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Immunocytochemical identification of osteogenic bone tumors by osteonectin antibodies

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Summary. 18 bone-forming tumours and tumour-like lesions were investigated immunocytochemically for the presence of osteonectin. A group of non-bone-forming skeletal tumours (five cartilage-forming tumours, four Ewing sarcomas and five extraskeletal sarcomas) served as controls. The studies showed that osteonectin antibodies react reliably with benign and malignant bone-forming tumours (two cases of fibrous dysplasia, three osteoid osteomas, 13 osteosarcomas). This finding was supported by protein blot studies. Osteonectin is formed by cells which do not yet possess the morphological phenotype of osteoblasts and may be regarded as a "differentiation marker" of the osteoblastic lineage. Only chondroid bone (tissue in which chondrocytes were surrounded by osteoid matrix containing type I and type II collagen) showed a positive reaction. All other primary skeletal tumours and extraskeletal soft tissue tumours were completely negative.

Key words: Bone tumors – Osteosarcoma – Osteonectin – Immunocytochemistry

Introduction

Approximately 90% of the total protein of bone consist of type I collagen. Although this is the only collagen type present in bone, it cannot be regarded as a bone-specific marker because it is also found in other tissues (von der Mark 1981). The specific properties of bone are therefore modified by its other, non-collagenous protein components. One of these proteins is osteonectin (Termine et al. 1981). It possesses a high affinity for hydroxyapatite and type I collagen, and one of its functions may be to connect the two molecules. Previous immunohistochemical studies have shown that osteonectin is a marker for the differentiation of bone matrix forming cells (osteoblasts) (Jundt et al. 1987; Bianco et al. 1988). From these results, the question arises of whether osteonectin is also produced by the cells of bone tumours, and whether it might be used as a marker for this tumour group.

To elucidate this question, immunocytochemical studies were performed to determine the presence of osteonectin in benign and malignant bone tumours. Mesenchymal tumours without bone formation served as controls. To ensure that cellular immunoreactivity was due to the presence of osteonectin, we studied extracts of osteosarcoma tissue by immunodetection of the protein on western blots following SDS polyacrylamide gel electrophoresis (SDS-PAGE).

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Material and methods

Two cases of fibrous dysplasia, three osteoid osteomas and 13 osteosarcomas were studied. Other primary skeletal tumours investigated were one chondromyxoid fibroma, one chondroblastoma, three chondrosarcomas, and four Ewing sarcomas. The group of soft tissue tumours included two fibrosarcomas, one rhabdomyosarcoma, one leiomyosarcoma and one meningeal sarcoma (Table 1).

Antibodies were raised in rabbits against intact osteonectin, as previously described (Termine et al. 1981). In addition, in some studies, an antibody raised against the amino terminal amino acid sequence of human osteonectin was also utilized. Antibody specificity was determined by western blots (see below) and Ouchterlony test with purified osteonectin.

For protein blot studies osteosarcoma tissue was homogenized in proteinase-inhibiting buffer (Termine et al. 1981), ultracentrifuged ($70000 \times g$) and the supernatant fraction lyophilized. Aliquots of these cell supernatants, conditioned medium from normal bone cells (Gehron Robey and Termine 1985) and guanidine HCl-EDTA extracts of normal human bone (Termine et al. 1981) were electrophoresed on SDS 4–20% polyacrylamide gradient gels, and separated polypeptides were elec-

Table 1. Investigated tumors

Diagnosis	No of cases	positive	negative
1. Osteosarcoma	13	13	0
2. Osteoid-Osteoma	3	3	0
3. Fibrous dysplasia	2	2	0
4. Chondroblastoma	1	0[a]	1
5. Chondromyxoidfibroma	1	0[a]	1
6. Chondrosarcoma	3	0[a]	3
7. Ewing-sarcoma	4	0	4
8. Fibrosarcoma	2	0	2
9. Rhabdomyosarcoma	1	0	1
10. Leimyosarcoma	1	0	1
11. Meningeal sarcoma	1	0	1

[a] chondroosteoid and intimately associated cells (chondroid bone) were positive

trotransferred to nitrocellulose. The resulting protein blot was incubated with a polyclonal antibody raised against a synthetic peptide coded for the amino terminal sequence of human osteonectin or with a polyclonal antiserum raised against the entire osteonectin molecule. Proteins reactive with these antibodies were localized by reaction with a peroxidase-labeled goat anti-rabbit IgG antibody.

