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Osteonectin – a differentiation marker of bone cells

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Summary. Bone matrix consists of type-I collagen and non-collagenous proteins. The latter represent only 10% of its total protein content. Since type-I collagen is also present in various other connective tissue sites (e.g., skin) it cannot be considered as bone specific. Among the non-collagenous components osteonectin – a 32 kilodalton (KD) glycoprotein linking mineral to collagen fibrils – is thought to be bone specific due to its biochemical properties. In the present study various skeletal and non-skeletal tissues were investigated for the presence of osteonectin by means of immunocytochemical methods. Two polyclonal antibodies against human and bovine osteonectin were applied. Immunocytochemically, osteonectin could be demonstrated in active osteoblasts and osteoprogenitor cells as well as in young osteocytes, while aged, quiescent osteocytes did not contain the protein, suggesting that the protein is a marker of the osteoblastic functional differentiation of bone cells. Osteonectin was absent in all non-skeletal tissues with the exception of chondrocytes in so-called mineralizing chondroid bone.

Key words: Bone matrix –Osteonectin – Osteoblasts – Immunocytochemistry – Differentiation – Human

Type-1 collagen is the principal protein component of bone matrix. Although it is the only type of collagen present it is not bone specific because it is also found in non-mineralizing connective tissue of the skin, lung, liver, eyes and tendons (Church 1981; Gay and Rhodes 1980; von der Mark 1981). Therefore, the specific quality of bone matrix as a mineralizing connective tissue is probably due to its 10% non-collagenous bone protein content. Based on this assumption a non-collagenous bone protein was extracted from fetal bovine bone that showed binding properties for collagen as well as for hydroxyapatite (the main mineral constituent of bone) and was therefore named osteonectin (Termine et al. 1981).

Since osteonectin must be regarded as a bone-specific protein according to present investigations (Termine et al. 1981) it is an obvious conclusion that it is exclusively produced by osteoblasts. Conversely, this would mean that the immunocytochemical demonstration of intracellular osteonectin speaks for an osteoblastic differentiation of the respective cells.

To check the importance of osteonectin as a cellular marker for differentiating osteoblasts, immunocytochemical studies with antibodies against human (hON) and bovine (bON) osteonectin were performed on bone matrix-forming and bone matrix-free tissues.

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Materials and methods

Antibodies were raised in rabbits immunized with bovine or human osteonectin as previously described (Termine et al. 1981). The osteonectin preparation used for immunization had been characterized as being homogeneous by gel filtration, SDS-Page and isoelectric focussing. The obtained antisera showed a single precipitation line with purified osteonectin or EDTA-Guanidin-HCl bone extracts in the Ouchterlony test (Termine et al. 1981).

Investigated tissues

Bone matrix-forming tissue obtained at surgery from 8 patients was investigated (4 × callus, 3 × reactive bone formation, 1 × myositis ossificans). After fixation in 4% buffered formaldehyde the material was embedded in paraffin. Adjacent connective tissue containing fat, blood vessels and skeletal muscle served as bone matrix-free control. The bone tissue was investigated undecalcified or after decalcification in EDTA (10%) or acidic solutions (Ossa fixona®, Röhm Pharma, Weiterstadt, FRG).

Immunocytochemical technique

All immunocytochemical studies were carried out using the PAP-technique (Sternberger 1979; van Noorden and Polak 1983) or the ABC-method (Hsu 1981) and gave identical results. The diaminobenzidine reaction was modified by addition of imidazole (0.1%, pH 7.2) according to Straus (1982). The sections were incubated overnight at 4° C with the primary antibody after optimal antibody dilution (1:1200) had been tested in preceding experiments. To reveal antigenic sites possibly masked by the preparation technique or by complex protein composition of the bone tissue, the sections were additionally treated with the enzymes trypsin (incubation for 30 min at 37° C in a solution of 100 mg trypsin and 160 mg $CaCl_2$ in 100 ml double-distilled water, pH 7.8, according to Brozman 1978), hyaluronidase

