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Original Articles

The Relationship Between Extracellular Matrix Vesicles and Calcospherites in Primary Mineralization of Neoplastic Bone Tissue

TEM and SEM Studies on Osteosarcoma

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Summary. Primary mineralization in neoplastic tissue was studied in osteosarcoma, correlating observations obtained by SEM to those found with TEM.

The process is characterized by extracellular matrix vesicles, distributed in the matrix between the forming neoplastic cells and the calcifying fronts. The occurrence of osmiophilic material and solitary hydroxyapatite crystals within the vesicles is followed by accumulation of apatite crystals, disappearance of the vesicular membrane and formation of calcospherites and calcified fronts. The process described here in neoplastic tissue is essentially similar to primary calcification in normal calcified tissues.

Key words: Matrix-vesicles – Calcospherites – Osteosarcoma – SEM, TEM.

Introduction

Primary mineralization is characterized by formation of extracellular matrix vesicles. These trilaminated membrane bound organelles, 0.05–0.45 μm in diameter, were defined with transmission electron microscope (TEM) in both normal and pathological conditions, and it is assumed that the vesicles, which bud from the cell membrane into the extracellular matrix, serve as initial loci of calcification (Anderson, 1976; Ali, 1977). Isolated matrix vesicles show high activity of alkaline phosphatase, pyrophosphatase and different ATPases (Anderson, 1976; Ali, 1977; Muhlrad et al., 1978; Sela et al., 1978), all of which enzymes are operative in the increase in concentration of inorganic phosphate both within the vesicles and in the milieu. In addition, pyrophosphatase and ATPase have an important role in removing the inhibitory effect of pyrophosphate and ATP on calcification (Ali, 1977). ATP has been shown to be potent in the stabilization of amorphous calcium phosphate, thus preventing crystallization of hydroxyapatite. The high levels of ATPase activity in the vesicle fraction are most probably operative at the initial formation

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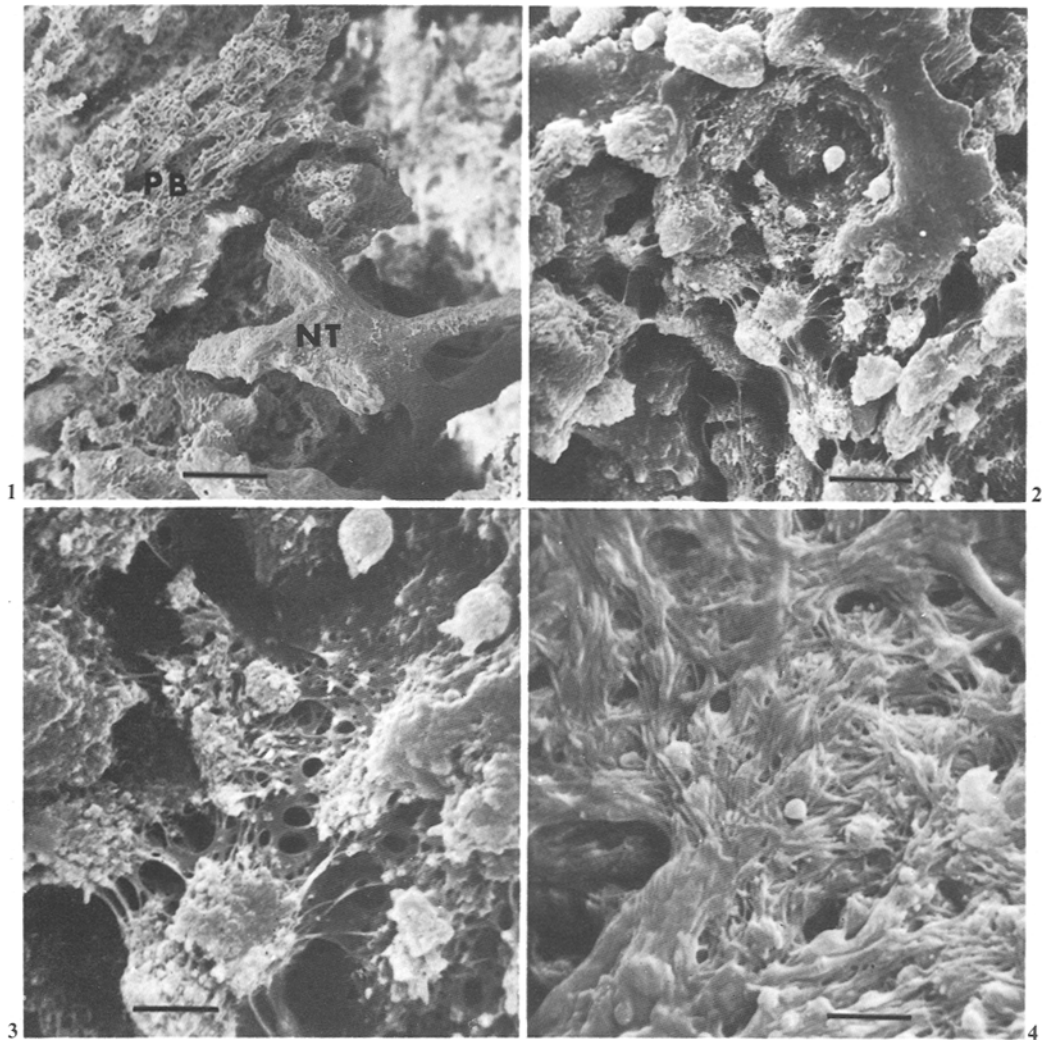

