with both methods, and decalcification did not influence the results. The sections were incubated overnight at 4 °C with the primary antibody after optimal antibody dilution had been tested in pilot experiments (Osteonectin 1:700, osteocalcin and BSP II 1:300, Decorin 1:100, PG 100 1:10). Negative controls were established by omitting the primary antibodies. The physis of a human fetal femur served as positive control.

Primary antibodies against osteonectin, osteocalcin, and bone sialoprotein II were kindly supplied by Drs. Termine, Gehron-Robey and Fisher of NIH, Bethesda, MD, U.S.A. Antibodies against decorin and PG 100 were those used in our previous studies (Bosse et al. 1993). All antibodies were polyclonal and raised in rabbits.

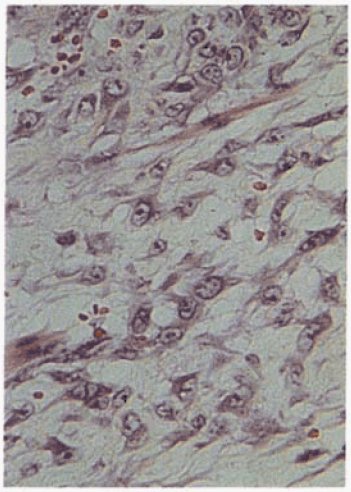
Results

The conventional histology of HO in pressure sores showed the typical zoning phenomenon with a broad zone of ossification at the periphery (Figure 1). The

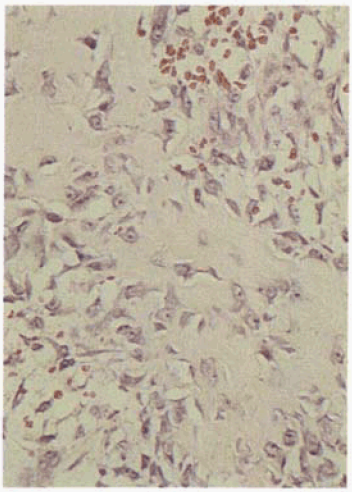
Figure 1. Cross section of heterotopic ossification with zoning phenomenon in a pressure sore



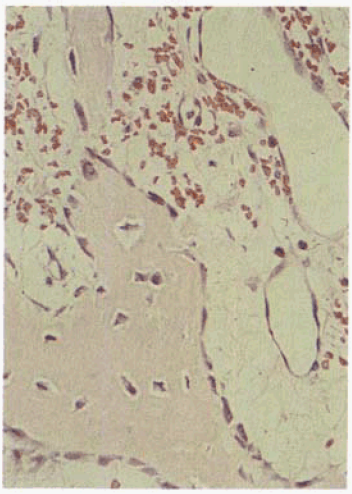
Low power view, EvG, ×3.



Marginal portion of heterotopic ossification with cellular pleomorphism, HE, ×350.



Intermediate portion with early trabeculation of ossifying areas, HE, ×350.



Mature area of bone formation with hypocellular stroma and sinuidal capillaries, HE, ×350.

fibroblastic proliferation varied in cellularity and pleomorphism. The intermediate portion showed spindle cells with early trabeculation of ossifying areas. The more mature areas were characterized by loops of trabecular bones with small osteoblasts and by a hypocellular stroma with numerous sinuidal capillaries.

Immunohistochemically the NCPs were expressed in all specimens of HO with a substantially divergent distribution pattern.

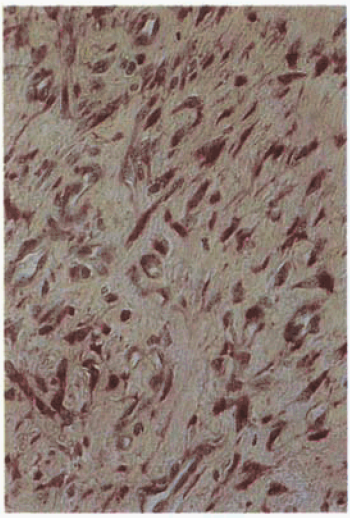
Osteonectin had a strong immunoreactivity in the vascular endothelium of granulation tissue (Figure 2) and was immediately adjacent to newly formed woven bone; at low magnification it gave the impression of a band-like seam around the trabecular board. A marked patchy immunoreactivity was observed not only in the cuboid osteoblast-like cells but also in the spindle-shaped cells located near the mineralization zones.

In mature bone the immunoreactivity was weaker and it decreased with the distance from the proliferation zone. Multinucleated giant cells were negative.

