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Effect of X-Ray Irradiation on Primary Mineralization in Rat Alveolar Bone

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Summary. The effect of X-ray irradiation on the process of primary mineralization in bone was studied by biochemical and ultrastructural methods. A single dose of 1500R was administered to the head region of rats. The animals were examined immediately after irradiation and 1, 2 and 3 weeks later. Fractions of isolated cells and extracellular matrix vesicles were prepared from the maxillary alveolar bone of irradiated and untreated rats by collagenase digestion and differential centrifugation. The protein content and activities of vesicular phosphatases were determined in both fractions. A continuous decrease in the activity of alkaline phosphatase could be observed in both cell and matrix vesicle fractions during a three-week follow up after irradiation. Acid phosphatase activity decreased only in the vesicle fraction.

Transmission electron microscopy of irradiated bone tissue revealed that many matrix vesicles were devoid of intact membranes and apatite crystals. Calcifying nodules were abundant in the matrix without their apparent fusion into larger mineralized structures. It is suggested that irradiation interferes with enzymatic processes associated with primary mineralization.

Key words: Bone – Irradiation – Matrix-vesicles – Osteoblast – Rat

It is widely accepted that extracellular matrix vesicles have an essential role in the initiation of primary mineralization in normal and pathological conditions (Anderson 1976). It has been shown that these organelles manifest high phosphatase activity, associated with the hydrolysis of potential inhibitors of calcification i.e. pyrophosphates, polyphosphonates and ATP (Ali et al. 1970). In addition, this enzymatic activity may result in high

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concentrations of calcium and phosphate within the vesicle and the eventual precipitation of crystaline mineral. The occurrence of matrix vesicles and their enzymatic activity in normal and healing alveolar bone have recently been demonstrated (Sela et al. 1978; Bab et al. 1979; Sela and Bab 1979; Muhlrad et al. 1981; Deutsch et al. 1982). In addition matrix vesicle calcification was studied in pathological tissues obtained from osteogenic neoplasms (Muhlrad et al. 1978; Sela et al. 1981), inflamed and wounded joints (Ali 1977; Stein et al. 1981; Bab et al. 1982), fracture callus (Ketenjian and Arsenis 1975) and ectopic calcifications (Boivin 1975; Kim 1976; Bab et al. 1981). These studies revealed that changes in the activity of matrix vesicles reflect alterations in the metabolic state of osteoblasts and chondroblasts.

Osteoradionecrosis is a frequent sequela following administration of therapeutic doses of irradiation. It has been suggested that this complication results mainly from damage to bone cells and vasculature (Cutler 1951). In addition, it was demonstrated that radiation injuries to differentiating cells inhibit the development of bone, cartilage and teeth (Furstman 1972) and induce a marked delay in the ossification process during healing of alveolar bone sockets (Horn et al. 1979). The detailed mechanisms operative in these instances are yet unknown. The purpose of the present study was to examine biochemical and morphological aspects of primary mineralization in alveolar bone after X-ray irradiation limited to the head region.

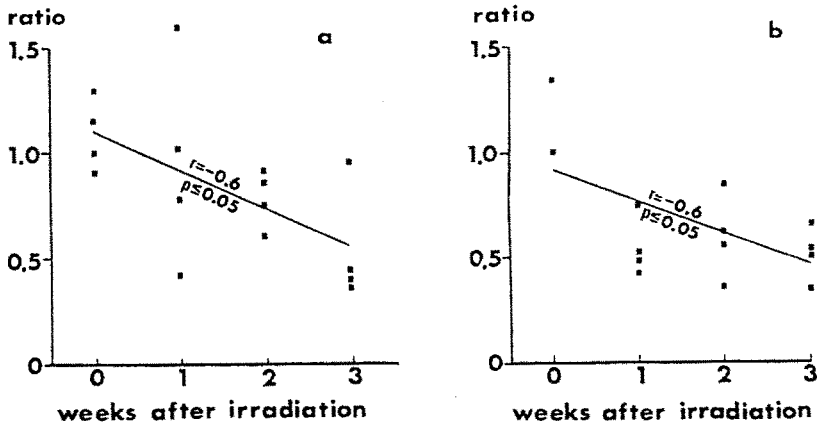
Materials and Methods

1152 male albino rats of the Hebrew University (Sabra) strain weighing 100–120 g each were divided into 16 experimental and 16 control groups. Each group comprised 36 rats, 33 of