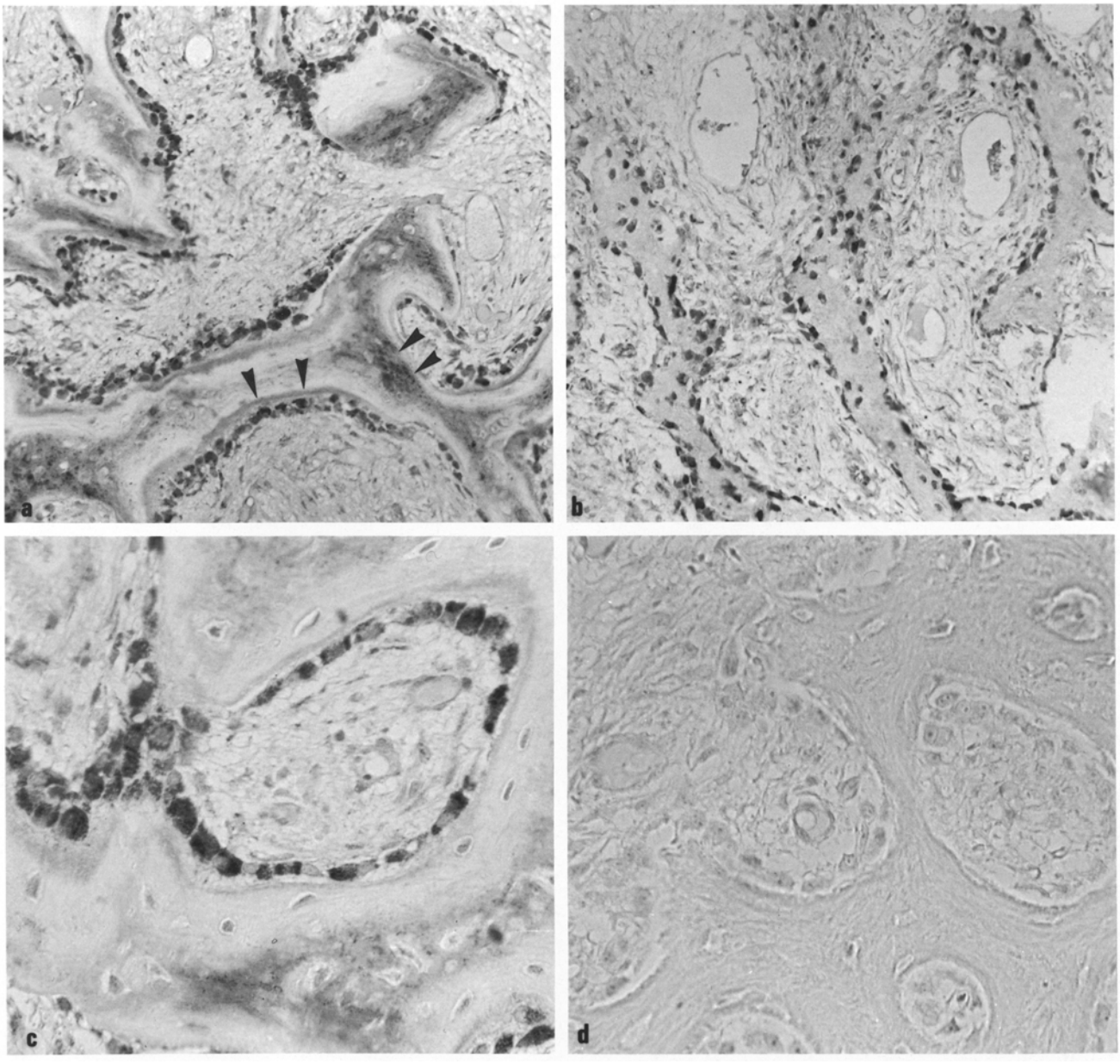

Fig. 1 a. Lamellar bone trabeculae (reactive bone formation). Positive immunocytochemical reaction of osteoblasts on bone surfaces (black-stained seams of osteoblasts). Faint staining of former mineralization fronts and centrally mineralized areas (*arrowheads*) of bone trabeculae. Anti-bovine osteonectin, PAP, DAB, no counterstaining, decalcified. ×125. **b** Woven bone of fracture callus with a positive immunocytochemical reaction of osteoblasts (on surfaces) and "walled-in" young osteocytes. Anti-bovine osteonectin, PAP, DAB, no counterstaining. ×80. **c** Positive immunocytochemical proof of osteonectin in the cytoplasm of osteoblasts forming seams on trabecular bone surfaces of lamellar bone. Faintly stained bone matrix at the former mineralization fronts as well as in the centrally located mineralized areas. Anti-human osteonectin, PAP, DAB decalcified, no counterstaining. ×310. **d** Negative control with non-immunserum. No staining of osteoblasts and bone matrix. PAP, DAB, decalcified, no counterstaining. ×310

(incubation for 30 min at 37° C, 30 IU/section, according to Tung et al. 1985) and collagenase (incubation for 30 min at 37° C in a solution of 0.25 collagenase in PBS, modified after Paul 1979).