Fig. 1. Freeze-fractured and dried osteosarcoma tissue. Note sponge like primary bone (*PB*) invading normal trabeculae (*NT*). Bar = 0.23 μm

Fig. 2. Mineral front exposed by sodium hypochlorite (organic material removed by 7% sodium hypochlorite) showing calcified globular nodules. Bar = 7.5 μm

Fig. 3. Higher magnification of the central area shown in Fig. 2. Note calcified globules constructed of minute calcospherites. Bar = 3.0 μm

Fig. 4. Freeze fractured and dried osteosarcoma showing calcified nodules embedded among and in, randomly oriented collagen bundles. Bar = 3.0 μm

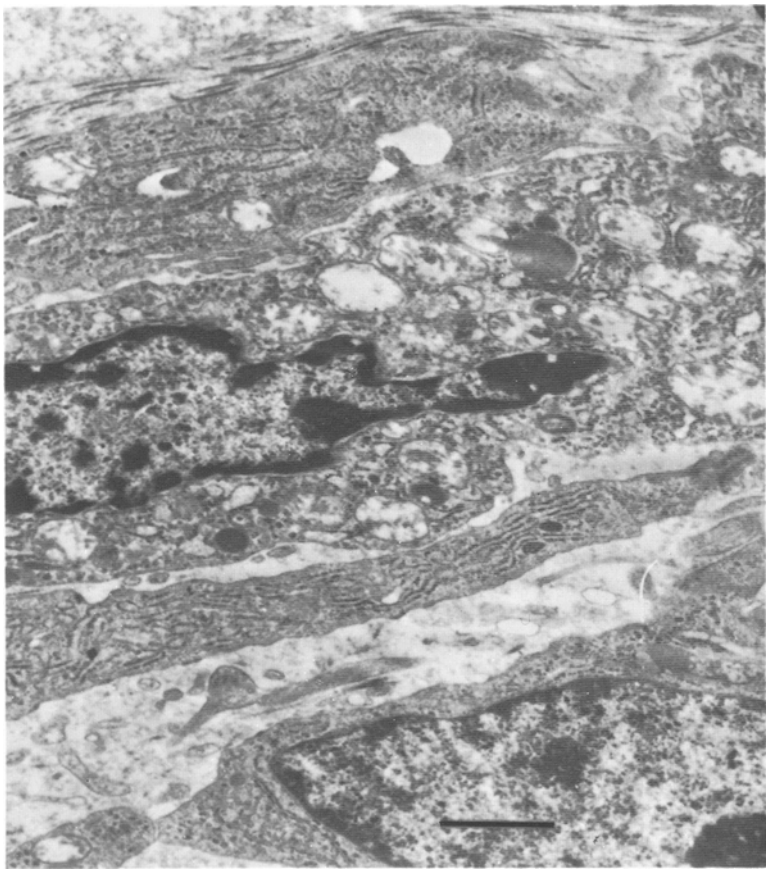

Fig. 5. Transmission electron microscopic view of forming neoplastic cells. Note lobulated nucleus containing prominent chromatin aggregates, long and irregular cell processes and abundance of endoplasmic reticulum. Bar = 1.22 μm

of crystalline mineral (Betts et al., 1975; Hsu and Anderson, 1977). Recently, the lipid spectrum of the vesicles has been characterized and it is known that lipids play a role in the increase of calcium concentration within the vesicle, by virtue of a lipid-calcium interaction (Wuthier, 1976).

