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Immunohistochemical Study of Osteonectin in Various Types of Osteosarcoma

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Polyclonal antibodies against osteonectin, a 32 kd noncollagenous bone protein, were applied for the histogenetic identification of variously differentiated osteosarcoma tissues. A strong positive reaction was found in matrix-producing osteosarcoma cells of the osteoblastic type, but pleomorphic or fibrosarcomatous osteosarcoma tissues reacted focally positive as well. Because the production of osteonectin depends on the osteoblastlike function of the individual tumor cell, a homogenous immunocytochemical staining of all tumor cells cannot be expected. Nevertheless, the immunocytochemical demonstration of osteonectin in osteolytic tumors that produce no or scarcely any matrix seems to be a valuable tool for establishment of their osteogenic origin. (Am J Pathol 1988, 132:233–238)

OSTEONECTIN IS A bone-specific, phosphorylated glycoprotein with a molecular weight of 32.000 kd.[1] It accounts for approximately one fourth of all noncollagenous bone proteins, which in turn make up 10% of the total protein content of the bone matrix.

The name osteonectin is derived from the high affinity of this protein for the collagenous bony constituents of the matrix and for the mineral hydroxyapatite. Osteonectin is produced by the osteoblast and has been demonstrated immunocytochemically in active, bone-matrix-producing cells (osteoblasts and young osteocytes) but not in dormant osteocytes or in inactive endosteal superficial cells.[2] For this reason, osteonectin can be regarded as a marker for the differentiation of bone-forming cells, and it was therefore logical to test its marker characteristics on bone tumor cells also.[3]

Osteosarcoma, the malignant variant of the osteoblastic lineage, is of particular interest in this context. Depending on their histologic pattern and their matrix production, osteosarcomas are subdivided into the following types: osteoblastic, fibroblastic, chondroblastic, anaplastic, small cell, and teleangiectatic.[4–6] If osteoid production by the tumor cells can be demonstrated histologically, there will be no problem in definitely classifying the respective tumor as an osteosarcoma. Diagnosis becomes difficult, however, if the tumor shows no matrix production, or if matrix production is scanty and hard to recognize. In such cases the immunohistochemical demonstration of osteonectin would be an important and helpful tool for establishing the definite diagnosis of osteosarcoma. To elucidate whether osteonectin immunohistochemistry would meet this requirement, this study investigated a number of osteosarcomas of varying degrees of differentiation.

Material and Methods

The antibodies were produced by the immunization of rabbits with bovine and human osteonectin, as recently described. The antisera showed a singular precipitation line with osteonectin or EDTA-guanidine-HCL bone extracts in the Ouchterlony test.[1]

Tumors Investigated

Osteosarcomas

Thirteen osteosarcomas in various stages of differentiation were studied (Table 1). The tumors were subtyped according to their main component as osteoblastic, fibroblastic, and anaplastic. The relative

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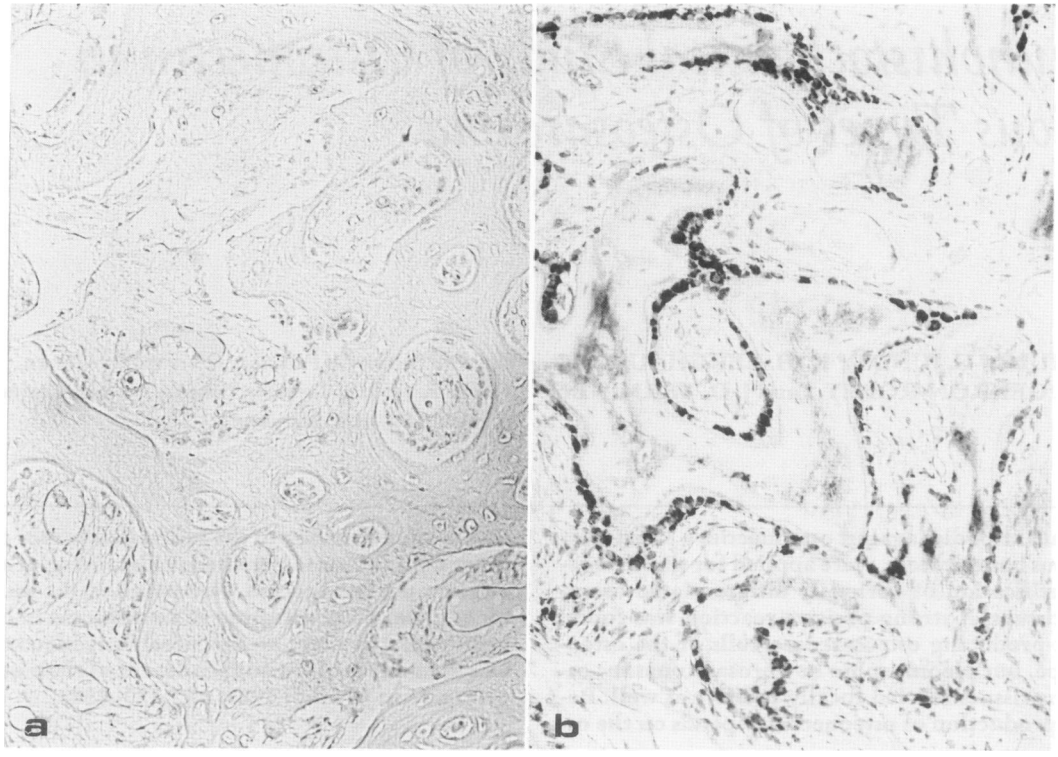


