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Case Report

Further Observations on the Relationship between the Matrix and the Calcifying Fronts in Osteosarcoma

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Summary. Human osteosarcoma biopsies were studied with the SEM using sequential etching with sodium hypochlorite solutions after removal of aluminium or gold coatings. Osteosarcomas differ from normal hard tissues in that the matrix never proceeds to complete mineralization, so that the specimens fragment on hypochlorite treatment. Details of the fibrillar pattern and calcospheritic type of mineralization pattern can be seen in hypochlorite etched, fractured surfaces and mineralizing fronts.

Key words: Bone mineralization – Scanning electron microscopy – osteosarcoma.

Introduction

The pattern of deposition of clusters of calcium phosphate crystals (calcospherites) in mineralizing matrices composed of collagen and proteoglycans in cartilage, fetal bone and dentine has attracted increasing interest in recent years. Evidence indicates that globular-calcification is nucleated around and inside extracellular matrix vesicles (Slavkin et al., 1976; Rabinovitch and Anderson, 1976; Anderson, 1976; Bonucci and Dearden, 1976; Ali, 1976; Felix and Fleisch, 1976). Matrix vesicles, of 500 to 3000 Å in diameter, are associated with globular calcification in both normal and pathological conditions (Anderson, 1976). It was surmised that the extracellular matrix vesicles bud from the cell membrane into the forming extracellular organic matrix, subsequently participating in min-

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eralization of the collagen and proteoglycan organic matrix. The relationship between the calcifying globular structures and the collagen-proteoglycan matrix was demonstrated in osteosarcomas with transmission electron microscopy (TEM) by Lee et al. (1975) and Williams et al. (1976). They showed that the extracellular tissue in cases of osteosarcoma was composed of an amorphous granular ground substance that was mixed with collagen fibres. In the matrix was scattered abundant clumps of randomly distributed hydroxyapatite crystals that were related to the collagen and the ground substance indiscriminately. In some instances, small circular structures of the order of 1000–2000 Å in diameter, and occasionally of larger size, were found in the extracellular matrix as well as in cellular cytoplasm.

Mineralization of primary bone and calcifying cartilage in a globular calcospheritic pattern has been described in scanning electron microscope (SEM) studies, in several mammalian species (Boyde and Hobdell, 1969b; Boyde, 1972); in human fetuses with achondrogenesis Type 1 (Ornoy et al., 1976); in cases of new bone formation during postextraction healing of tooth sockets in rats (Sela and Jaffe, 1977); and in benign and malignant neoplastic bone formation in pathologic conditions (Sela, 1977b). The purpose of the present SEM study was to examine the relationship between the matrix and the calcifying components in osteosarcoma.

Materials and Methods

The specimens for SEM examination were obtained from 10% neutral formol saline-fixed bone-biopsies of osteosarcomas, which were transferred to 70% ethyl alcohol for storage and to water before freezing in CCl_2F_2 at –155° C. They were fractured under liquid nitrogen (–196° C) and freeze dried at –70° C in Edwards Speedivac-Pearse tissue dryer (Edwards High Vacuum Ltd., Manor Royal. Crawley, Sussex, United Kingdom). After preparation – the details follow – the specimens were examined in a Cambridge S410 SEM operated at 10 kV.

Variation of Etch Duration

One group of samples was treated for different periods with 7% sodium hypochlorite (we shall hereinafter use the term "etch" to describe this treatment). Eight specimens were etched for 1, 2, 4, 8, 16, 32, 48 and 66 min respectively, 17 specimens were etched for 90 min and another 6 specimens for 128 min each. The specimens of this group were coated with gold in a vacuum device (Sputter-coating Unit E–5000, Polaron Ltd., Holywell Industrial Estate, Watford, Herts, United Kingdom).