TEM studies of bone producing malignant neoplasms focused attention on the cellular characteristics and structure of the intercellular matrix (Hirota, 1959, 1960; Ghadialli and Mehta, 1970; Kay, 1971; Hirota and Morimoto, 1971; Lee et al., 1975; Williams et al., 1976). Further examination revealed the formation of matrix vesicles in osteosarcoma and these were isolated and characterized biochemically by Muhlrad and associates (1978). Examination of osteosarcoma and other bone producing neoplasms with the scanning electron microscope (SEM) revealed fibrillar and calcospheritic patterns of mineralization; the diameters of the calcospherites ranged between 1–3 μm, their surfaces were constructed of minicalcospherites 0.1–0.3 μm in diameter. (Sela, 1977;

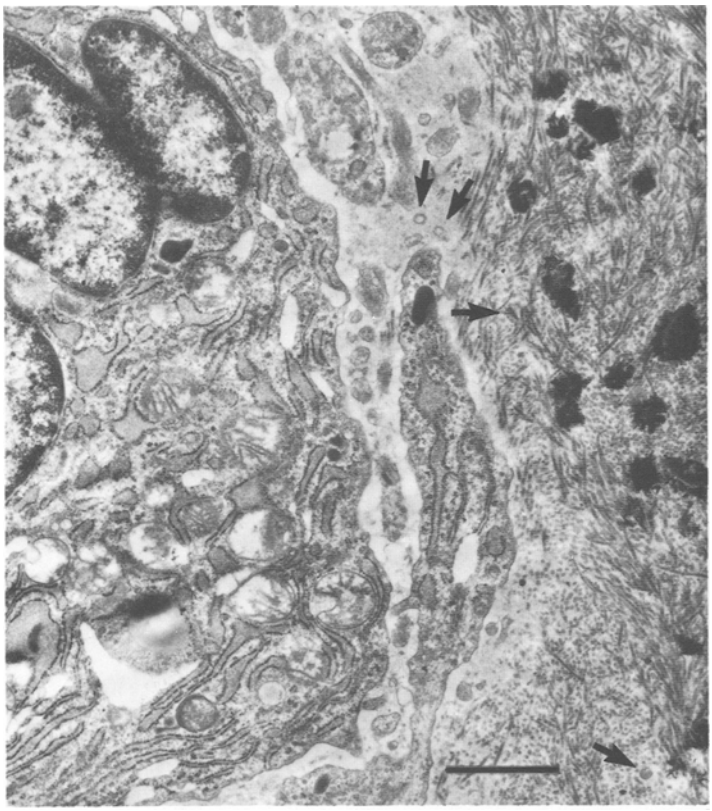

Fig. 6. Neoplastic cell, matrix and mineralized globules. Note lobulated nucleus, rich endoplasmic reticulum and long irregular processes. Matrix vesicles (*arrows*) are embedded in haphazardly scattered collagen fibers. Bar = 1.40 μm

Sela and Boyde, 1977; Boyde and Sela, 1978). We present here a correlative TEM and SEM study on the ultrastructural features of primary calcification in osteosarcoma.

Materials and Methods

Scanning Electron Microscopy

The specimens were obtained from biopsies of osteosarcomas. Tissue samples were freezed in CCl_2F_2 at −155° C, fractured under liquid nitrogen (−196° C) and freeze dried at −70° C in an Edwards Speedivac-Pearse tissue dryer. (Edwards High Vacuum Ltd., Manor Royal, Crawley, Sussex, England). They were then gold coated in a vacuum device (Sputter-coating Unit E-5000; Polaron Ltd., England) and examined with the SEM (Stereoscan S4–10, Cambridge Scientific Instruments Ltd., England). SEM examination was repeated after partial or complete removal of the organic matrix from the specimens by treatment with 7% sodium hypochlorite, performed by a method described in detail previously (Sela and Boyde, 1977).