Figure 1—Human callus serving as positive control. **a**—Negative control (non-immune rabbit serum, no counterstaining, ×80) **b**—Positively reacting cuboidal osteoblasts on the surfaces of newly formed trabeculae. (anti-osteonectin, DAB, no counterstaining, ×80)

amount of bone matrix formation (0–100%) was estimated in histologic sections of the osteosarcomas to obtain an adequate approach to the osteoblastic potential of the respective tumors.

Nonosteogenic Tumors

Nonosteogenic tumors were immunocytochemically studied as controls. This group included 3 chondrosarcomas, 4 Ewing's sarcomas, 2 fibrosarcomas, 1 rhabdomyosarcoma, and 1 leiomyosarcoma.

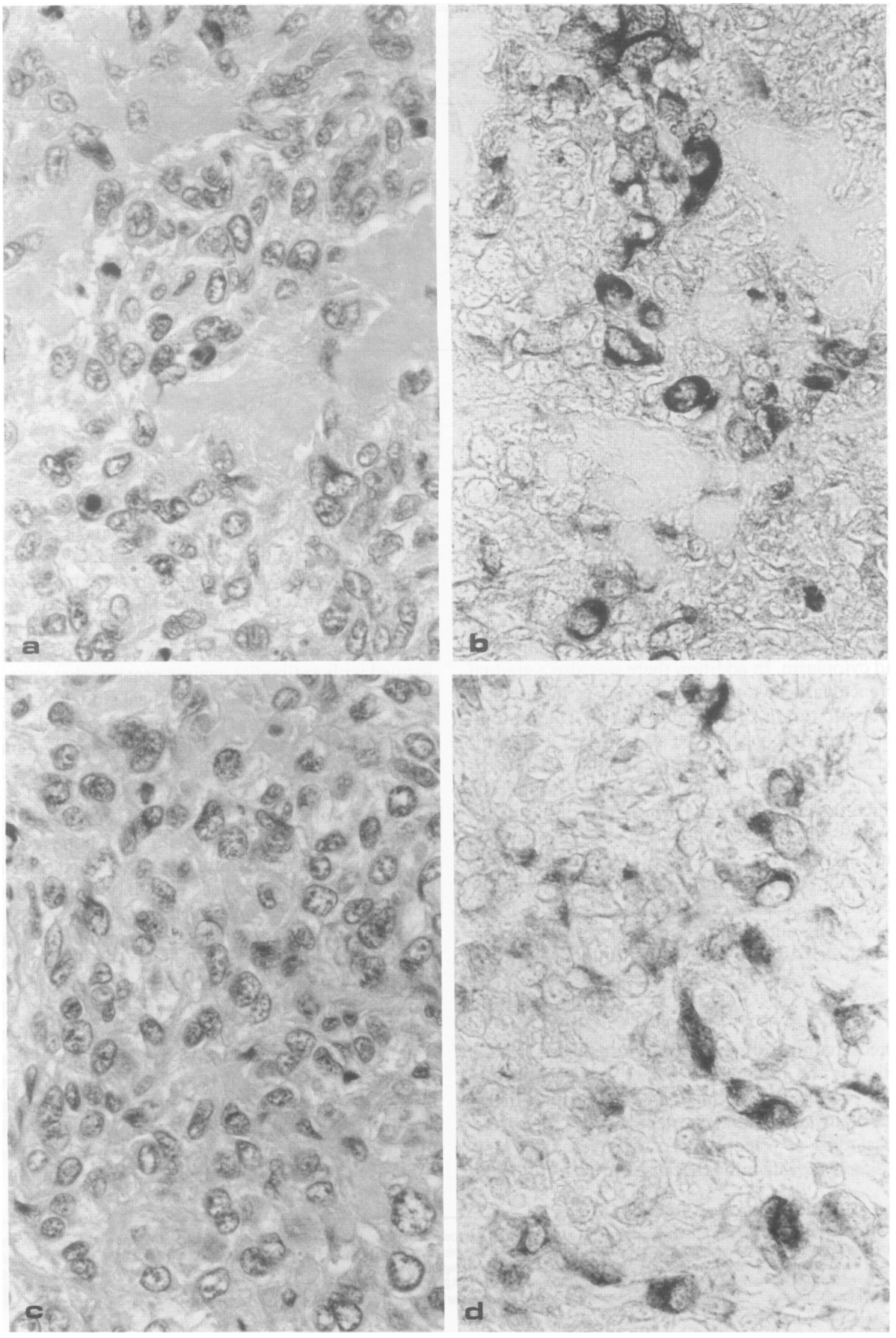
Immunocytochemical Technique

After fixation in 4% buffered formaldehyde the tumor tissue was embedded in paraffin and, if necessary, demineralized in 10% EDTA solution or Ossafixona (Röhm Pharma, Weinheim, FRG) in the case of massive osteoid formations with calcifications. All immunocytochemical studies were performed by using the PAP technique[7,8] or the avidin-biotin complex method.[9] Identical results were obtained with both antibodies. Diaminobenzidine modified by the addition of imidazole[10] was used for the staining reaction. The deparaffinized slides were incubated overnight with the primary antibodies in a dilution of 1:1000 at 4 C. Trypsinization of the slides did not improve the immunocytochemical reactions. Slides of human callus tissue (Figure 1) were included in all immunocytochemical incubations as positive controls.[2]

Results

All osteosarcomas reacted positively with the antibody against human and bovine osteonectin. The intensity of the reaction differed and showed a clear-cut quantitative dependence (number of marked cells) on the differentiation of the tumor tissue (Table 1). The classical osteoblastic osteosarcomas composed of tumor cells and matrix components in nearly equal quantities revealed a strongly positive marking of malignant osteoblasts in the immediate surroundings of osteoid islands within the tumor tissue (Figure 2a, b). A topographically unordered and rather disseminated marking was seen in the tumor cells of osteoblastic