Results

Bone matrix-forming tissue

Callus and myositis ossificans structures consist mainly of trabecular, sometimes centrally mineralized osteoid with osteoblast seams on the surface and densely packed osteocytes. An intense granular reaction of the cytoplasm of the osteoblasts was seen with both antibodies (Fig. 1). The cytoplasmic reaction of the osteocytes was of varying intensity and seemed to correlate with cell size and cell location. "Young" osteocytes situated close to the osteoid trabecular surface in large, round lacunae showed a stronger antibody binding than centrally located, smaller osteocytes in spindle-shaped lacunae where marking by the osteonectin antibodies was weak or absent (Fig. 2).

Outside of the bone and adjacent to the osteoblast

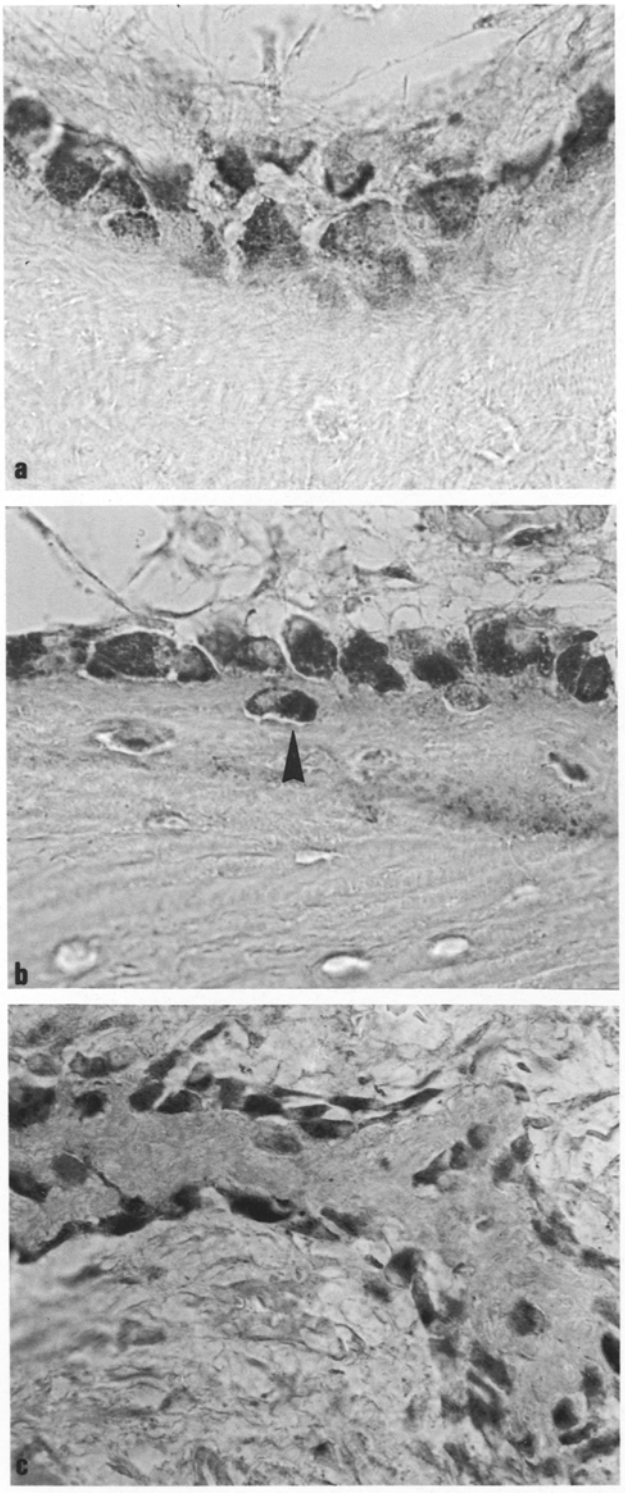

Fig. 2 a. Osteonectin in the cytoplasm of active, matrix-producing osteoblasts as well as in the above-located preosteoblasts. No staining of quiescent osteoblasts in the deeper bone. Anti-human osteonectin, PAP, DAB, decalcified, no counterstaining. × 500. **b** Positive immunocytochemical proof of osteonectin in active osteoblasts and young osteocytes (*arrowheads*) in the osteoid seam of lamellar bone. Anti-human osteonectin, PAP, DAB, decalcified, no counterstaining. × 500. **c** Osteonectin in the cytoplasm of osteoblasts and "walled-in" osteocytes of woven bone trabeculae. Anti-human osteonectin, PAP, DAB, decalcified, no counterstaining. × 500

seams, spindle-shaped cells with the morphological appearance of fibroblasts were found to be marked. The intensity of the marking in the cytoplasm of these cells decreased according to their distance from the osteoblast seams. Fibroblasts located in the center of the spongy "medullary spaces" remained unmarked (Fig. 3).

Formation of osteoid matrix in the form of homogeneous deposits of an intercellular substance between rounding cells was especially observed in early-stage callus tissue. This "fresh" matrix formation was also accompanied by an intense antibody reaction in the respective cells (Fig. 3).

Tissues free of bone matrix