All studies in immunocytochemistry were performed on formalin-fixed material (4% buffered formalin) after embedding in paraffin. If necessary, the specimens were decalcified in EDTA solution (10%) or acids (Ossafixona by Röhm Pharma, Weinheim, FRG). Either the PAP-technique (Sternberger 1979; van Noorden and Polak 1983) or the ABC-method (Hsu et al. 1984) were used. Identical results were obtained with both methods. Prior treatment of the slides with enzymes (Brozman 1978) had no influence on the results.

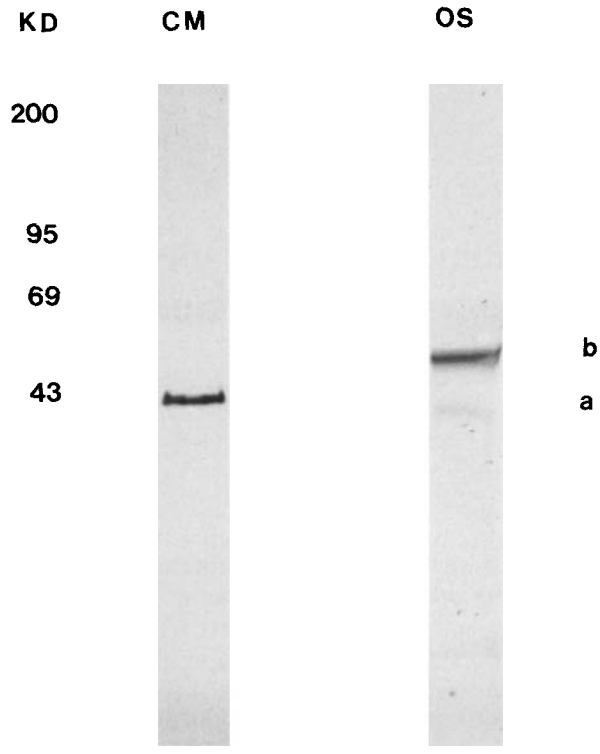

Fig. 1. Protein blot experiments (*CM*=conditioned medium of fetal human bone cells; *OS*=osteosarcoma cytosol): Strongly positive reaction in *CM* and *OS* using a polyclonal antibody against the entire osteonectin molecule with different molecular weight reaction products (*a*+*b*) in CM and OS samples. The sequence-specific antibody produced a similar reaction product that, however, was very faint in the OS-sample (data not shown)

Results

When the sequence-specific (osteonectin peptide) antibody preparation was used, a single polypeptide in samples of conditioned medium taken from fetal human osteoblast cell cultures and from human mineralized bone matrix, reacted strongly. The osteonectin present in these samples had an apparent molecular weight of 43000 daltons in this gel electrophoresis system. A diffuse band in the osteosarcoma sample also was slightly positive. However, its molecular weight was distinctly and reproducibly larger than in the cell culture and tissue extract samples. The blot was then washed and re-used with a polyclonal antibody raised against the entire osteonectin molecule. In this case, the osteonectin present in the cell culture medium and human bone extract was intensely positive with the same apparent molecular weight, and the immunoreactive material in the osteosarcoma extract also became intensely positive (Fig. 1). The biochemical basis behind the shift in apparent molecular weight in the osteosarcoma extract is not known and has not been observed in any other situation.

The two cases of fibrous dysplasia that we studied showed a distinctly positive reaction with the osteonectin antibodies. This reaction occurred chiefly in cells immediately adjacent to newly formed woven bone or cells located within the trabeculae of woven bone (Figs. 2a and b). The reaction differed in degree, sometimes covering the whole cytoplasm and sometimes showing a granular intracytoplasmic distribution. Not only did cuboidal, osteoblast-like cells react in this fashion (Fig. 2b) but also the spindle-shaped cells which were also present on the surface of the woven bone trabeculae (Fig. 2a, bottom). The intensity of osteonectin reactivity decreased with the distance of the cells from woven bone.

