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UNIVERSITY OF BERGEN
1981

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THESIS
UNIVERSITY OG BERGEN
1981

Universitetet i Bergen
1981

Acknowledgements

The present investigations were performed at Department of Oral Pathology and Forensic Odontology, School of Dentistry, University of Bergen, during the years of 1975-1980.

I wish to express my sincere gratitude to my teacher in pathology Professor Gisle Bang who introduced me to the field of bone biology and pathology and has given me inestimable support, constructive criticism and ready help during the course of these years.

I am further greatly indebted to Professor Flora Hartveit, Professor Svein Thunold and Professor Ole Didrik Lærum who have provided me with excellent working condition at the Gades Institute, Department of Pathology, University of Bergen.

I would also like to thank Prosector Helge Dalen for valuable help, support and for providing excellent laboratory facilities and equipment at the Laboratory of Clinical Electron Microscopy.

I wish to extend my gratitude to my co-authors Docent Bengt C.Magnusson and Cand.odont Anne Christine Johannessen for most inspiring and rewarding co-operation to this study.

I am indebted to Mr. T. Christensen for preparation of so many photographic prints, to Mr. J. Høydal for carrying out the freeze-drying of the dentin and to Mr. T. Jacob Raa and Mr. A.Nilsen for careing for the animals.

I would also express my thanks to Mrs. Rita Eriksen for valuable technical help, and Cand.odont Paul Riordan who has

helped with correcting the language of the manuscripts. Mrs. Turid Davidsen gave me excellent technical assistance in operating procedures, processing of the light microscopical and electron microscopical material and conscientious secretarial work for which I am grateful. I will also thank Mrs. Liv Wiese-Hansen and Mrs. Berit Birkeland who have performed part of the typing and to the Bergen University Library for providing the literature required.

Financial support has been received from the Norwegian Research Council for Science and Humanities (grant no. C.51.34-3), J.L. Meltzers Høyskolefond, A/S Norsk Dental Depot's fond for Odontologisk Forskning and Dr.med. F.G. Gades Legat.

Bergen, January 1981

Rune Nilsen

The following papers are referred to by Roman numerals in the text.

I Electron microscopy of induced heterotopic bone formation in guinea pigs.
Nilsen, R.
Archs oral Biol.: 1977: 22: 485-493

II Electron microscopic study of mineralization in induced heterotopic bone formation in guinea pigs.
Nilsen, R.
Scand. J. Dent. Res.: 1980: 88: 340-347

III Enzyme histochemistry of induced heterotopic bone formation in guinea pigs.
Nilsen, R. & Magnusson B.C.
Archs oral Biol.: 1979: 24: 833-841

IV Enzyme histochemical studies of acid phosphatase isoenzymes in induced heterotopic bone formation in guinea pigs.
Nilsen,R. & Magnusson, B.C.
Submitted to Scand. J. Dent. Res.

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.
Nilsen, R. & Magnusson, B.C.
Submitted to Scand. J. Dent. Res.

VI Microfilaments in cells associated with induced heterotopic bone formation in guinea pigs.
An immunofluorescence and ultrastructural study.
Nilsen, R.
Acta path.microbiol.scand.Sect.A, 1980: 88: 129-124

VII X-ray microanalytical studies of initial mineralization in induced heterotopic bone formation in guinea pigs.
Johannessen, A.C., Nilsen, R. & Bang, G.
In press Acta Odont. Scand.

Introduction

Bone is the principal mineralized tissue of vertebrates. Dentin, dental cementum and cartilage resemble bone to a great extent, and the matrices consist of collagen and several other proteins together with cementing substances. The ratio between collagen and the cementing substances is higher in bone, dentin and cementum, than it is in cartilage. The basic processes in formation and mineralization of the different matrices seem to be the same (26). The cells producing the matrices resemble each other, although odontoblasts of the dentin and cementoblasts of the acellular cementum are not enclosed in their own matrices. Osteoblasts initially surrounded by the newly formed unmineralized matrix are called osteoid osteocytes. Following mineralization of the matrix they are named osteocytes.

The bone is a dynamic tissue in which resorption and formation of the matrix are constantly taking place. The main type of cell engaged in the resorption of bone is the osteoclast, which is a multinucleated giant cell. The corresponding cell types in cartilage, dentin and dental cementum are chondroclasts, dentinoclasts and cementoclasts.