Table 1. Ratios of enzymatic specific activities and protein content in cell and matrix vesicle fractions between irradiated rats and paired normal controls (IR/N ratios)

Assay	Weeks after irradiation							
	0		1		2		3	
	Cell[a]	Vesicle[b]	Cell	Vesicle	Cell	Vesicle	Cell	Vesicle
Alkaline phosphatase	1.19[c] ±0.17	1.09 ±0.09	0.59 ±0.12	0.95 ±0.25	0.60 ±0.10	0.77 ±0.06	0.51 ±0.06	0.54 ±0.13
Acid phosphatase	1.18 ±0.17	1.44 ±0.33	0.72 ±0.17	1.37 ±0.22	0.92 ±0.11	0.96 ±0.12	1.08 ±0.48	0.77 ±0.12
ATPase	1.23 ±0.03	1.15 ±0.26	0.90 ±0.05	1.27 ±0.28	1.30 ±0.49	0.48 ±0.02	1.71 ±0.43	1.31 ±0.16
Protein	0.95 ±0.06	1.06 ±0.08	1.61 ±0.34	1.28 ±0.25	0.84 ±0.11	0.79 ±0.13	1.08 ±0.20	1.13 ±0.34

[a] Cell fraction
[b] Matrix vesicle fraction
[c] Mean of 4 experiments ±SEM (standard error of mean)



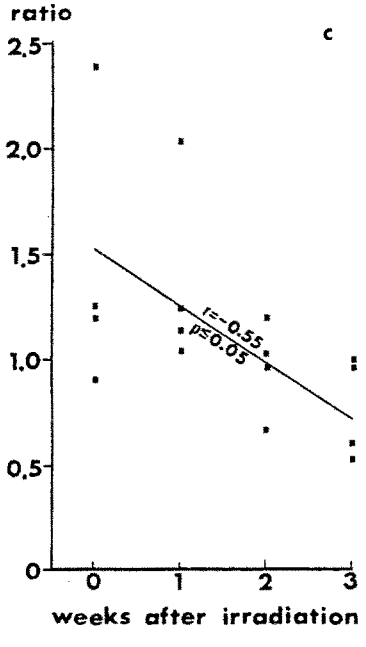


Fig. 1a–c. Regression curves between time after irradiation and enzymatic specific activities.
a alkaline phosphatase activity in matrix vesicle fraction.
b alkaline phosphatase activity in cell fraction.
c acid phosphatase activity in matrix vesicle fractions

these served for biochemical experiments and 3 for electron microscopy. Irradiation of experimental animals was performed in a protective lead shield with the head region left exposed. A single dose of 1500R was administered with a Philips 250 KV X-ray machine. The rats were killed immediately after irradiation and 1, 2 and 3 weeks later. Four pairs of groups, each pair comprising an experimental and a control group, were studied at each time period. Fractions of cells (osteoblasts, osteoclasts and blood cells) and matrix vesicles were prepared as described previously (Bab et al. 1979). In short, maxillary alveolar bone was dissected from anesthetised rats and the molar teeth extracted. Specimens from each group were pooled in cold Gey's solution, cut into 1 mm pieces and subjected to collagenase digestion and differential centrifugation. Material from both groups of each pair were processed simultaneously. The cellular and vesicular fractions thus obtained were subjected to analysis of their phosphatase activity and protein content. The activities of alkaline phosphatase, Mg^{2+}-ATPase