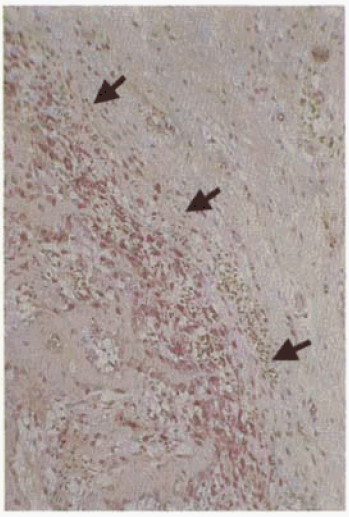
Osteocalcin was observed only in the osteoblastic rim of mature bone (Figure 3), not in the granulation tissue, nor in the osteoblastic and spindle-formed preosteoblastic cells around the newly-formed trabecular bone.

Bone sialoprotein II was predominantly found in preosteoblastic cells with granular intracytoplasmic distribution. The immediately adjacent spindle cells showed a faint immunoreactivity (Figure 4), and in the mineralized mature bone an extracellular immunoreactivity was also observed, as in the granulation tissue and in the osteoclasts.

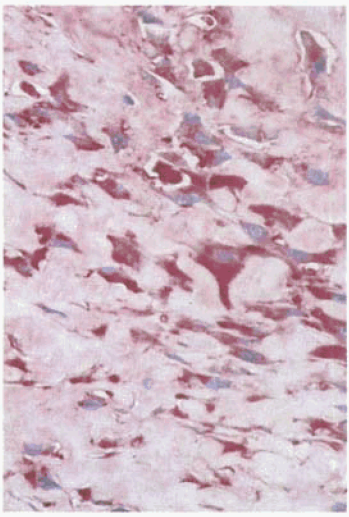
Figure 2. Immunohistochemical demonstration of osteonectin



In the granulation tissue of the ulcus, APAAP, ×350.

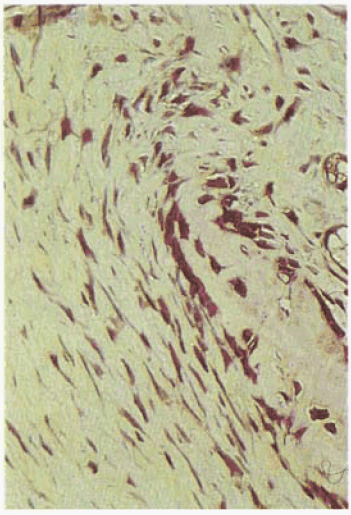


In the early trabeculation of ossifying areas with a band-like seam (arrows), APAAP, ×85.

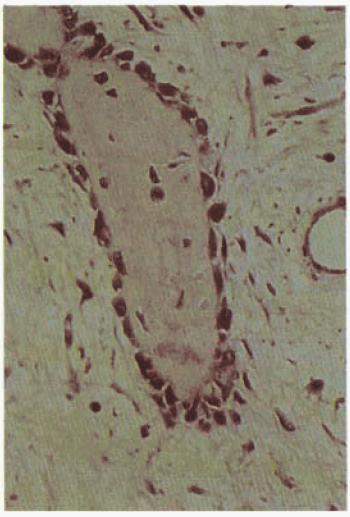


In osteoblasts and spindle-shaped cells in the ossification front, APAAP, ×430.

Figure 3. Osteocalcin-immunoreactivity



In early woven bone in osteoblasts and in the immediately adjacent fibroblastic cells, APAAP, ×350.



In mature bone only in the osteoblasts in a rim-like pattern, APAAP, ×350.

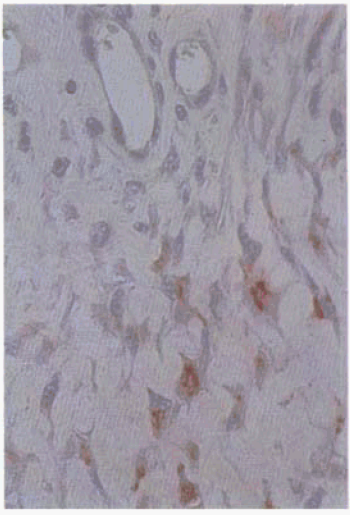


Figure 4. *Bone sialoprotein II-immunoreactivity* with a granular intracytoplasmic distribution in preosteoblasts and fibroblastic cells, APAAP, ×350.

Decorin was mostly seen in the stroma of HO (Figure 5). Fibroblast-like cells and osteoblasts in the proliferation zones were positive too, but less strongly so.

PG 100 showed a strongly positive immunostaining in the fibroblastic stroma, especially in areas near to preostoblasts and osteoblasts (Figure 6). In contrast, zones of mature bone exhibited little or no immunoreactivity in osteoblasts and osteocytes. However, osteoclasts were strongly positive, as their precursor cells were detectable as large mononuclear cells in the stroma. Decorin and PG 100 were clearly visible in the granulation tissue too.