Many attempts have been made to identify the origin of cells of the hard tissues. In particular the origin and fate of osteoclasts and their relationship to the osteocytes have given rise to various hypotheses (27, 39). Several investigators have proposed that there are two

different cell lines; one for the matrix-producing cells and one for the matrix resorbing cells (20, 25, 27, 29, 44). These authors suggest that osteoclasts derive from blood monocytes. A recent report, (36) has also proposed T-lymphocytes as the source for osteoclasts. Still others have described osteoblasts and osteoclasts as deriving from one common precursor cell and then developing into two different cell lines (40, 42). Bordier (19) on the other hand, has interpreted from studies on the effect of calcitonin that there is only one line of differentiation into bone cells including the following steps: Stem cell ---→preosteoclast ---→ osteoclast---→ preosteoblast---→ osteoblast---→ osteocyte.

It is generally agreed that the hard tissue forming cells are responsible for the synthesis of the organic matrix. The matrix consists mainly of collagen but other organic substances such as phosphoproteins, proteoglycans, glycoproteins and phospholipids constitute a considerable portion (6, 22, 28, 50, 51). The formation of the hard tissues includes synthesis of the organic matrix followed by its subsequent mineralization.

The mechanisms of mineralization have been extensively investigated during the last half century and many theories have been proposed (13, 50). From 1923 to 1928 James C. Watt (52) published several articles in which he suggested that bone cells secreted granules which in turn made up the "heavily calcified areas". His theory was neglected for more than 30 years, while Robison's (41) alkaline phosphatase theory from the same period initiated a

considerable proportion of the studies regarding alkaline phosphatases and mineralization of hard tissue. His theory implied that the enzyme splits organic phosphate compounds and thus increases the local phosphate concentration facilitating the crystal formation. The alkaline phosphatase has since then been shown to be a group of isoenzymes including ATPases and pyrophosphatases as well as more unspecific alkaline phosphatase (23, 33, 43, 47). All these subgroups have been proposed to be involved in the mineralization process in some way. It has further been suggested that alkaline phosphatases are involved in the secretion and maturation of collagen (17, 41).

Until 1970 the prevailing view was that the nucleating mechanism reflected properties of the macromolecules in the extra-cellular matrix. Both the interior and the surface of collagen fibrils were believed during a long period to be the first sites of crystal formation (22, 42). Initial crystal formation between the collagen fibrils has been proposed to be associated with calcium binding glycoproteins and other noncollagenous proteins (16, 50, 51). Degradation of some of these macromolecules has thus been suggested to be an event leading to crystal formation (12, 48) and some authors (38, 45, 48) have proposed that lysosomal enzymes may mediate this degradation.

Since 1970 Watt's theory (52) has been taken more seriously, mainly due to the discovery and increasing understanding of the matrix vesicles. These extra-cellular membrane-bound vesicles were first described in 1967 by Bonucci (18) and Anderson (4). The last 10 years have

given an increasing knowledge of matrix vesicles (5, 6) and they have been shown to have different alkaline phosphatases (5, 6, 7, 25, 46). Many authors have suggested that the first crystals of hard tissues appear in these vesicles (5, 7, 14, 15).

Thyberg (48), who studied guinea pig epiphyseal plate, described two types of matrix vesicles. He found that the initial crystals were always found outside the vesicles, forming small clusters which grew radially throughout the interfibrillar ground substance, without any specific orientation to collagen fibrils. This is in accordance with Urist (50) who suggested that the interfibrillar proteinaceous structure is the substratum for mineralization. Thyberg (48) suggested that matrix vesicles type 1 be interpreted as extruded lysosomes. The occurrence and role of lysosomal enzymes in the matrix vesicles have, however, been disputed by other investigators (1, 2, 35).

Due to the possible role of the matrix-producing cells in the mineralization of hard tissues, attempts have been made to analyse the intra-cellular amounts of minerals in matrix forming cells, using different types of calcium binding dyes (8, 21, 30), as well as microanalytical methods (37). Some groups have also proposed, from studies using calcium binding dyes, that accumulation may occur in the cell body (21, 30), while others have suggested that it is a function of matrix vesicles (1, 2, 3).