Cartilage. Chondrocytes and cartilagenous intercellular substance failed to show a positive reaction with osteonectin antibodies with the exception of the transitional zones between chondroid and osteoid tissue, which is called chondroid bone (Beresford 1981). In these zones, where the chondroid matrix exhibits a homogeneous condensation already in hematoxylin-eosin-stained sections, the chondrocytes revealed a distinct and sometimes very intense granular reaction of their cytoplasm. After trypsination, osteonectin marking was also apparent in the chondroid matrix of these transitional zones (Fig. 4).

Other tissues. Interstitial connective tissue from various body regions, adipose tissue as well as smooth and skeletal muscle were additionally investigated as bone matrix-free controls. Here, the reactions with osteonectin antibodies were completely negative, in the cells as well as in the intercellular spaces (Table 1).

Discussion

Because of its ubiquitous presence in the body, type-I collagen cannot account for the specific physicochemical properties of the bone matrix, especially not for its affinity for mineralization. The study of non-collagenous bone proteins therefore suggested itself in an attempt to find an explanation for this phenomenon. A number of non-collagenous bone proteins has been isolated in the meantime, and their chemical structure has been partly elucidated (Table 2). Their functional importance, however, is still largely unknown. One of these proteins, a 32 KD-glycoprotein, was found to have a strong affinity for calcium hydroxyapatite molecules as well as for collagen fibrils, thus linking the two structures. For this reason it was given the name osteonectin. Its probable function is to maintain bone structure by contributing to the stabilization of the hydroxyapatite molecule and to its organization at the fibrillar collagen matrix (Otsuka et al. 1984; Fisher and Termine 1985).

As far as the bone specificity of osteonectin is concerned it is not important whether it induces mineralization of the bone matrix alone or in combination with other proteins. The decisive question is whether it is present exclusively in bone matrix or bone cells. Biochemical studies of connective tissue from sites remote from the skeleton (e.g., skin) have not led to the extraction of a protein with similar properties (Otsuka et al. 1984; Fisher and Termine 1985). Our immunocytochemical investigations have confirmed these results in all non-osteogenic tissues. The fact that osteonectin was demonstrable in all cells identified as osteo-