The three osteoid osteomas were also positive. At low magnification the reaction product gave the impression of a band-like seam around the trabeculae (Fig. 3a). Nearly all cuboidal, osteoblast cells around the newly formed trabeculae of the spongiosa revealed intense cytoplasmic staining (Fig. 3b). Extracellular osteoid matrix was only weakly stained. Positive reactions were also ob-

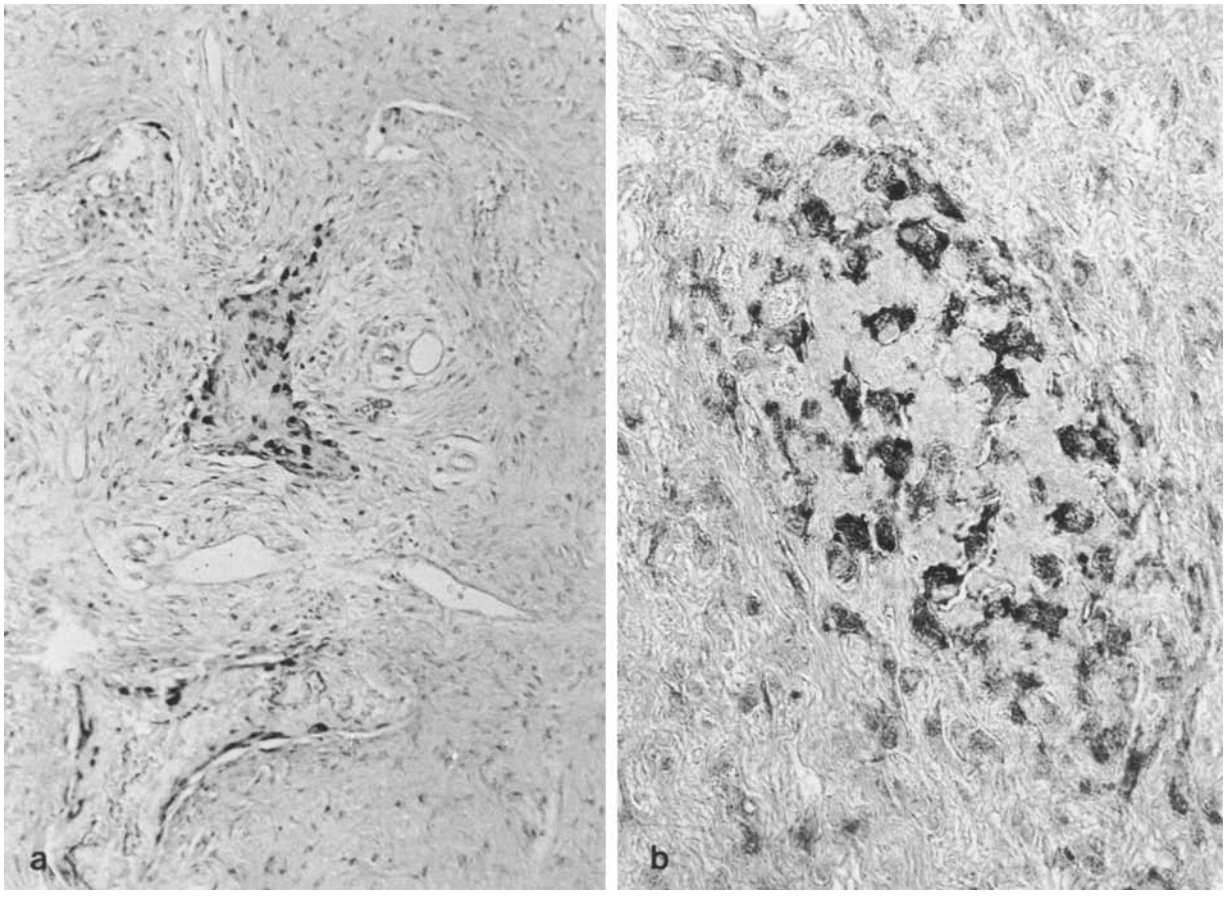

Fig. 2. Fibrous dysplasia. Positive reaction in cells of woven bone trabeculae, as well as in spindle-shaped (**a**, *bottom*) and cuboidal cells (**b**). ABC-Peroxidase, DAB, **a:** 125× **b:** 300×

served in oval to spindle-shaped cells located centrally in the nidus area, whereas the multinucleated giant cells were completely negative (Fig. 3c).