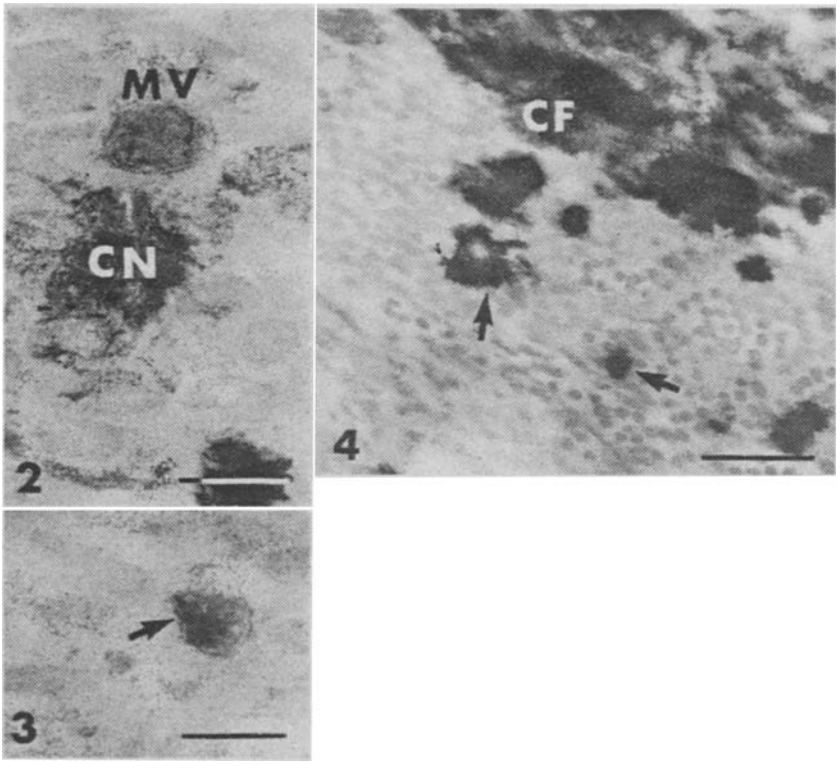


Fig. 2. High power electron-micrograph of matrix vesicle (*MV*) from rat alveolar bone immediately after irradiation. Note initial mineral deposites in vesicle and calcifying nodules (*CN*). Original magnification ×90,000; *Bar* = 0.08 μm

Fig. 3. Electron-micrograph of matrix vesicle from rat alveolar bone immediately after irradiation showing apatite crystal penetrating the vesicular membrane (*arrows*). Original magnification ×63,200; *Bar* = 0.12 μm

Fig. 4. Calcifying front (*CF*) from normal rat alveolar bone. The mineralizing matrix contains calcifying nodules (*arrows*). Original magnification ×24,500; *Bar* = 0.41 μm

and acid phosphatase in the fractions were assayed as outlined previously (Sela et al. 1978). Protein determination was performed according to Bradford (1976). Enzymatic activities were expressed as n mol Pi liberated per mg protein per min (specific activity). Protein content was expressed as mg protein in fraction per gram dissected bone. Ratios of enzymatic activities or protein content were calculated between respective cell or vesicle fractions obtained from each pair of groups of irradiated and normal animals (IR/N ratios). Linear regression curves were plotted to demonstrate the relationship between changes of these ratios and time. Correlation coefficients were considered significant at $p < 0.05$.

Specimens of alveolar bone for transmission electron microscopy were separated from untreated and irradiated rats following intracardial perfusion with 3% glutaraldehyde in 0.02 M TES, pH 7.2, and further fixed in the same solution at 4° C. This was followed by post-fixation in osmium tetroxide in 0.1 M cacodylate buffer pH 7.2, and *en bloc* staining with 0.5% uranyl acetate in veronal buffer pH 5.1 in ice. Embedding in low viscosity resin (Spurr 1969) was preceeded by dehydration in a graded series of alcohols and propylene oxide. Ultrathin sections were restained with uranyl acetate and lead citrate and examined with a Philips EM400 Electron microscope.