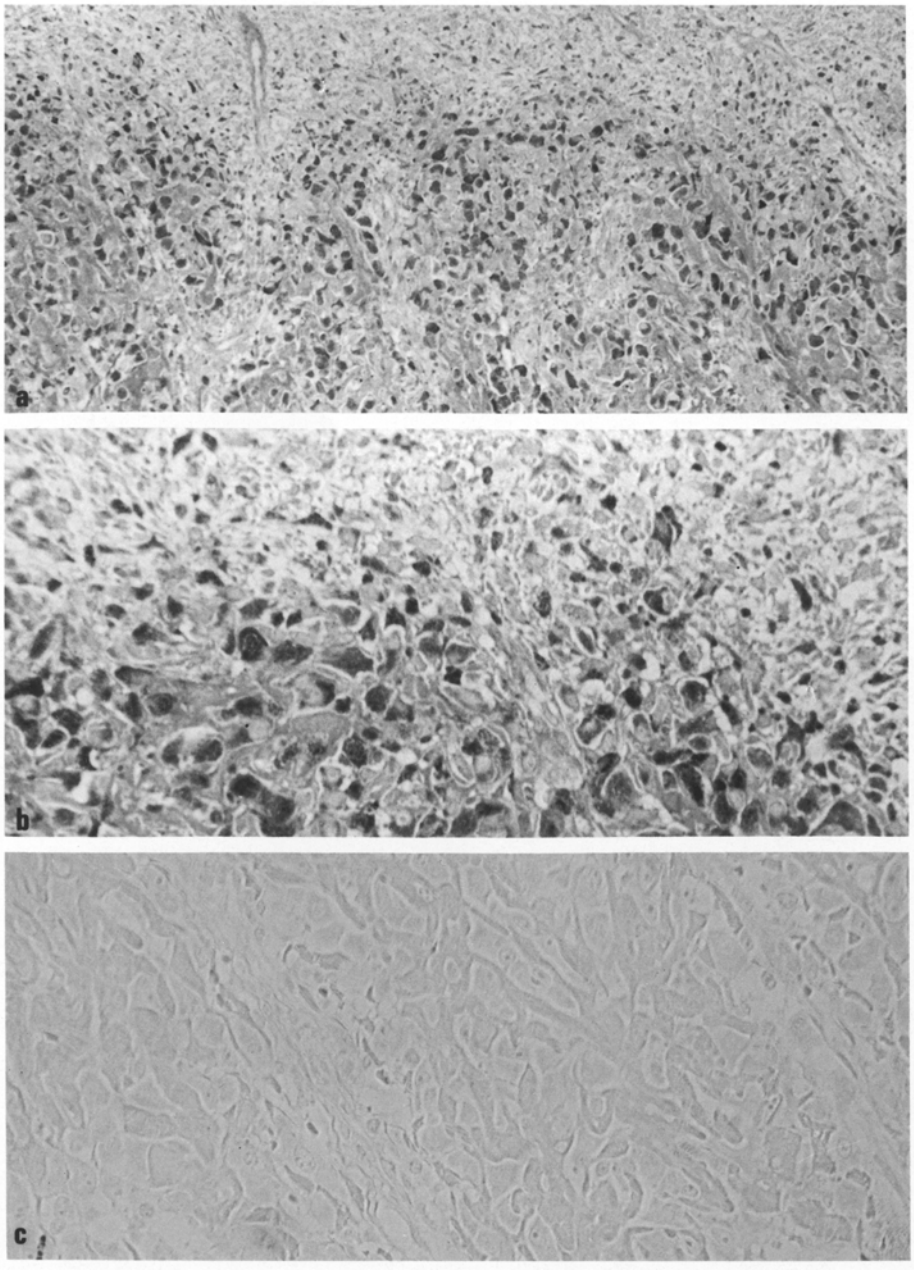

Fig. 3. a Immature osteoblastic tissue of fracture callus. Early stage of matrix production by differentiation of osteoblasts from osteoprogenitor cells. Increasing maturation from top to bottom with strongly positive reaction by osteonectin antibodies. Anti-bovine osteonectin, PAP, DAB, no counterstaining. × 80.
b Detail of immature fracture callus with strongly positive-reacting osteoblasts and moderate marking of newly formed osteoid deposits between the cells. Anti-bovine osteonectin, PAP, DAB, no counterstaining. × 310.
c Negative control: no reaction of either osteoblasts or osteoid in the same area (as above) of immature fracture callus. Non-immunserum, PAP, DAB, no counterstaining. × 310

blasts already by staining with hematoxylin and eosin is to be considered as a positive result. The cytoplasmic labeling of osteoblasts in woven and lamellar bone was partly very intense with both antibodies, thus confirming the expectation that osteonectin as a bone-specific protein is produced by osteoblasts. Based on the same assumption, demonstration of non-collagenous bone proteins (osteonectin, bone proteoglycan, bone sialoprotein) was recently used to identify osteoblastic properties of cultured bone cells (Whitson et al. 1984).