Sequential Etching of Aluminium Coated Specimens

Three subgroups of 5, 8 and 9 specimens were etched with a 7% NaOCl solution for 7, 10 and 13 min respectively, freeze-dried, mounted on copper stubs and coated with aluminium in a vacuum evaporation device. Following examination with the SEM, the specimens were further etched for another minute with 7% NaOCl (which also removes the Al coating), freeze-dried. re-coated and re-examined with the SEM; then etched for 30 s with 0.1 N HCl, washed, freeze-dried, re-coated and re-examined with the SEM. Another 1 min of 7% NaOCl, and SEM examination completed the treatment.

Removal of Gold by Amalgamation

Eight specimens were sputter-coated with gold and examined with the SEM. This was followed by removal of the gold coating by amalgamation with mercury for 24 h (Sela, 1977); a 30 s etching with 7% NaOCl solution, washing, freeze-drying, re-coating and SEM examination. The same procedure was repeated six times before a 1 min etching with 0.1 N HCl washing, freeze-drying, re-coating and examination with the SEM.

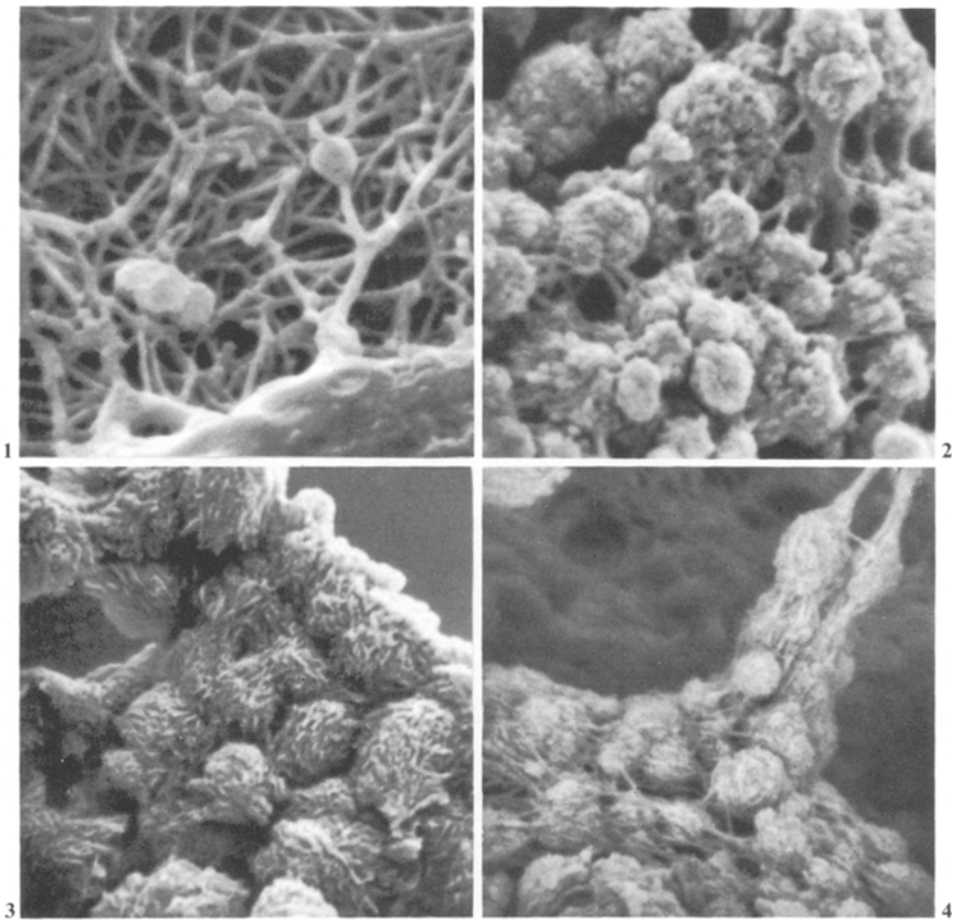

Fig. 1. Freeze-fractured and -dried osteosarcoma showing randomly oreinted collagen fibril network, with spherical knobs suggestive of calcospherites. Width of field = 4 μm.