All 13 osteosarcomas were positive. The degree of the reaction differed in the tumour portions examined and correlated with the degree of matrix formation, but was also clearly demonstrable in matrix-free regions (Fig. 4a, b). The reaction was strongest in the predominantly cuboidal cells on the surface of the tumour osteoid but occasional spindle-shaped cells and bizarre pleomorphic tumour cells also reacted positively (Fig. 4c). In general, the extent and intensity of the reaction varied from 5% to more than 50% of the investigated tumour tissue. Tumour portions with chondroid differentiation were negative whereas osteoid was weakly positive.

All other skeletal tumours investigated were negative. None of the Ewing sarcomas showed a positive reaction. The chondromatous tumours were likewise negative but presented a special feature; single positively reacting cells located near the mineralization zones were seen in the areas of chondroblastoma that consisted of fibroblastic spindle-shaped cells (Fig. 5a). The immediately adjacent multinucleated osteoclastic giant cells were negative (Fig. 5b).

Chondrosarcomas revealed no marking of tumour cells. They were negative, as was the chondroid matrix. However, cartilage with a positive cytoplasmic reaction was observed in the matrix zones that displayed an osteoid-like staining behavior under the light microscope (Fig. 5c). This reaction was also found in the chondromatous portions of the osteosarcomas.

All soft tissue tumours examined were negative. These findings were also confirmed after prior treatment with enzymes.

Discussion

Osteoblasts differ from other connective tissue cells by their ability to form bone matrix. 90% of the latter consist of type I collagen, the remaining 10% of non-collagenous proteins. The bone matrix stores calcium salts in the form of hydroxyapatite,

