ARTICLE V

Enzyme histochemical studies of induced heterotopic cartilage and bone formation in guinea pigs with special reference to acid phosphatase. A light and enzyme electron microscopic study.

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Running title: Acid phosphatase in bone formation

Abstract - Heterotopic bone and cartilage formation was studied in a bone induction model in abdominal muscles of guinea pigs using demineralized allogenic dentin as inductive material. The light microscopical study showed high activity of acid phosphatase (AcP) and leucine aminopeptidase (LAP) in young matrix-producing cells. The highest activity was seen in cells surrounded by unmineralized matrix. In the surrounding matrix there was strong staining of AcP,especially in matrix with high glycosaminoglycan content. Electron microscopical studies of AcP in unfixed frozen tissue showed intracellular activity mainly located in lysosomes. Extracellular AcP was found in matrix vesicles in the newly formed hard tissue. It was most pronounced in areas of cartilaginous differentiation. By means of energy dispersive x-ray microanalysis in a scanning transmission electron microscope, the deposits were confirmed to contain lead precipitates. With the exception of some lysosomal staining, prefixation with formaldehyde prevented demonstration of the enzyme activity. Both AcP and LAP may be involved in degradation of glycosaminoglycans or other organic components of the matrix which enable the matrix to mineralize.

Key words: Acid phosphatase, electron microscopy, leucine aminopeptidase, microanalysis, minralization, bone.

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Introduction

Studies of the function and importance of acid phosphatase (AcP) have shown that this enzyme can be found in matrix-producing areas of bone and cartilage (13, 40,41). Both enzyme histochemical (17, 18) and biochemical studies (5, 38) have produced evidence of two groups of AcP in normal hard tissue forming areas, one concerned with resorption and another possibly involved in matrix production or mineralization. The hypothesis that removal of glycosaminoglycans or other non-collagenous proteins from extra-cellular matrix precedes the mineralization of hard tissues has been suggested (14, 21). Lysosomal enzymes have been proposed as mediators of this reduction of glycosaminoglycans (19, 29, 31,33). Electron microscopical investigations have shown lysosome-like extra-cellular bodies, matrix vesicles type 1 (25, 37). Mineralizing bone matrix exhibits less of these and more of type 2 matrix vesicles compared with cartilage (25, 28, 37). Other authors, however, have not been able to demonstrate two types of matrix vesicles in bone or cartilage. The majority of investigators (1, 2, 4, 22) have suggested from enzyme electron microscopical studies that the amount of extra-cellular AcP was negligible. A few investigators have, however, found AcP staining in matrix vesicles of the epiphyseal plate (34) and healing alveolar sockets (32).

It has been clearly shown that demineralized and freeze-dried allogenic dentin implanted in the abdominal muscles

of guinea pigs induces bone formation in the host tissue (7, 8, 25). This model was found suitable for enzyme histochemical studies of hard tissue formation (26).

The present investigation was undertaken to study the extra- and intra-cellular distribution of acid phosphatases, leucine aminopeptidase, some dehydrogenases, alkaline phosphatase and ATPase, prior to mineralization in areas with heterotopic bone and cartilage formation. The localization of acid phosphatase was also studied by electron microscopy and energy dispersive x-ray micro-analysis.

Material and Methods

Eighteen randomly bred young male guinea pigs were used. They were fed a standard mixed diet supplemented with swedes and hay, (Norwegian standard for guinea pigs and rabbits, Statens Institutt for folkehelse, Oslo, Norway). During Nembutal ® anesthesia 4 dentin implants from demineralized and freeze-dried allogenic teeth were implanted intra-muscularly as described previously (7, 25).

Three animals were killed with ether after 7, 10, 14, 18, 21, 28 days, respectively. The implants were excised with some surrounding tissue and placed in Histocon ® (26) for about 6 h before being frozen at -140°C in pre-chilled isopentane (Kebo, Sweden).

LIGHT MICROSCOPY

Frozen sections, 7 µm thick, were cut, as far as possible these were serial sections. Selected sections were stained with haematoxylin and eosin, the elastin van Gieson technique or Alican Blue with pH 0.5 or 2.5. The following incubations for enzymes were carried out: AcP (azo-dye method) (9), LAP (24), $NADH_2$ - and $NADPH_2$ - diaphorase (15), glutamate dehydrogenase (15), glucose-6-phosphate dehydrogenase (3), nonspecific alkaline phosphatase (azo-dye method) (12), ATPase (calcium-cobalt method at pH 9.4) (30).