Fig. 2. Mineral front exposed by hypochlorite etching for 129 min, showing large spherical regions with smaller spherical substructure. Further etching for 2 min resulted in no change establishing the mineralized nature of the surface. Width of field = 8.5 μm

Fig. 3. Mineral front of anorganic osteosarcoma showing linear substructure of the surfaces of the large calcospherites. 128 min hypochlorite treatment. Width of field = 8 μm

Fig. 4. Calcospherites are joined by mineralized fibrils in this anorganic preparation. 128 min hypochlorite treatment. Width of field = 8 μm

Removal of Gold by Cyanide

Six specimens were gold coated and examined with the SEM; this was followed by removal of the gold with a 10% sodium cyanide solution (Sela and Boyde, 1977). The specimens were etched with a 7% NaOCl solution for 1 minute, washed, freeze-dried, gold coated and examined in the SEM. The same procedure was repeated 6 times.

Results

Freeze-fractured and freeze-dried surfaces of osteosarcomata showed a predominance of areas in which the matrix fibres could be distinguished over those

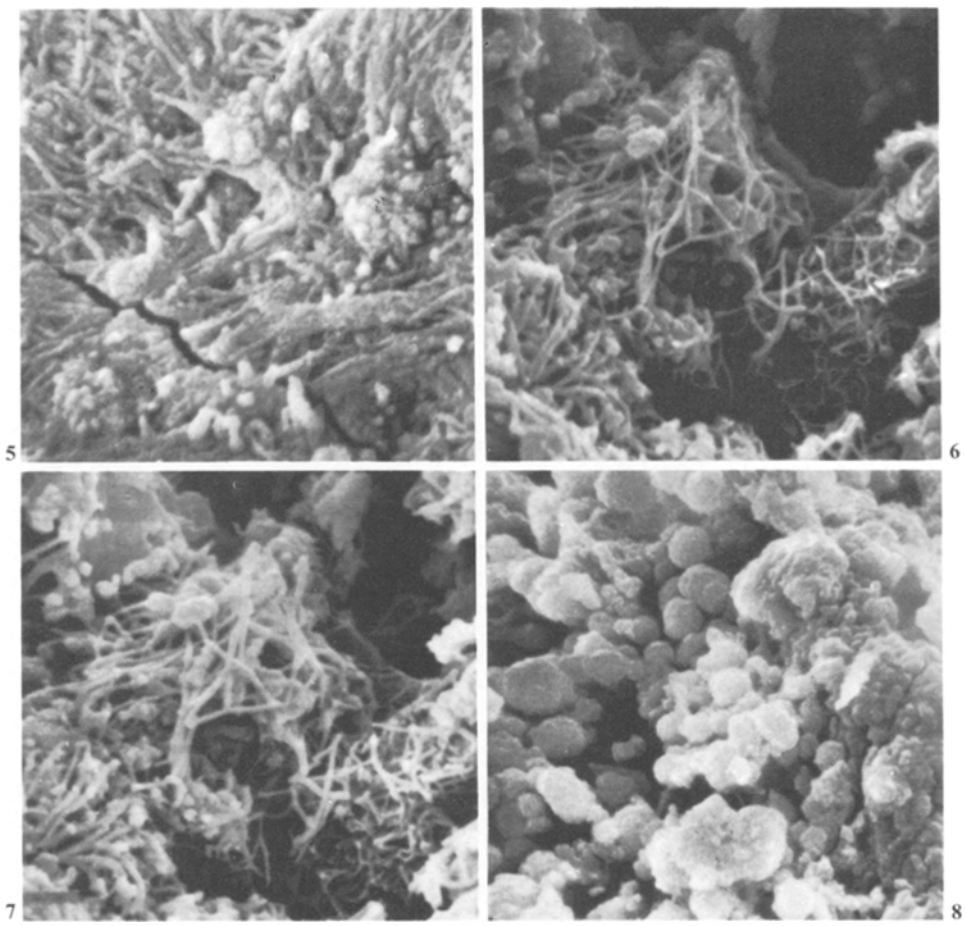

Fig. 5. Fractured surface etched with hypochlorite for 90 min shows both mineralized collagen fibrils and spherical mineral particle clusters unrelated to collagen pattern. Width of field = 8 μm