Another observation was, however, that osteonectin is not generally present in the bone cell cytoplasm but is apparently produced in dependence on function. This is suggested by the fact that osteonectin was found solely in "walled-in" osteocytes of the bone matrix, whereas osteocytes in small, spindle-shaped lacunae at some distance from the trabecular bone surface did not show any cytoplasmic marking. This finding corresponds in its functional aspect to earlier electron-microscopic observations that large young osteocytes have a higher organelle content (rER, Golgi fields) than mature "old" osteocytes (Schulz et al. 1974).

Accordingly, osteonectin is present in bone cells (osteoblasts, osteocytes) if active matrix synthesis takes place. The finding that reactivity with osteonectin antibodies was also observed in cells of fibroblastic appearance seems to call for a different interpretation. These spindle-shaped cells were always seen in close proximity to an osteoblastic seam. This topographic relation to active matrix-producing osteoblasts and the decreasing intensity of osteonectin reactivity with increasing distance from the bone surface leads to the assumption that these spindle-shaped cells are newly recruited precursor cells of osteoblasts. They probably represent an intermediate stage between the so-called "deter-

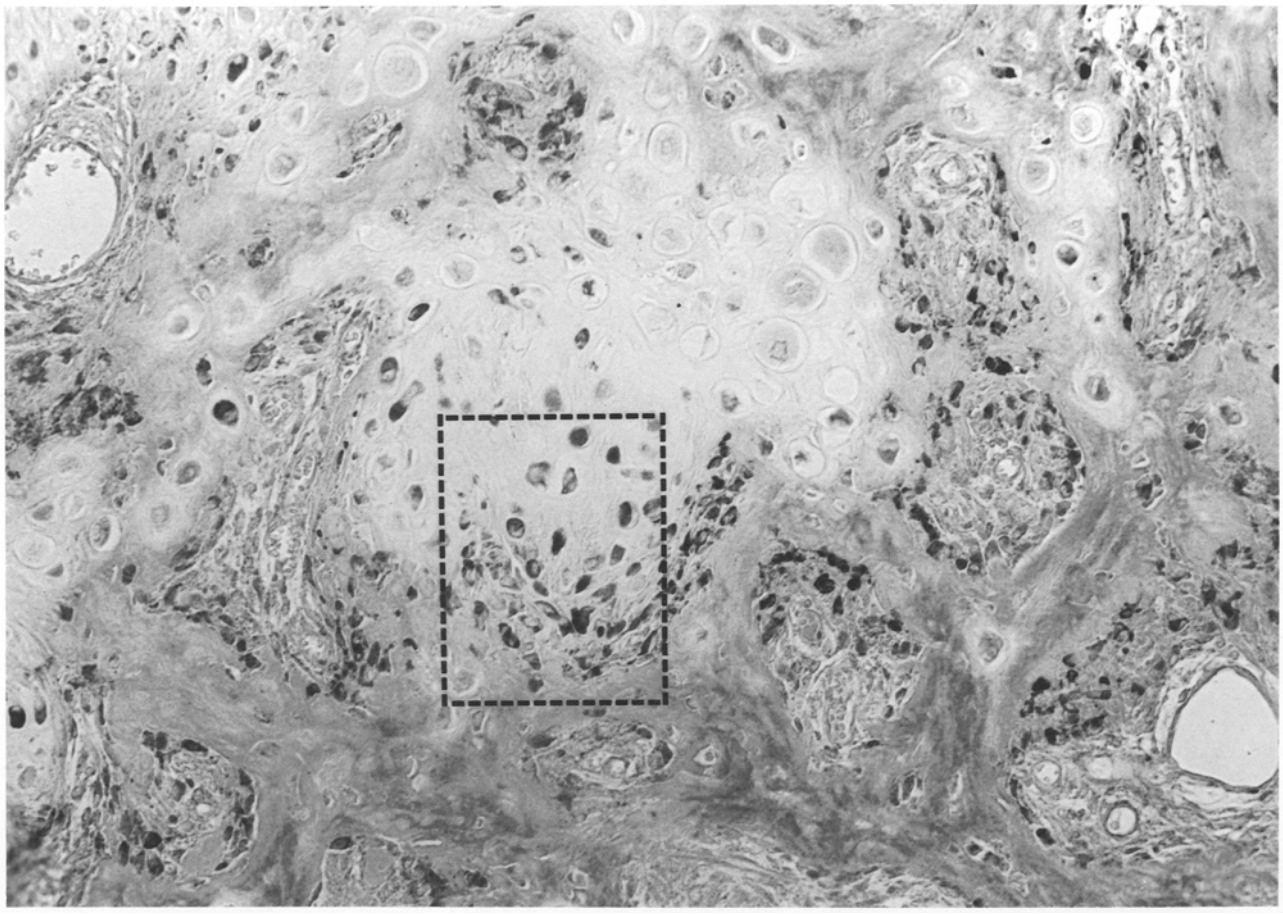

Fig. 4. Fracture callus with cartilage (*center*), chondroid bone (*encircled*) and woven bone (*bottom*). Centrally located chondrocytes do not react with anti-osteonectin, while chondrocytes in the area of chondroid bone adjacent to woven bone structures are strongly stained to the same intensity as osteoblasts of woven bone trabecular (lower part at the right and left). Cartilagenous matrix is negative, while woven bone matrix shows a distinct reaction. Anti-bovine osteonectin, PAP, DAB, no counterstaining. ×125

Table 1. Immunocytochemical findings in human tissues with polyclonal human and bovine osteonectin antibodies

Positive for osteonectin	Negative for osteonectin
Bone tissue	
osteoblasts	
developing osteoprogenitor cells	endosteal lining cells (non-functioning osteoprogenitor cells)
young active osteocytes	old (quiescent) osteocytes
osteoid[a]	
mineralized bone[b]	
Cartilage	
chondrocytes of chondroid bone	chondroblasts, chondrocytes
mineralizing chondroid bone matrix[a]	cartilagenous matrix
	non-skeletal tissues
	stromal connective tissue
	fibroblasts
	collagenous intercellular substance
	smooth muscle tissue
	striated muscle tissue
	fat cells
	nerves

[a] Reaction intensified after trypsination of paraffin-embedded tissue;