As controls for all enzyme histochemical methods, sections were incubated in the appropriate media without substrate. Som sections were also treated in distilled water at 90°C for 10 min. before incubation in the complete media. All sections were mounted in glycerin jelly.

ENZYME ELECTRON MICROSCOPY

Frozen sections, 10 µm thick, were cut, mounted on gelatine coated Perspex ® slides and incubated for AcP according to Barka & Anderson (9) using Na- β - glycerophosphate as the substrate. Some sections were fixed in 1% formaldehyde (freshly prepared) for 30 min. prior to washing (15 min.) and incubation. Controls were incubated in a medium lacking the substrate. After 3 rinses in 0.1 M cacodylate-buffer containing 7% sucrose, the sections were fixed for 2 h in 2% glutaraldehyde in the same buffer, dehydrated and embedded in Epon ® according to Morgan et al. (23).

The actual areas were selected out light microscopically and thin sections (60-80 nm) were cut with a diamond knife, collected on formvare-coated coppergrids and examined unstained in a Philips 300 transmission electron microscope operating on 60 kV.

ENERGY DISPERSIVE MICROANALYSIS

The same electron microscopical grids used for ordinary electron microscopy were also studied in a Philips 500 scanning electron microscope using the scanning transmission mode (STEM). An energy dispersive spectrometer (Kevex®) was attached to the microscope. During the analysis the beam was stationed on the structure of interest, and x-ray spectra were obtained showing the elemental composition in the selected spot. The microscope was operated on 50 kV, spotsize 64 nm and counting time 100 sec. live time. The dead time was approximately zero.

Results

After 10 days areas of dentin resorption appeared, showing dentinoclasts and macrophages with intense AcP staining and also moderate to intense dehydrogenase activity. Osteoid and early cartilage were first found after 14 days (Fig 1).

The amount of these new tissues increased from 21 days on, and the osteoid seemed to mineralize to a greater extent. From this time the implanted dentin also seemed to be remineralized, mainly in areas with mineralized bone. Both at 14, 16, 18 and 21 days cartilaginous differentiation of the matrix was seen and these areas exhibited hypertropic cells, and extra-cellularly small amounts of collagen (Fig.1) and large amounts of glycosaminoglycans (Fig. 2).

In connection with unmineralized cartilage, areas of osteoid and bone also often appeared.

When the sections were incubated for AcP, intense enzyme activity was found in the young chondrocytes, somewhat lower activity in the osteoid osteocytes and almost negligible activity in osteocytes of mineralized bone (Fig.3). The matrix around the young chondrocytes also exhibited an intense staining for AcP. The extracellular activity of AcP was much lower in the osteoid, which seemed also to contain less glycosaminoglycans as shown by the Alcian Blue stain, and more collagen, than the cartilage matrix. When incubating sections for the different dehydrogenases, a high metabolic activity was shown in the young chondrocytes and osteoid osteocytes. The $NADPH_2$ - diaphorase (Fig.4) and glutamate dehydrogenase were especially intense in the young chondrocytes. Alkaline phosphatase stained more strongly the young matrix-producing cells than the osteocytes surrounded by mineralized matrix. In the cells of unmineralized newly formed tissue ATPase activity was strong. The osteoblasts, osteoid osteocytes and chondrocytes of unmineralized cartilage were intensely stained by LAP (Fig.5). In the osteocytes of mineralized bone the LAP-activity was low. Unmineralized cartilage showed areas with an extra-cellular granular staining of LAP.

In the ultrastructural studies of the frozen sections, the cellular structures were preserved, which permitted localization of both precipitates and cellular organelles.

The electron microscopical studies showed intra-cellular electron-dense precipitates in lysosomes (Fig.6) and occasionally along the endoplasmic reticulum (Fig.7). Small extra-cellular precipitates were found in the un-mineralized newly-formed tissue, greatest amounts in areas of new matrix production showing cartilaginous differentiation (Fig. 8). The precipitates were located in small round matrix vesicle-like structures (Fig.9). The sections fixed in formalin showed less precipitates than the unfixed specimens, although some precipitates could still be found located intra-cellularly in lysomes. The controls all showed negative staining for enzymes.