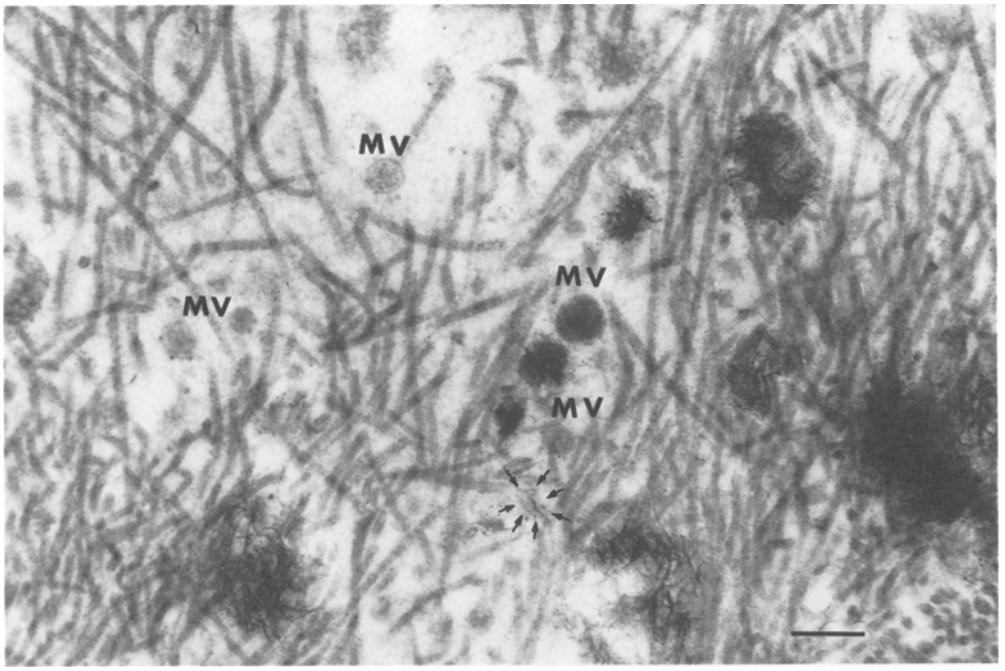

Fig. 7. Matrix vesicles (*MV*) and calcifying nodules in haphazardly distributed collagen fibers. Note osmiophilic material and solitary hydroxyapatite crystals in matrix vesicles close to the mineralizing nodules (*arrows*). Further accumulation of apatite crystals is accompanied by the absence of the vesicle membrane. The arrangement of some of the outermost spicules resembles a feather-like pattern. Bar = 0.22 μm

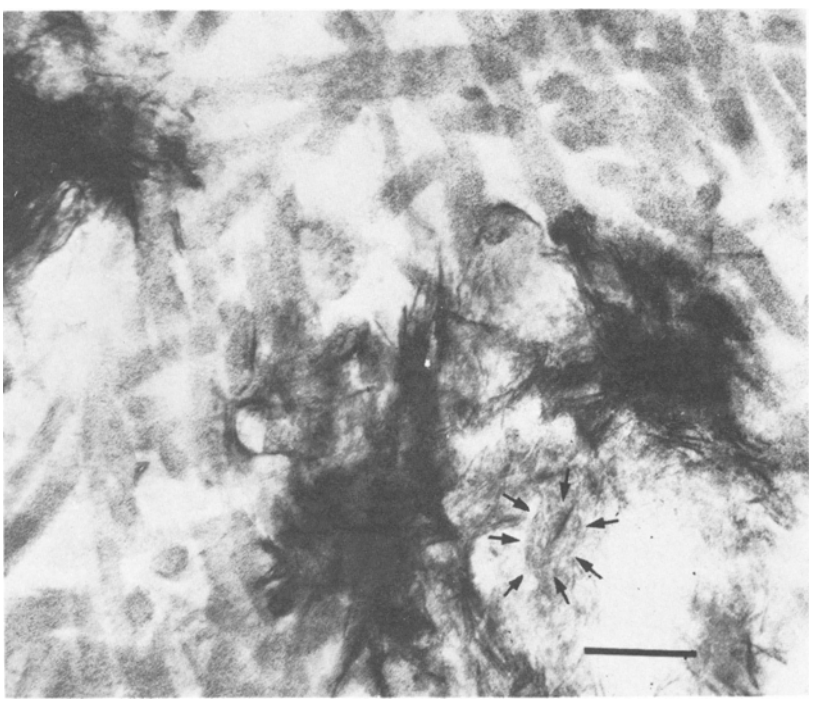

Fig. 8. High magnification of partially fused calcified nodules. Note matrix vesicle containing hydroxyapatite crystal (arrows). Bar = 0.20 μm

Fig. 9. Calcifying front showing attachment of calcospherites. Note apatite crystals arranged in a feather-like appearance on the surface. Bar = 0.22 μm

Transmission Electron Microscopy

The specimens were fixed in 4% glutaraldehyde solution in 0.1 M sodium cacodylate buffer, pH 7.2. Post fixation was performed for 1 h at 4° C in 1% osmium tetraoxide containing the same buffer. Dehydration in a graded series of alcohols and propylene oxide was followed by embedding in epon, and ultrathin sections were stained with 3% uranyl acetate for 5 min and lead citrate, and examined with Philips EM 400 electron microscope.