Figure 2—Immunocytochemical results with osteoblastic osteosarcoma tissue. **a**—Classical osteoblastic osteosarcoma with approximately 50% of osteoid as a component of tumor tissue. (H&E, ×780) **b**—Same type of differentiation with immunocytochemical marking of numerous osteoblastic osteosarcoma cells containing osteonectin as indication of their functional activity. The tumor cells are often connected to osteoid islands. (anti-osteonectin, DAB, no counterstaining, ×780) **c**—Osteoblastic osteosarcoma with sparse (10%) osteoid formation. (H&E, ×780) **d**—Same tumor with disseminated marking of some functionally active osteosarcoma cells producing osteonectin. (DAB, no counterstaining, ×780)


a
b
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d

Table 1—Intensity of the Immunohistochemical Reaction of Osteonectin (ON) Antibodies in Relationship to Matrix Formation of Different Types of Osteosarcoma

Case	Age	Sex	Localization	Type of osteosarcoma	Amount of matrix* (%)	Reaction intensity with anti-ON
1	21	M	Tibia	Osteoblastic	<10	+
2	34	M	Femur	Osteoblastic	50	+++
3	35	F	Tibia	Osteoblastic	>80	+++
4	43	F	Femur	Osteoblastic	50	+++
5	-	-	-	Osteoblastic	50	+++
6	23	F	Lung metastasis	Fibroblastic	0	+(+)
7	16	M	-	Osteoblastic	50	+++
8	-	-	-	Osteoblastic	50	+++
9	43	F	Lung metastasis	Osteoblastic	50	+++
10	44	M	Femur	Osteoblastic	50	+++
11	19	M	Humerus	Osteoblastic	50	+++
12	22	F	Femur	Anaplastic	<10	+(+)
13	12	F	-	Osteoblastic	50	+++

* Area of osteoid in relation to total tumor tissue.
-, data not known.
+, low.
++, moderate.
+++, strong.

osteosarcomas with clearly discernible but less pronounced osteoid formation of the diffuse interstitial osteoid deposition type (Figure 2c, d).