The energy dispersive x-ray microanalytical spectrum from measurements of the electron-dense precipitates showed a distinct peak corresponding to the Kα peak of lead (Fig. 10). There was no difference between the intra-cellular and extra-cellular deposits. According to the microanalytical spectrum calcium and phosphorous was found in only small amounts in these electron-dense structures. The matrix around the precipitates was completely free of lead and showed only a low background spectrum containing very small amounts of calcium and phosphorous.

Discussion

The results demonstrate large amounts of osteoid, unmineralized cartilage and bone in connection with the implants, as well as resorption of the dentin matrix. This is in keeping with previous studies on induced heterotopic bone formation (7, 8, 25, 28). The model was well suited for enzyme histochemical studies of mineralizing bone making possible intra-sectional comparisons between the reaction types.

The present study clearly showed the occurrence of AcP and LAP in cells of the matrix-producing areas, confirming previous reports (17, 18, 26, 34). Intense AcP activity in the resorption area containing macrophages and dentinoclasts confirms the generally accepted localization of AcP (16, 41).

Staining of AcP was intense in the cells of the unmineralized cartilage and moderate in osteoid osteocytes. Evidence of degeneration or osteocytic osteolysis (11) has not been encountered in the present or previous studies of this model (25, 26), and the activity of the dehydrogenases and ATPase showed metabolically active matrix-producing cells.

Removal of macromolecules is proposed to occur before or in connection with initial mineralization of cartilage (10, 35, 36). Cartilaginous matrix exhibited a high amount of AcP while the osteoid matrix had only a little.

This agrees well with the fact that the content of proteoglycan in osteoid is lower than in unmineralized cartilage. Loss of proteoglycans in some of the hard tissues has been proposed to be mediated by different lysosomal enzymes (19, 29, 31). The occurrence of intense actitivity of AcP and LAP in the matrix of young cartilage containing abundant glycosaminoglycans, which is a part of the proteoglycans, suggests that lysosomal enzymes have a function in the degradation of proteoglycans.

The finding of acid phosphatase in type 1 matrix vesicles in the extra-cellular unmineralized matrix is in contrast to the results of Ali et al. (1) and Matsuzawa & Anderson (22) who regarded lysosomal enzyme activities in matrix vesicles as negligible.

The present study showed that fixation with formaldehyde destroyed much of the AcP activity in matrix-producing cells. This concurs with results from a light microscopical study of isoenzymes of AcP using the same experimental model (27), and supports the view that there is a special isoenzyme in matrix-producing areas, distinct from that of resorbing cells. The effect of the fixative may explain some of the different results of AcP reported in the literature. The sensibility of AcP to aldehydes has been shown in other cell types (6). The present study may indicate that the extra-cellular AcP in matrix vesicles type 1 derives from lysosomes as suggested by Thyberg, Nilsson & Friberg (37) in studies on metaphyseal bone.

The LAP stained the cytoplasm of matrix-producing cells strongly, supporting our previous findings (26), and suggesting that it is involved in synthesis or preparation of the matrix and not in resorption of bone as proposed by Lipp (20).

Urist (39) claimed that, after the matrix has been laid down an alteration must occur before crystal formation can commence. The present study indicates that AcP from matrix vesicles type 1, and LAP may contribute to the initial mineralization by being involved in this alteration.

Legends to figures

Fig.1 Induced hard tissue formation 14 days postoperatively showing osteoid (O) and unmineralized cartilage (C) closed to the implanted dentin (D).
Van Gieson, X 112

Fig.2 Induced hard tissue formation 14 days postoperatively showing abundant extra-cellular glycosaminoglycans in the areas with cartilaginous differentiation (C) compared with the outer osteoid zone (O).
The picture shows the same area as fig.1. Dentin (D).
Alcian blue pH 2.5, X 112

Fig.3 Induced hard tissue formation 21 days postoperatively showing intense acid phosphatase staining intra- and extra-cellularly in the unmineralized cartilage (C) and in dentinoclasts (open arrows). Dentin (D).
Moderate staining in osteoblasts and osteoid osteocytes (arrowheads). HE X 224

Fig.4 Induced hard tissue formation 21 days postoperatively showing $NADPH_2$-diaphorase with intense activity in cells of the unmineralized cartilage (open arrow).
Dentin (D), X 224

Fig.5 Induced hard tissue formation 21 days postoperatively showing leucine aminopeptidase with intense staining of cells with cartilaginous differentiation (open arrow).
Dentin (D) X 470