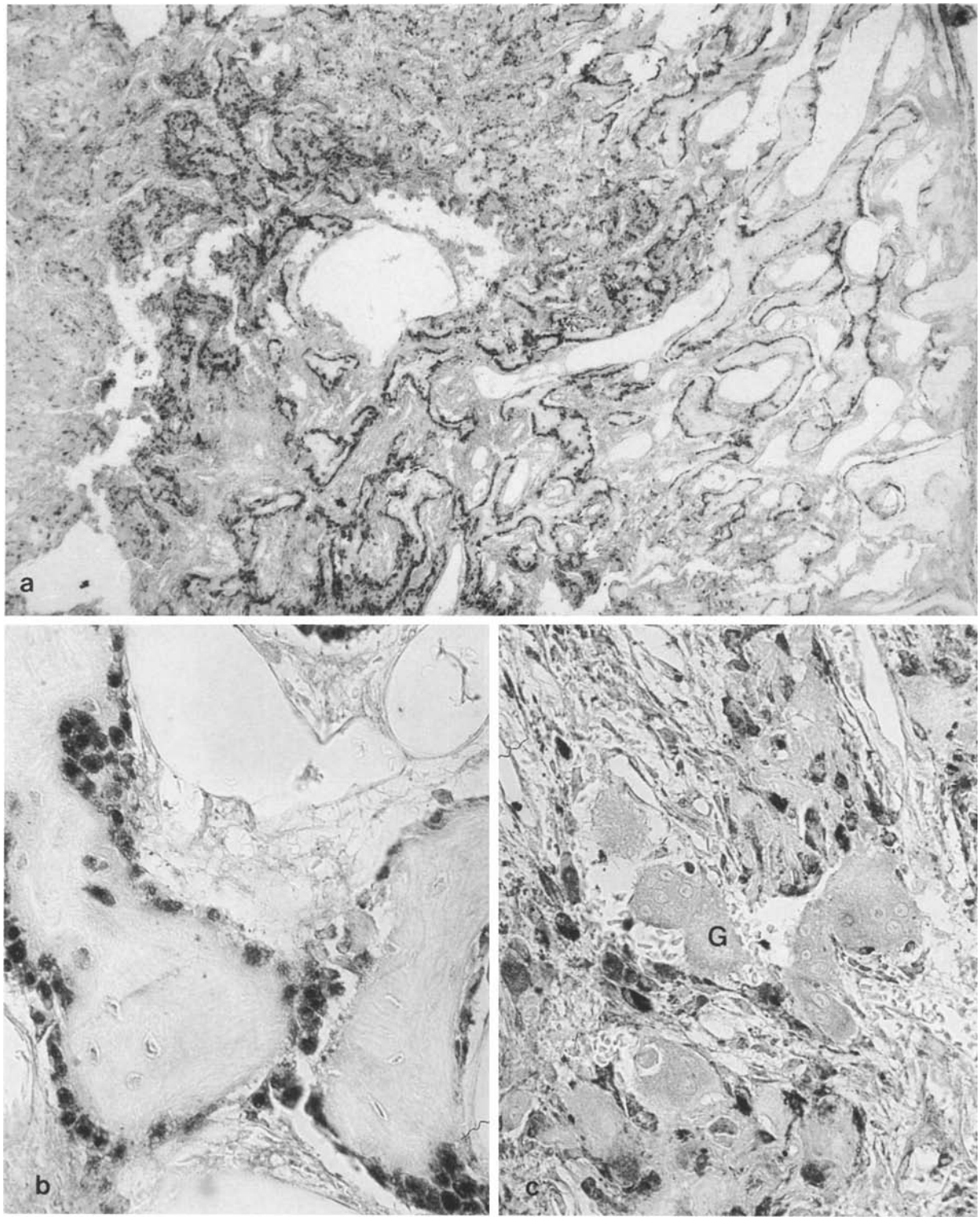

Fig. 3. Osteoid-osteoma. Seam-like positive reaction in osteoblast-like cells covering bone trabeculae (**a**). Cuboidal osteoblasts at trabeculae surfaces showing an intense cytoplasmic staining with anti-osteonectin (**b**). Centrally located spindle-shape cells of the nidus are also positive. Multinucleated giant cells (*G*) are completely negative (**c**). ABC-Peroxidase, **a:** 75×, **b+c:** 300×