The nonmatrix-forming tumors also showed a positive reaction. In the anaplastic tumor type, some hyperploid tumor osteoblasts with giant nuclei were found to display a strong marking of the frequently broad cytoplasm. In addition there was also a gradual difference in the cytoplasmic marking of the atypical osteoblastic tumor cells (Figure 3a, b): The nonmatrix-forming fibroblastic type also showed a disseminated and intensive cytoplasmic marking of some tumor cells. There were also numerous other tumor cells in which the intensity of the cytoplasmic immune reaction was distinctly weaker and of gradual difference (Figure 3c, d).

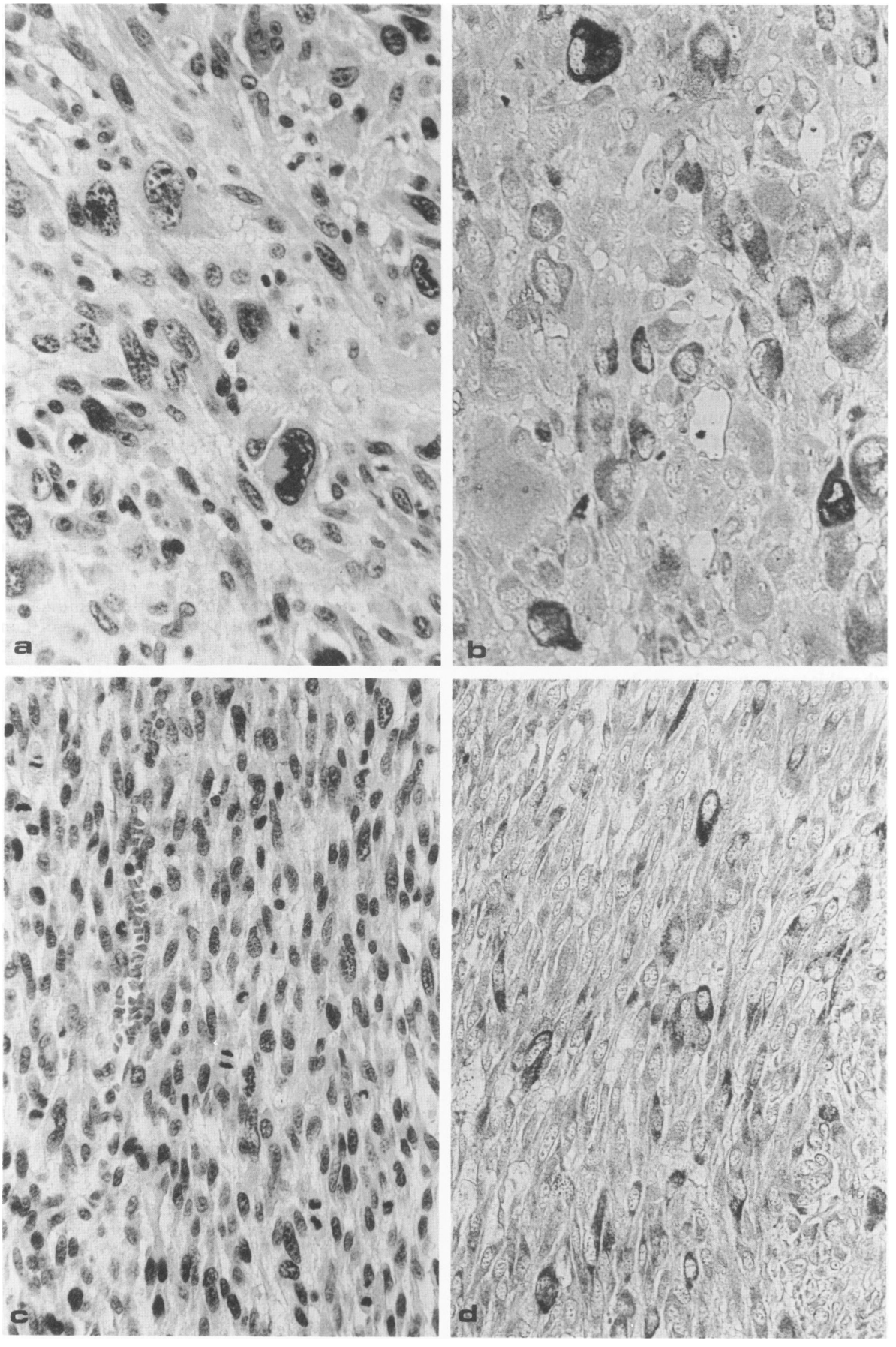
No tumor of the control group, including chondrosarcomas, Ewing's sarcomas, fibrosarcomas, rhabdomyosarcomas, and leiomyosarcomas showed a positive reaction with both osteonectin antibodies.