[b] reaction positive after demineralization and trypsination

mined osteoprogenitor cells" (Friedenstein 1973) and already completely developed active osteoblasts.

Therefore the demonstration of osteonectin can reveal the gradual osteogenic differentiation of cells that do not show this property on purely histological examination. This statement, however, is restricted by the fact that certain chondrocytes also reacted with osteonectin antibodies, but this occurred only in regions of chondroid tissue (especially in callus) showing a direct transition to bone matrix (osteoid). Previous investigations have shown that these transitional zones are also heterogeneous with regard to their collagen content. Cartilage-specific type-II collagen is present in combination with type-I collagen, so that the term "chondro-osteoid" was coined for these zones (Beresford 1981). They are able to mineralize like the enchondral ossification zones of the epiphyses, thus permitting conclusions as to the importance of osteonectin for tissue mineralization. In the extracellular matrix osteonectin can also be demonstrated by immunocytochemical methods. This applies to the chondro-osteoid zones as well as to the unmineralized osteoid of the bone trabeculae. However, immunoreactivity was only found or intensified after the histological sections had been pretreated with proteases (trypsin). This well-established immunocytochemical technique (Brozman 1978) reveals hidden antigenicity caused by fixation. Treatment with hyaluronidase or collagenase did not lead to

Table 2. Non-collagenous proteins of bone

Protein	MW	Part (%)	Function	References
Glycoproteins				
Glycoprotein	24 KD	—	—	Termine et al. 1981
Osteonectin	32 KD	25	linking collagen and hydroxyapatite	Termine et al. 1981
Glycoprotein	62 KD	—	—	Termine et al. 1981
Bone Sialo-Protein (BSP)	70–80 KD	24	—	Fisher et al. 1983b Herring et al. 1977 Quelch et al. 1984
Osteocalcin	6 KD	25	binding to hydroxyapatite, coupling factor between osteoblasts and osteoclasts	Price et al. 1976 Price and Bankol 1981
α_2-HS-Glyco-protein[a]		1–9	—	Quelch et al. 1984
Proteoglycans[a] protein core	38 KD	0,1	—	Fisher et al. 1983a
Chondroitin-sulfate	40 KD		—	
Bone Morphogenic Protein (BMP)			inductive effect on osteoprogenitor cells	Nakagawa and Urist 1977

[a] Higher concentrations in immature (neonatal) bones than in adult bone matrix according to references

better antibody-binding, suggesting that proteoglycans or collagen do not mask the presence of osteonectin in osteoid.

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