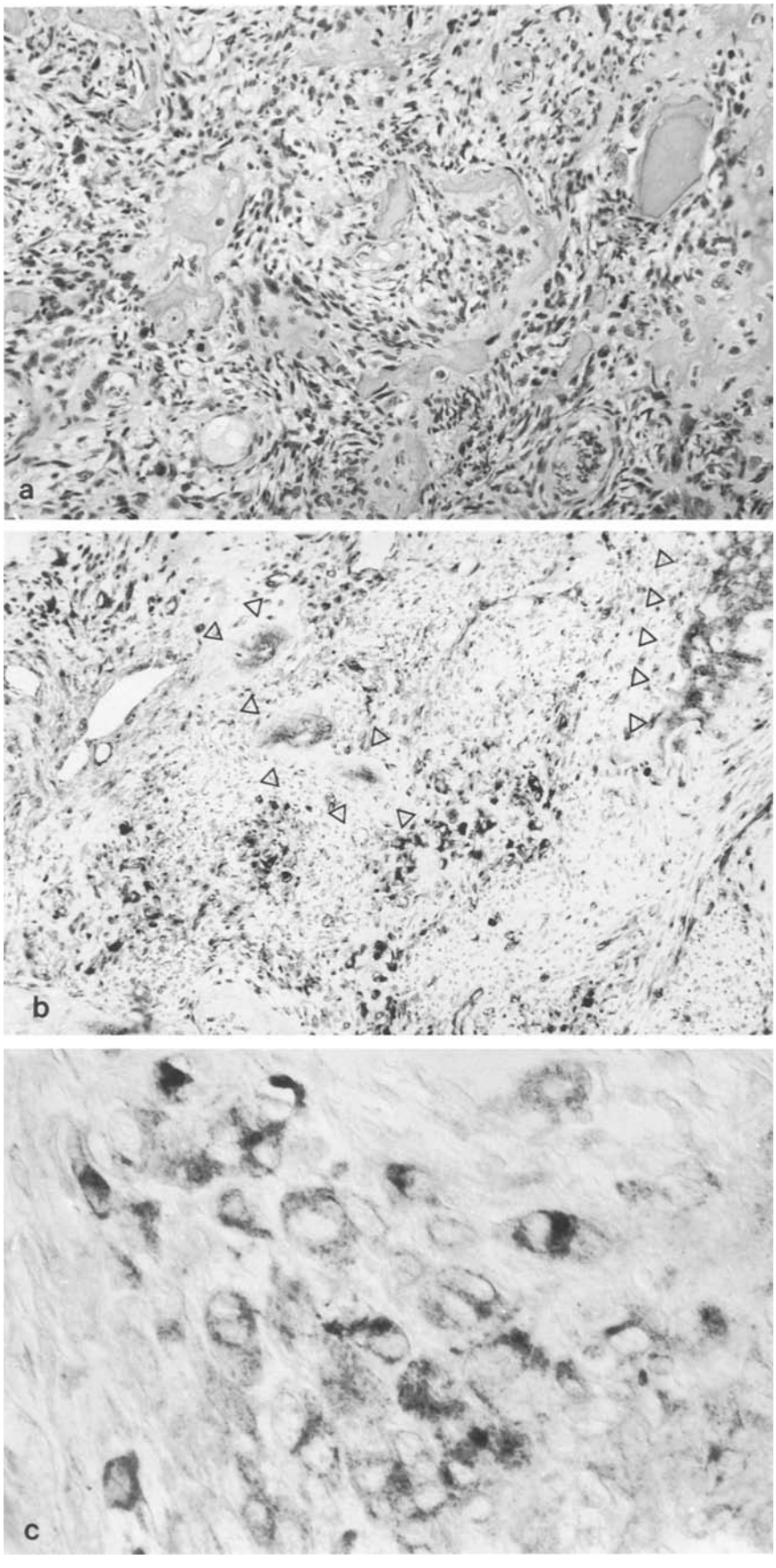

Fig. 4. (**a**) Classical osteosarcoma with tumour osteoid and both polygonal and spindle-shaped tumour cells. HE, 125×. (**b**) Positive immunoreaction in osteoblastic tumour cells with anti-osteonectin. Poorely mineralized matrix (*arrowheads*) is weakly stained. ABC-Peroxidase, DAB, 125×. (**c**) Positively reacting osteoblastic tumour cells. Anti-osteonectin, ABC-Peroxidase, DAB, 300×