Figs. 6 and 7. Fractured surface etched with sodium hypochlorite for 2 min (**Fig. 6**) and a further 1 min (**Fig. 7**). No change has occured, establishing the fact that the fibrillar features are mineralized. Widths of fields = 8.5 μm

Fig. 8. Debris from treatment of osteosarcoma with hypochlorite, collected on millipore filter and freeze-dried, showing dense clusters of clacospherites. Width of field = 8.5 μm

areas in which the fibres were obscured by the mineralization process. Such preparations showed the collagen to be arranged as a randomly oriented web of single fibrils (Fig. 1) Oriented bundles of fibrils were uncommon.

Anorganic specimens showed spherical features in the range of 1 to 3 μm in diameter which showed substructures of either smaller protruberances about 0.1 to 0.3 μm diameter (Fig. 2) or linear features in the same size range as the matrix fibrils (Figs. 3 and 4). In contrast to studies of normal hard tissues, it was very difficult to be sure that one was seeing a mineralizing front proper in anorganic osteosarcoma material because NaCOl treatment led to fragmentation of the specimens. However, the sequential etching procedure used here was of value in allowing us to determine that an exposed surface was a mineral front, rather than just one stage in a dynamic dissolution process.

Etched fractured surfaces (Figs. 5–7) could be positively identified during early stages of matrix dissolution. They showed a mixture of appearances of mineralized fibrils and mineral particle clusters (mini-calcospherites), both of which are atypical of normal tissues in which it is difficult to distinguish either the matrix fibrils or features of the mineralization process in a fractured surface. This observation shows that osteosarcoma matrix mineralization rarely proceeds to a state of near completion; or that, if it does, then such areas are preferentially evaded during the propagation of a (freeze) fracture.

Clusters of mineralized material could be collected on filters from the supernatant hypochlorite used to dissolve the organic matrix of the specimens (Fig. 8).

Discussion

The calcifying globules in developing bone and cartilage are the result of precipitation of calcium salts in cellular extrusions, which, in later stages, become detached from the cell membrane, serve as initial loci of calcification, and/or adhere to the surrounding calcifying structures (Anderson and Reynolds, 1973; Bonucci, 1970, 1971). Anderson (1976) believes that the production of vesicles is probably a differentiating function of bone and cartilage cells, whether they are benign or malignant, and that vesicle-mediated calcification is common in normal tissues such as cartilage, bone, dentine and deer antler (primary bone), as well as in pathologic conditions, namely calcification in arteriosclerosis and in malignant tumours like osteosarcoma and chondrosarcoma. It is probably true, however, that the globular type of calcification is only characteristic of rapid mineralization in young hard tissues and that calcification in a collagen-dependent pattern is associated with the mature process of ossification (Boyde and Hobdell, 1969a; Boyde, 1972).

The present studies suggest that the matrix patterns and mineralization patterns in osteosarcomata are a) very variable and b) not quite typical of fetal or adult bone or calcifying cartilage. Thus, although spherical mineral particle clusters reminiscent of the "calcospherites" found in cartilage and dentine are the commonest features found on anorganic specimen surfaces, these are sometimes linked by linear cylindrical features which are identified as mineralized collagen fibrils. Thus, the mineralization process is like that in fetal bone in being independent of the distinction between collagen and ground substance,

yet also, and in a presumably later stage, like that in adult bone in that it appears to spread preferentially along and in collagen fibrils. In most cases, however, the separate mineralization centres within the matrix fail to unite to an extent comparable with that of the normal calcifying connective tissues and, as a result, the specimens fragment to a remarkable degree during the process of solution of the organic matrix. In other words, whereas it is simple to prepare anorganic specimens of normal hard tissues, osteosarcomata fall apart.

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