The first report of matrix induced bone formation appeared in 1965 (49). Since then Urist and his group have carried out extensive studies on the bone inductive

mechanism of various matrices. Experimental studies have shown that dentin as the inducing matrix in abdominal muscles of guinea pigs gives a high yield of new bone (9, 10, 11). Bone formation that occurs de-novo in post-foetal life should therefore be an unique experimental model for studying formation and mineralization of hard tissues.

Using the advantages of this experimental bone induction system the present investigations were undertaken to study:

1. The cellular differentiation and cellular ultrastructure in early heterotopic bone formation.
2. The ultrastructure of the initial crystal-formation in heterotopic bone formation.
3. The distribution of actin microfilaments in cells involved in induced heterotopic bone formation.
4. The distribution of hydrolytic and oxidative enzymes in the different cell types and tissues appearing in the heterotopic bone forming area with special emphasis on acid phosphatase.
5. Energy dispersive microanalysis of the amount and distribution of calcium and phosphorous during the initial phases of heterotopic bone formation.

Methods used in the present studies

A. Experimental method:

Demineralized freeze-dried allogenic dentin was implanted in abdominal muscles of young male guinea pigs (9, 10). The implants with the surrounding tissue were taken out after 7 to 28 days and prepared according to the histological methods outlined below.

B. Histological methods:

1. Transmission electron microscopy (TEM) on undemineralized tissue (I, II, VI). This method was also combined with selected area electron diffraction (I).

2. Energy dispersive microanalysis (XMA) combined with scanning transmission electron microscopy (STEM) on frozen, freeze-dried and vacuum-infiltrated material (VII).

3. Enzyme histochemistry on unfixed frozen tissue:

Light microscopical studies included:

oxidative enzymes ($NADH_2$- and $NADPH_2$ diaphorases, glucose-6-phosphate dehydrogenase), alkaline phosphatases (non-specific alkaline phosphatase and APTases), acid phosphatases and leucine aminopeptidase (III, IV, V).

Electron microscopical studies were performed for acid phosphatase (V). This method was also combined with x-ray microanalysis on the STEM mode (V).

4. Immunofluorescence on unfixed frozen sections (VI) with an indirect technique using a human anti-actin serum.

General summary and conclusions

The experimental model gave a high yield of newly formed hard tissue. Bone was found most frequently, but cartilage was also observed (I, III, V). This is in keeping with previous light microscopical studies on this model (9, 11). The cellular differentiation revealed two separate lines of cells. One cell line ended in macrophages or multinucleated giant cells (dentinoclasts and osteoclasts) resorbing the implanted dentin or the newly formed bone. The other cell line differentiated to osteoblasts or chondroblasts and ended as osteocytes or chondrocytes (I, V). There was no evidence of a common origin of osteoblasts and osteoclasts (I). The resorbing cells seemed to constitute one single cell line originating from monocytes and not T-lymphocytes as proposed by Milhaud & Labat (36). Resorption of the implanted matrix seemed to appear before the onset of new matrix production (I) and the close relationship observed between matrix-producing cells and resorbing cells (I,III) may suggest that the resorbing cells stimulate the differentiation of matrix forming cells. The close contact between these two groups of cells (I,III), the occurrence of tight junctions between projections from different matrix-producing cells (I,II) together with the high amounts of actin in these projections (VI) suggested that the cells of the hard tissues constitute a unique, complexly interacting cellular system.

Both mononucleated cells, macrophages and the highly differentiated multinucleated giant cells were involved in resorption of the implanted dentin matrix as well as the newly formed hard tissue (I). The lysosomal enzymes seemed to be highly involved in the resorption process, and a distinct EDTA sensitive and tartrate resistant isoenzyme of acid phosphatase was found in these cells (IV). The leucine aminopeptidase, however, which has previously been interpreted as an enzyme of resorptive cells (34) showed only low activity(III). The ruffled border of osteoclasts and dentinoclasts seemed to form a closed area by means of the clear sealing zone (I), which also contained increased amounts of actin (VI). The actin may contribute to the sealing function of this part of the cell.