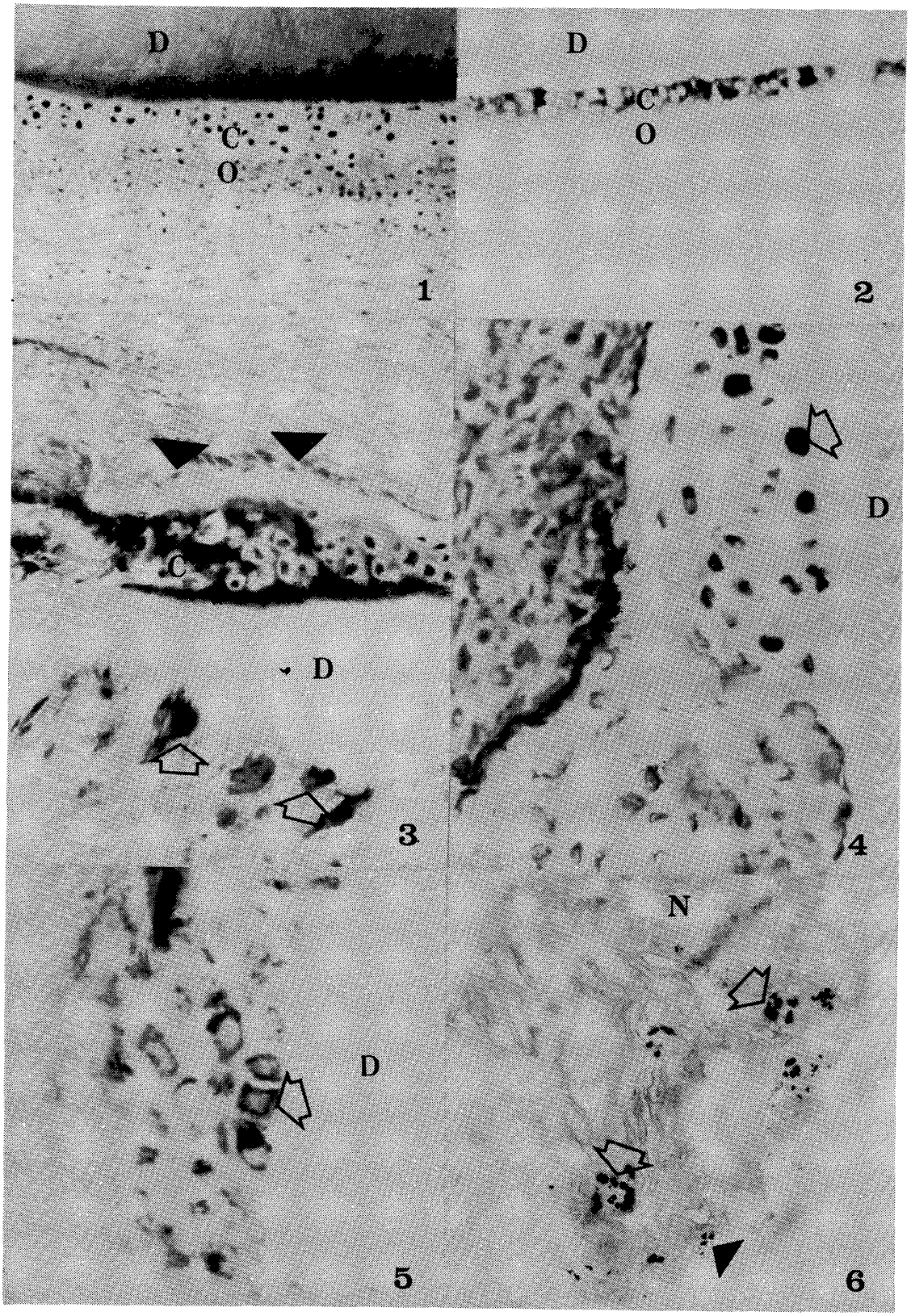
Fig.6 Electron microscopical picture of an osteoid osteocyte having precipitates for AcP mainly located in lysosomes (open arrows). Note some precipitates near the plasma membrane (arrowhead). Nucleu (N). Unstained. X 13600

Fig.7 Electron microscopical picture showing AcP in lysosomes and along the endoplasmic reticulum (open arrow) of an osteoid osteocyte. Unstained. X 23000