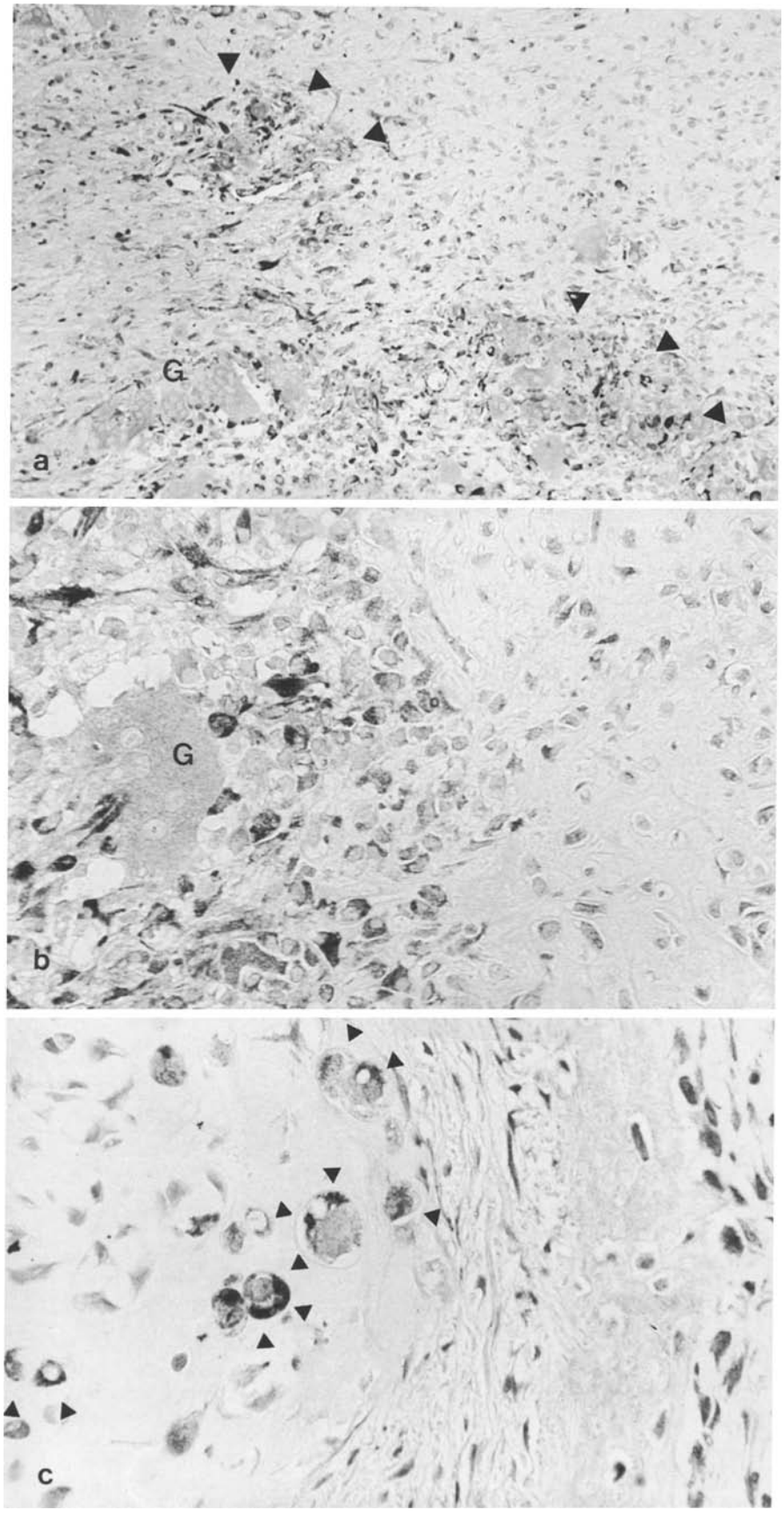

Fig. 5. (**a**) Chondroblastoma. Positively reacting spindle-shaped cells near to mineralization areas (*arrowheads*). ABC-Peroxidase, DAB, 125×. (**b**) Chondroblastoma. Multinucleated giant cells (*G*) near mineralization areas are negative. ABC-Peroxidase, DAB, 300×. (**c**) Chondrosarcoma. "Chondro-osteoid" next to reactive bone formation in chondrosarcoma. Positive reaction (*arrowheads*) in centrally located tumour cells surrounded by chondroosteoid. Tumour cells in purely chondroid matrix (*left*) are negative. ABC-Peroxidase, DAB. Counterstaining with haematoxylin, 300×

thus forming mineralized bone. With the isolation and characterization of the non-collagenous matrix proteins, it becomes possible to identify specific cellular products of osteoblasts, aside from type I collagen which is also found in many other connective tissues (Fisher and Termine 1985; von der Mark 1981).

One of the main components of the non-collagenous bone proteins is osteonectin. It has a molecular weight of 38000 daltons, is a phosphorylated glycoprotein and was isolated from bovine bone tissue in 1981 by Termine et al. Antibodies against osteonectin have meanwhile been successfully used for the characterization of bovine and human bone cells cultures (Gehron Robey and Termine 1985; Whitson et al. 1984) for the study of dental tissue (Tung et al. 1985) and for fetal bone as well as reactive bone lesions (Jundt et al. 1987; Bianco et al. 1988). In this context it has been shown that the capability for osteonectin synthesis already exists in cells which by purely morphological definitions are not yet osteoblasts but can be regarded as osteoprogenitor cells (Friedenstein 1973).

Even under pathological conditions, osteoblasts usually retain their ability to form osteoid. This permits their definite morphological identification. But if this ability is incompletely developed, as may be the case in malignant bone tumours, it can be very difficult to classify a tumour as being an osteosarcoma, because other areas in the lesion may mimic the picture of other mesenchymal tumours such as fibrosarcoma, malignant fibrous histiocytoma, synovial sarcoma, or chondrosarcoma. This problem arises in particular if the diagnosis must be established on the basis of a biopsy, as has recently been proposed in some therapeutic plans (Rosen et al. 1982; Winkler et al. 1984). It would therefore be helpful to have other non morphological criteria that might demonstrate the osteoblastic origin of a tumour.

Immunohistochemical studies with antibodies against type I collagen have shown that this collagen type is demonstrable in tumours other than osteosarcoma. In addition, osteosarcoma cells seem not only to lose their ability to produce osteoid but also the ability to synthesize type I collagen. In fibrosarcomatous areas they may react only with antibodies against type III collagen (Remberger and Gay 1977; Roessner et al. 1983). Attempts to produce antibodies against osteosarcoma cells did result in antibodies, but these reacted not only with osteosarcomas but also with other soft tissue tumours (Bruland et al. 1986). Antibodies against non-collagenous bone proteins such as osteonectin may not pose such problems because in vivo they are predominantly demonstrated in osteoblastic cells (Jundt et al. 1987; Otsuka et al. 1984; Termine et al. 1981; Whitson et al. 1984).