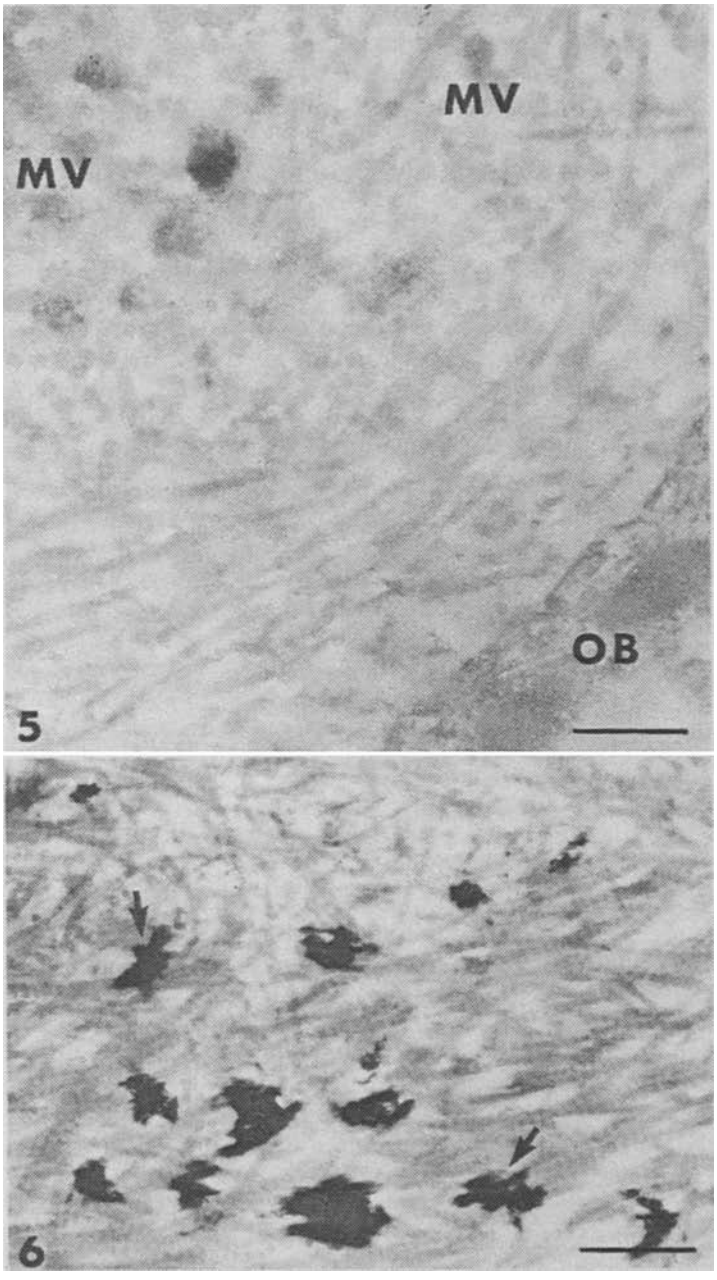


Fig. 5. Matrix from rat alveolar bone 2 weeks after irradiation. *MV*-matrix vesicles devoid of crystaline mineral and indistinct membrane details; *OB*-osteoblast. Original magnification $\times 33{,}000$; *Bar* = 0.23 μm

Fig. 6. Matrix from rat alveolar bone showing abundance of calcifying nodules similar to those found in normal bone (*arrows*). Original magnification $\times 12{,}000$; *Bar* = 0.63 μm

Results

The ratios of specific activities of the different phosphatases and protein content between irradiated and normal (untreated) animals (IR/N ratio) are shown in Table 1. The IR/N ratios of alkaline phosphatase specific activity in the matrix vesicle and cell fractions showed a significant inverse correlation with time after irradiation ($r = -0.6$; $p < 0.05$ for both fractions) (Fig. 1a, b). In addition, a significant inverse correlation was recorded between the IR/N ratios of acid phosphatase specific activity in the vesicle fraction and time after irradiation ($r = -0.55$; $p < 0.05$) (Fig. 1c). No significant trend of changes could be identified in the ratios of acid phosphatase specific activity in the cellular fraction. The ratios of ATPase specific activity and protein content of both cellular and vesicular preparations showed inconsistent alterations during the experimental period.