Fig.8 Electron microscopical picture of young chondrocyte showing extra- and intra-cellular staining for AcP. Unstained. X 10000

Fig.9 Electron microscopical picture of a matrix vesicle type 1 showing precipitates for AcP (open arrow). Unstained. X 28000

Fig.10 Energy dispersive x-ray microanalysis using scanning transmission electron microscopy of a matrix vesicle showing a K_{α} peak of Pb and a K_{α} peak of Cu. 64 nm spotsize. 50 kV.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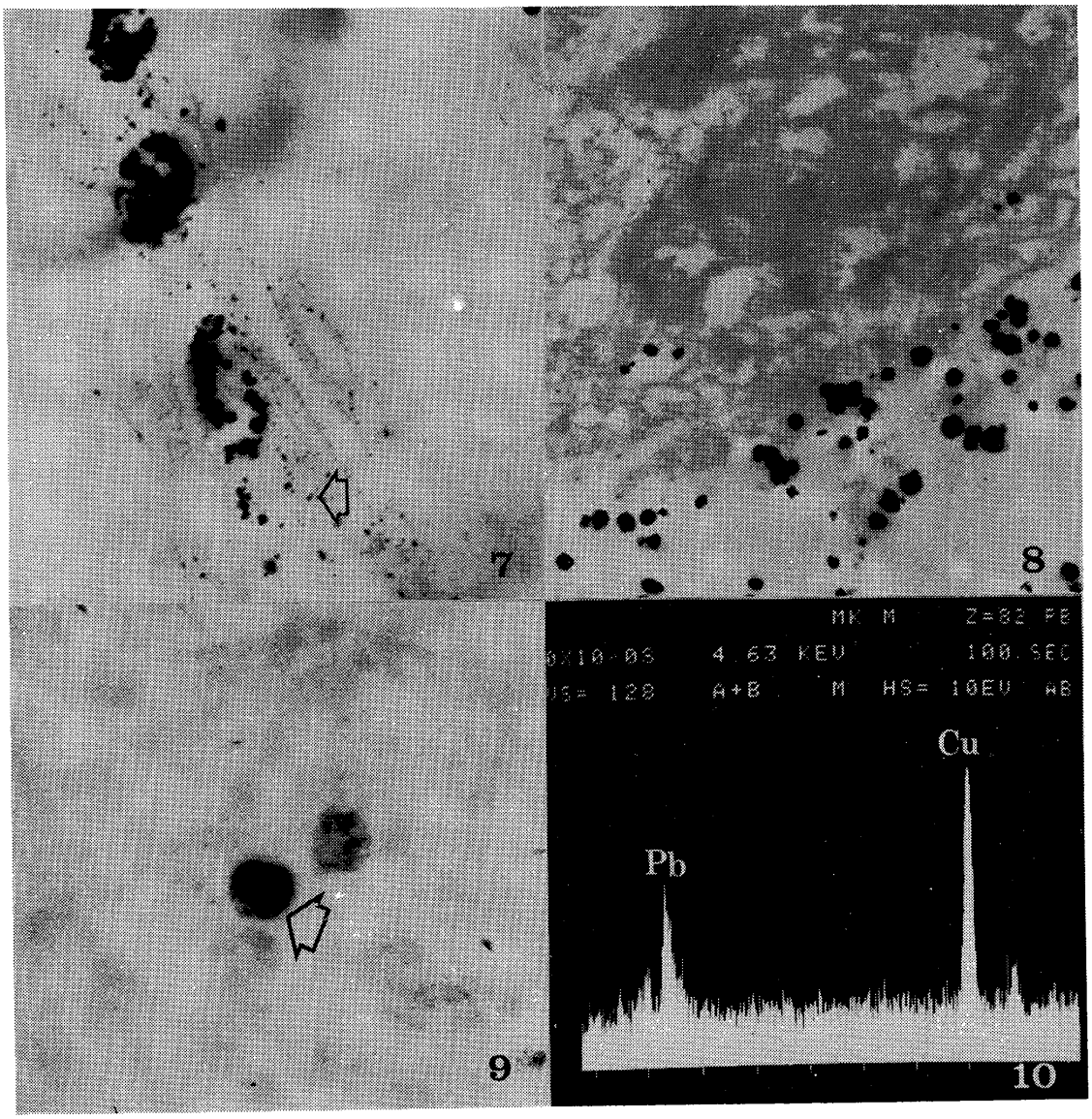
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