Observations

Examination of the tissue obtained from the osteosarcoma with the SEM revealed primary sponge-like bone invading normal trabeculae (Fig. 1). Higher magnifications disclosed calcified globular nodules, 1 to 3 μm in diameter (Fig. 2). These globules or calcospherites were constructed of minute calcospherites, the diameters of which ranged from 0.1 to 0.3 μm (Fig. 3). The calcospherites were embedded in randomly oriented collagen bundles that were partially calcified (Fig. 4). TEM examination of the tissues revealed neoplastic cells with irregular long processes and occasional indistinct cell boundries. The cells had a rich endoplasmic reticulum and large, lobulated nuclei with prominent chroma-

tin aggregates and nucleoli (Figs. 5, 6). The extracellular matrix contained randomly scattered collagen fibers and large quantities of mineralized spherical nodules, mixed with amorphous material (Figs. 6, 7). Abundant extracellular matrix vesicles between 0.05 and 0.45 μm in diameter were observed. They were found in the matrix, between the cells and the calcifying fronts, occasionally located among the calcified nodules. Some vesicles contained osmiophilic material (Fig. 7), in others, solitary hydroxyapatite crystals were present frequently extruding through the vesicular membrane (Figs. 7, 8). Both vesicles with osmiophilic material and with hydroxyapatite crystals were scanty around cells and abundant at the calcifying fronts (Fig. 7). Crystal accumulation was accompanied by absence of the vesicular membrane (Figs. 7, 8). In many instances mineralization was characterized by formation of round structures with diameters ranging from 0.1 to 0.8 μm. These consisted of hydroxyapatite crystals which had accumulated around initial foci within the vesicles. The outermost spicules, extruding from the surfaces of the round calcified structures, were arranged in a feather-like pattern (Figs. 7, 8, 9). In some areas spherical nodules were fused together to form the calcified front proper (Fig. 9).

Discussion

The ultrastructure of osteosarcoma was studied with both scanning and transmission electron microscopes and observations made were correlated in order to obtain comprehensive understanding of the initial stages of neoplastic mineralization. TEM revealed typical calcifying fronts surrounded by malignant cells with large lobulated pleomorphic nuclei containing abundant aggregated chromatin and nucleoli. These cells contained a rich endoplasmic reticulum and had long irregular processes. The organic matrix was mainly composed of haphazardly arranged collagen fibers. These observations are in agreement with previous studies on osteosarcoma (Hirota, 1959, 1960; Ghadially and Mehta, 1970; Kay, 1971; Lee et al., 1975; Williams et al., 1976). Neoplastic primary calcification was characterized by large quantitites of matrix vesicles distributed among the cells, collagen fibers and calcifying fronts. These observations corroborate with studies of previous investigators (Ascenzi and Bonucci, 1971; Anderson, 1976) who stated that matrix vesicles originate from the growing cells in calcifying tissues. It has been shown in the present study that further maturation of the vesicles occurs as the calcifying front is approached manifested by the occurrence of both osmiophilic material and hydroxyapatite crystals within the vesicles. It has been suggested that the osmiophilic material is a calcium binding lipid (Anderon, 1976; Wuthier, 1976). The increase in quantity of vesicles containing apatite crystals and/or osmiophilic material in the present study is in direct relationship to their distance from the cells. The process of maturation in these tumors is similar to that found in other calcifying tissues and occurs by the growth of apatite crystals, accompanied by disappearance of the vesicle membrane, most probably due to its rupture (Anderson, 1976). Further maturation is accompanied by production of round calcified nodules, the diameters

of which are somewhat greater than those of the vesicles and similar to those of the minicalcospherites observed with the SEM in the present work and in previous studies (Sela, 1977; Sela and Boyde, 1977; Boyde and Sela, 1978). As in other calcified tissues, the nodules of mineralization in osteosarcoma are distributed alongside collagen fibers and production of calcospherites is the result of conglomeration of minicalcospherites. Further information obtained by TEM has revealed that the outermost apatite crystals of the spheric structures are arranged in a feather-like pattern.

It is concluded that the ultrastructural features of primary mineralization in osteosarcoma are basically similar to those found in other calcified tissues. It should be pointed out that the neoplastic tissue exhibited larger quantities of matrix vesicles and calcifying nodules than found previously in normal bone healing (Sela and Yaffe, 1977; Sela et al., 1978). It is suggested that the extensive production of vesicles is a result of the high cellular activity of the neoplastic tissue and evidence of the increased rate of primary calcification. The relatively poor ossification of the neoplastic tissue, as compared to normal bone healing can be attributed to the fact that primary mineralization in normal bone precedes a remodelling process by osteon formation that could not be demonstrated in osteosarcoma. These observations are supported by our previous study on the enzymatic activity of vesicles isolated from osteosarcoma. The high levels of activity of alkaline- and pyro-phosphatase were attributed to either increased synthesis by the tumor tissue per se or to the occurrence of isoenzymes (Muhlrad et al., 1978).

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