Discussion

Osteosarcomas originate from osteoblasts whose function is the production of new bone. However, definite proof of this production is only possible in the case of highly differentiated osteosarcomas.[6] Even the classical type of osteoblastic osteosarcoma[11,12] may pose diagnostic difficulties because the interstitial matrix deposits, if not mineralized, are sometimes difficult to distinguish from the deposits of a hyaline, collagenous ground substance present in malignant soft tissue tumors such as synovial sarcoma, fibrosarcoma, malignant fibrous histiocytoma, or hemangiopericytoma.[13,14] For better characterization of tumor osteoid, immunohistochemical studies were performed with antibodies against proteins that are typical for bone. Immunohistochemical studies with collagen antibodies have shown that type I collagen is present in osteosarcoma cells as well as in bone matrix.[15,16] This demonstration is not bone-specific, however, because collagen type I occurs also in numerous other nonmineralizing connective tissues.[17-19] Only after the isolation of bone-specific, noncollagenous matrix proteins such as osteocalcin[20] and osteonectin[1] did specific immunologic identification of bone matrix constituents become possible.

First immunofluorescent studies on native tissues (frozen sections from calf bones) revealed that osteonectin was present mainly in bone matrix.[1] In immunocytochemical studies on human bone and callus tissue the authors were able to demonstrate the intracellular presence of osteonectin in actively bone-forming osteoblasts.[2] Subsequent immunocytochemical studies on a broad spectrum of bone and soft tissue tumors showed that osteonectin is demonstrable in bone-forming tumors only, but not in chondrosarcomas or soft-tissue sarcomas.[3]

Figure 3—Nonmatrix forming types of osteosarcoma. a—Anaplastic osteosarcoma lacking osteoid production. (H&E, ×780) b—Immunocytochemical proof of osteonectin. Intensive marking in the cytoplasm of huge pleomorphic atypical osteoblasts revealing functional activity as osteogenic cells. Lesser marking of scattered disseminated tumor cells. Many tumor cells remain completely negative. (anti-osteonectin, DAB, no counterstaining, ×780) c—Fibroblastic osteosarcoma lacking any osteoid production as well. (H&E, ×312) d—Small number of fibroblastic osteosarcoma cells showing varying intensity of intracytoplasmic marking by osteonectin antibodies as expression of their osteogenic function. (anti-osteonectin, DAB, no counterstaining, ×312)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Because the production of osteonectin depends on the functional state of an osteoblast, no homogenous immunocytochemical marking of all tumor cells was found in the investigated osteosarcomas. It was therefore necessary to examine various differentiation types more closely with regard to the ability of the tumor cells to produce osteonectin. The results obtained from 13 osteosarcomas in varying degrees of differentiation showed that osteonectin could always be demonstrated reliably in those osteosarcoma cells located within or in the immediate neighborhood of osteoid islands.

Osteonectin also has been found in tumor tissues that contain no, or only a slight amount of, matrix, such as anaplastic osteosarcomas or fibroblastic osteosarcomas or their respective components.[6] The identification of osteonectin in the fibroblastic and anaplastic types of osteosarcoma is of special importance because it can prove that these cells belong histogenetically to the osteosarcoma tissue. This statement becomes relevant when it must be determined whether the biopsy from an osteolytic tumor with fibrosarcomatous or anaplastic differentiation should be considered to be one of the known partial components of an osteosarcoma.[4,11,22]

A special differentiation type of osteosarcoma described in recent years is small-cell osteosarcoma,[5] which may simulate Ewing's sarcoma histologically.[23] Whether this tumor type also produces osteonectin remains an open question for the present because no such tumor has approved in the authors' case material to date.

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