Transmission electron microscopy revealed features of primary mineralization, i.e. matrix vesicles and calcifying nodules, in both normal tissue and irradiated bone immediately after treatment. Initial mineral deposits were identified in some vesicles (Fig. 2). In other instances the crystals penetrated the vesicular membrane (Fig. 3). Fusion of calcifying nodules resulted in formation of calcifying fronts proper (Fig. 4). One to three weeks after irradiation most of the vesicles in the experimental bone were devoid of crystaline mineral and showed disintegrating membranes (Fig. 5). Electronmicrographs of irradiated bone obtained after 3 weeks showed an abundance of small calcifying nodules 0.07–0.3 μm in diameter similar to those found in normal bone, without apparent evidence of their fusion into larger mineralized structures (Fig. 6).

Discussion

In the present study a single dose of 1500R X-ray irradiation was administered specifically to the head region in rats. The jaw bones served as a source for cells and matrix vesicles. Protection of the other parts of the body was contrived in order to minimize radiation induced systemic changes that have been previously reported to affect different metabolic pathways in bone (Phillips and Kimeldorf 1966). The biochemical analysis of cellular and vesicular fractions reported herein revealed a continuous decrease in the activity of alkaline phosphatase during the three week period after irradiation. This finding is in agreement with the results of Wilkins and Regen (1934) who examined changes in the enzymatic activity of irradiated bone tissue in puppies. They showed that the decrease in the activity of alkaline phosphatase started a few days after exposure; lowest levels of activity were recorded 1–3 weeks afterwards. Studies in bone and cartilage demonstrated that a large proportion of alkaline phosphatase activity is associated with matrix vesicles (Ali et al. 1970; Bab et al. 1979). It may thus be concluded that the present decrease in the activity of this enzyme in cells and matrix vesicles represents respective changes on the tissue level. The mecha-

nism of inhibition of alkaline activity by irradiation could be associated with synthesis of the enzyme or with a direct effect on the molecule. Cohen and Gong (1963) argued that the depressing effect of X-ray irradiation on alkaline phosphatase activity is a result of a damage to the osteoblasts. A study of bone tissue in organ culture revealed that these cells were most sensitive to irradiation (Nijweide et al. 1978).

Examination of acid phosphatase revealed a decrease in activity in the matrix vesicle fraction during the three week period after irradiation. This decrease occurred concomitantly with the descent in the activity of alkaline phosphatase. These results appear to disagree with studies on tissue after irradiation that showed an increase in the activity of acid phosphatase associated with increased lysosomal activity of osteoblasts, macrophages and mononuclear leukocytes (Vaes 1965; Furstman 1972). The present suppression of acid phosphatase activity in the vesicle fraction suggests that this enzyme might be a component of matrix vesicles and is not merely associated with lysosomal contamination of the fractions. Other findings in preparations of isolated and purified matrix vesicles are in support of this conclusion (Bab et al. 1982).

The present biochemical and morphological observations did not show apparent irradiation induced changes in the amount of matrix vesicles. It may therefore be suggested that the inhibition of alkaline phosphatase activity was associated with damage to the vesicular membrane. This inhibition could result in the absence of apatite crystals from vesicles and in growth failure of calcifying nodules and completion of the mineralization process. A recent study on the effect of irradiation of bone during would healing (Horn et al. 1979) demonstrated variable interferences with cellular activity, in particular with the production of calcifying matrix. It is thus suggested that the retardation of bone formation induced by irradiation may be partly linked to alterations in the process of primary mineralization.

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