The cells involved in new matrix production, such as osteoblasts and young osteoid osteocytes (as well as their cartilaginous counterparts), exhibited morphological and enzyme histochemical evidence of high metabolic activity (III,V). An EDTA resistant and tartrate sensitive acid phosphatase was found in the new matrix-producing area (IV). The high amounts of actin observed in these young matrix-producing cells suggested highly active synthesis of matrix components and their transport out of the cell (VI). Two types of matrix vesicles were found extra-cellularly. Type 1 vesicles resembling the intracellular lysosomes appeared most frequently in the areas of cartilaginous differentiation (I). This corresponded to

the areas having high extra- and intra-cellular acid phosphatase activity (IV,V). Acid phosphatase was found extra-cellularly in type 1 matrix vesicles and intra-cellularly in lysomes and endoplasmic reticulum.

Type 2 vesicles whose content were like the intra-cellular matrix (I,II), seemed to bud off from the young matrix-producing cells (I). These vesicles were found most frequently in bone. Type 2 matrix vesicles may be one mechanism by which organic material can be exported from the cell. Channels from the endoplasmic reticulum to the surface, an evident exocytic activity, may be another mechanism of transport out of the cell (I).

The present study showed that the initial crystals appeared in a few matrix vesicles type 2, while most crystals appeared in the space between the collagen fibrils without any relationship to matrix vesicles (II). Alterations of the newly formed matrix into "a calcifiable matrix" as proposed by Urist (50) seemed to be important events which may be mediated by means of enzymes from the matrix-producing cells. Acid phosphatases, alkaline phosphates and leucine aminopeptidase may be involved in this process. The cartilaginous tissue had the highest amounts of type 1 matrix vesicles and constituted also the area of highest extra-cellular activity for acid phosphatase (I, V). Extra-cellular precipitates were found in structures resembling matrix vesicles type 1 (V). Intra-cellularly acid phosphatase was observed in lysosomes and

along the endoplasmic reticulum (V). Incubation for ultrastructural localization was performed on unfixed frozen tissue due to this isoenzyme's aldehyde sensivity (IV, V). A lysosomal nature was suggested for the matrix vesicle type 1 (I, V), as previously proposed by Thyberg (48). Other authors, however, have disagreed with the view that acid phosphatase or other lysosomal enzymes are found in matrix vesicles (6, 35). X-ray microanalysis combined with scanning transmission electron microscopy (STEM), which was performed on the precipitates of acid phosphatase, seemed to be a useful tool also in enzyme electron microscopical studies (V).

The x-ray microanalytical study (VII) on unfixed and freeze-dried tissue showed that the matrix-producing cells were highly involved in accumulation of the minerals. Calcium seemed to appear first in the cytoplasm of osteoblasts, then it was found in the osteoid matrix extracellularly. A high Ca/P ratio could mean that calcium accumulates in the osteoblasts and especially in the osteoid osteocytes. It may then be transported out of the cell bound to organic components. Both glycoproteins (16) and phosphoproteins (51) have exhibited very high calcium-binding capacity. It seems reasonable that the intra - cellular calcium is bound to such an organic component and secreted into extra-cellular space. Vesicles may be one means of this transport out of the cell. Such active transport may account for the high amount of actin found in these cells (VI). The substrate specific ATPase which was found in these young matrix-producing cells (III) has been

proposed to be involved in the transmembranous transport of calcium in the plasma membrane, intra-cellular vesicles and mitochondria (24). The high Ca/P ratio which was observed inside the cell in the present study (VII) has also been seen inside fractions of matrix vesicles from cartilage (53). The finding of high amounts of calcium and phosphorous in the cytoplasm of osteoblasts and young osteocytes (VII) is, however, in contrast to other microanalytical studies (3,6), where it was suggested that matrix vesicles, and not the cell bodies, accumulate calcium; this accumulation was interpreted as active calcium accumulation. Other studies have proposed that mitochondria might be involved in calcium accumulation (32). Although the present study showed that the matrix-producing cells accumulate calcium and phosphorous, it is not impossible that some calcium and phosphorous also may pass outside the cell from blood vessels to the site of initial mineralization. This has, in any case, been shown in dentin formation (31).

The present studies indicate that the highly specialized hard-tissue forming cells are the central co-ordinators of mineralization. The young osteocytes (osteoid osteocytes) seem to be involved in and possibly responsible for making the newly formed matrix calcifiable. They may also be responsible for accumulation of calcium and phosphorous for initiating crystal formation.

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