Figure 6. Immunohistochemical demonstration of PG 100

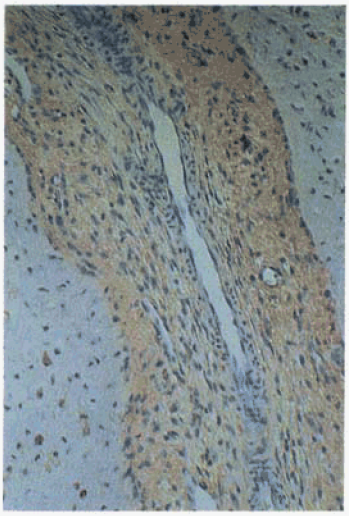
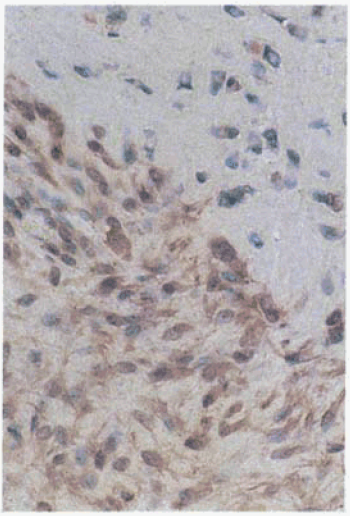
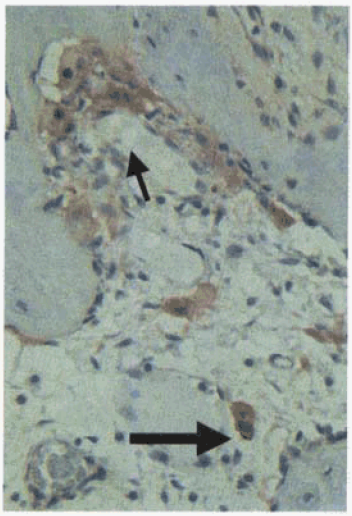


Figure 5. Immunohistochemical demonstration of *decorin* especially within the stroma, ABC, 140×.



In fibroblastic cells of the fibrous stroma next to the proliferating ossification zone, ABC, 350×.



In osteoclasts (short arrow) and osteoclast precursor cells (long arrows) in more mature stages of heterotopic ossification, ABC, 350×.

Discussion

HO has been associated with a number of different conditions, and is seen most commonly following hip surgery, neurologic injury or severe burns (Sawyer et al. 1991). It is obvious that mesenchymal cells are able to differentiate in an osteogenic direction, and that heterotopic bone undergoes systematic histologic progression from initiation to osteoid formation by calcification (Ackerman 1958). However, the mechanisms of the induction process of HO and the stimulating agents are not precisely known (Ekelund 1991).

Bone cell ontogeny has been an active area of investigation and, in recent years, increasing attention has been paid to the group of NCPs of bone. Selection of newly synthesized mineralized matrices as sources of NCPs and the use of molecular biology technique have led to an information explosion in this field (Mizuno et al. 1992). Despite detailed knowledge concerning the biochemistry and the exact function of these proteins has not yet been elucidated. In our study we have therefore focused our interest on the immunohistochemical demonstration of the NCPs in heterotopic ossification. This ectopic bone shows several properties of orthotopic bone and therefore represents an excellent model for studying bone matrix components.

Decorin and the other NCPs exhibited a substantially divergent distribution pattern. Decorin was detectable in the perivascular matrix of granulation tissue, as well as in the stroma of HO. The ossification zone stained very strongly.

In contrast, all the other NCPs were predominantly detectable intracellularly with accumulation in fibroblasts and preosteoblasts. They were found predominantly in the activity centers of early osteogenetic areas. Mature types of HO showed a more discrete expression pattern for those proteins with weaker reaction in the narrow osteoblastic rims and, apparently, markedly reduced proliferation activity. All NCPs were markedly reduced in osteoblasts, osteocytes and so-called osteolining-cells in mature forms of HO. Here PG 100 and bone sialoprotein II immunostaining predominated in osteoclasts. Our results indicate an increased expression of noncollagenous proteins as a major parameter in the pathogenesis of HO. Fibroblasts were found to be osteoprogenitor cells. The biological activity of NCPs may be correlated to the step-by-step development of HO, with a maximum in matrix-forming cells in the early stages of osteoneogenesis and a second peak for PG 100 and bone sialoprotein II in osteoclasts.

Acknowledgements

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