However, recent investigations have shown that proteins whose amino acid sequences are highly similar to those of osteonectin are found outside bone. These include a platelet-associated protein (Stenner et al. 1986), a protein found in cultivated endothelial bovine cells (Sage et al. 1986), a basement membrane protein (BM 40) identified in the mouse EHS-tumour (Dziadek et al. 1986) and a protein named SPARC that is secreted by embryonic mouse parietal endodermal cells (Mason et al. 1986). Comparing the amino-acid sequences of SPARC and osteonectin, differences are found at the N-terminal region. Recent results of DNA-analysis indicate that at the DNA-level this variable region is encoded by one exon (Findlay et al. 1988). The functional aspects of this finding remain to be clarified.

Distribution of osteonectin was investigated in various tissues at the m-RNA and protein level. Osteonectin m-RNA was demonstrated in osteoblasts, in osteosarcomas and chondrosarcomas of rat and mouse as well as in human and chicken fibroblasts (Young et al. 1986). However, in fetal bovine tissue at the protein level, a 500- to 1000-fold higher concentration of osteonectin was found in bone than in other tissues using a sensitive radioimmunoassay system (Gehron Robey et al. 1987).

These differences in protein concentration probably explain that osteonectin can only be demonstrated immunocytochemically in specialized cells (osteoblasts and osteoprogenitor cells) of human tissue (Jundt et al. 1987; Bianco et al. 1988). These results prompted us to investigate whether osteonectin antibodies might also be suitable markers for benign and malignant osteoblastic tumor cells.

Our results show that all the osteosarcomas we studied reacted with the osteonectin antibody. That this immunoreactivity was due to the presence of osteonectin was suggested by data from western (protein) blots. A positive immunocytochemical reaction was not only found in the osteoid-producing parts of the tumour but also in the fibrosarcomatous and pleomorphic areas. All soft tissue tumours of similar morphological structure but different histogenesis were negative. There were two exceptions, however, which concerned the cartilage tumours. The chondroblastoma and some of the chondrosarcomas displayed areas with foci of positive osteonectin reaction. These areas

showed also light microscopic peculiarities. In the chondroblastoma, spindle-shaped and oval cells in the neighborhood of mineralized areas were positive. The same feature has been observed in reactive bone lesion (callus) in morphologically similar cells which are located in the neighborhood of newly formed trabeculae and are probably determined osteoprogenitor cells (DOPC) (Friedenstein 1973). Since chondroblastomas often show mineralized areas and sometimes also formation of new bone (Dahlin and Unni 1986; Gravanis and Giansanti 1971) it may be assumed that the spindle-shaped oval cells that reacted positively with anti-osteonectin are DOPC-like cells. In the chondrosarcomas, chondrocyte-like tumour cells surrounded by an osteoid-like matrix were seen on light microscopy. It was only in those areas in which the transition of chondrosarcoma tissue to mineralizing "chondroid" bone occurs (Beresford 1981) that an occasionally well defined osteonectin marking was observed. This indicates a switch-over of cellular metabolism to the production of bone matrix proteins, similar to the process of enchondral ossification. This "switch-over" can also be proven by the fact that a combination of cartilage-specific type II collagen and bone-typical type I collagen occurs in chondroid bone (Beresford 1981).

Despite these two exceptions it can be stated that the demonstration of osteonectin in tumours has proved to be a valuable factor in the diagnosis of bone-forming tumors, in particular of osteosarcomas. In addition, osteonectin has been detected in pleomorphic and fibroblastic subtypes of osteosarcomas (Schulz et al. 1988). In combination with the light microscopic findings, a positive osteonectin reaction makes it possible for the pathologist to classify a tumour as an osteosarcoma even if only a small amount of material is available. However, the purely chondroid component of an osteosarcoma cannot be distinguished immunocytochemically from a chondrosarcoma by means of the osteonectin antibody, so that this differential diagnostic decision must still be based on the well-known clinical, pathological and radiological aspects of a tumour (Dahlin and Unni 1986; Huvos 1979; Schajowicz 1981).

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Note added in proof:

Meanwhile osteonectin/SPARC/BM-40 immunoreactivity was demonstrated in decidua and some carcinomas.
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