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Early Ultrastructural Changes in Osteocytes from the Proximal Tibial Metaphysis of Mice after the Incorporation of ^{224}Ra [1, 2]

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Osteocytes from the proximal tibial metaphysis of mice were examined electron microscopically at various time intervals after the incorporation of 1, 1.5, or 5 μCi/kg of body weight ^{224}Ra. The animals were sacrificed 2 hr, 24 hr, or 5 days following administration of the short-lived bone-seeking radionuclide. Only the younger, osteoblast-like osteocytes from the undecalcified trabecular bone were studied. Compared with the osteocytes from bone tissue of control animals, about half of the cells examined in specimens from animals treated with ^{224}Ra showed ultrastructural alterations. The chromatin in the nuclei of many osteocytes was condensed. The mitochondria showed various signs of damage such as loss of cristae, swelling, and dissociation or disruption of both limiting membranes. Additionally, giant mitochondria were found. The rough-surfaced endoplasmic reticulum was sometimes dilated and formed large cisternae. The Golgi complex was vacuolated. The pericellular spaces of lacunae containing severely damaged osteocytes were enlarged. It is thought that the early ultrastructural changes in osteocytes from the trabecular bone of mice resulted from the effects of direct irradiation upon the cells. Probably, all osteocytes which showed lesions were situated within the range of the α radiation emitted from the incorporated ^{224}Ra which was randomly distributed in the mineralized bone matrix of the trabeculae. The mitochondria of the osteoblast-like osteocytes appeared to be the cellular organelles most sensitive to the effects of ^{224}Ra administration.

INTRODUCTION

Radium-224 is a short-lived bone-seeking radionuclide with a half-life of 3.64 days. This α-emitter is still used in human medicine for the therapy of certain bone diseases, although the occurrence of osteosarcomas in ^{224}Ra-treated patients has been reported (*1*).

Quantitative autoradiography has revealed that ^{224}Ra, although being called a bone volume seeker, is deposited 2–48 hr after the injection into mice in the

[1] Dedicated to Prof. Dr. med. Dr. h. c. mult. Erich Letterer on the occasion of his 80th birthday.

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endosteal and periosteal surface regions of the compact mineralized bone rather than in its central volume regions.[3] Various early effects of the incorporated radionuclide upon osseous tissue from the proximal tibial metaphysis of rats and mice have been reported in histochemical (*2*), light (*3*), and electron microscopic investigations (*4*). Gössner (*3*) observed that osteocytes in the metaphyseal trabecular bone of mice were undergoing necrosis 3 days after the incorporation of 25, 75, or 150 μCi/kg of body weight ^{224}Ra. Osteocyte destruction leading to the disappearance of cells reached a peak on the 7th day following ^{224}Ra administration.

In the present study, osteocytes from the proximal tibial metaphysis of mice were examined electron microscopically at short time intervals after the incorporation of small amounts of ^{224}Ra, in order to obtain information about the effects of the incorporated radionuclide upon the ultrastructure of the bone cells. It was of interest to learn which of the cellular organelles in osteocytes were most sensitive to the short-lived α-emitter. It was not intended to carry out a statistical evaluation of ^{224}Ra-induced ultrastructural alterations in osteocytes. This was impossible because the numbers of examined cells were too low, due to the fact that osteocytes make up only a small volume fraction in trabecular bone tissue.

Only a particular type of osteocytes from the undecalcified trabecular bone were studied. These were the osteocytes of the formative period in which the cells have been described as resembling osteoblasts (*5–8*).

Jande and Bélanger (*5*) observed in the trabecular bone of the lower jaw of young rats three types of osteocytes which could be distinguished by characteristic differences in ultrastructure and function. Younger, osteoblast-like osteocytes associated with bone formation were located in the peripheral regions of the trabeculae close to the endosteal bone surface. Further inwards in the bone formative osteocytes were absent and resorptive osteocytes associated with osteolysis appeared. Toward the middle of each trabecula, where the older cells were present, degenerative osteocytes were prominent and cell death occurred naturally.

Similar different types of osteocytes have been found in the diaphyseal trabecular bone of the tibia of young chickens (*6*), the endosteal cortical bone of the tibia of rats (*7*), and in the osteons and interstitial bone of the femoral shaft of young adult rabbits (*8*). The fine structure of osteocytes displaying osteoblastic and osteoclastic activity has also been described in the diaphyseal compact bone of the femur of adult mice (*9*).

To avoid a false interpretation of ultrastructural changes in osteocytes from the proximal tibial metaphysis of mice after the incorporation of ^{224}Ra, therefore, only the younger, osteoblast-like osteocytes were considered in this study.

MATERIALS AND METHODS

Thirty-two female NMRI mice (Neuherberg strain), 3–4 weeks old and weighing approximately 21 g, were used for the experiments. They were divided

[3] B. Hindringer and W. Gössner, Microscopic distribution of shortlived α-emitting bone seekers studied by quantitative autoradiography. In *Contamination by Bone-Seeking Radionuclides and Radioprotection*, pp. 340–359. Société Française de Radioprotection, Paris, 1971.

TABLE I

Groups of Mice Sacrificed at Short Time Intervals after the Incorporation of Small Amounts of ^{224}Ra

Group of mice	*Incorporated amount of ^{224}Ra ($\mu Ci/kg$ of body weight)*	*Time interval between the injection of ^{224}Ra and sacrifice of the mice*
I	1	24 hr
II	1	5 days
III	1.5	2 hr
IV	5	24 hr
V	5	5 days

into eight experimental groups consisting of four animals each. Twenty mice received activities of ^{224}Ra of either 1 (groups I and II), 1.5 (group III), or 5 (groups IV and V) $\mu Ci/kg$ of body weight by single intraperitoneal injections (Table I). The incorporation of each amount of ^{224}Ra, injected into a mouse, was confirmed by whole-body measurement of each animal before sacrifice. Additionally, autoradiograms of the left tibiae of all ^{224}Ra-treated animals were made according to the method described previously.[3]

Radium-224 (Thorium X) was used as commercially supplied (Amersham Buchler GmbH & Co. KG, Braunschweig, Germany) in the form of a radium chloride solution containing 2–6.2 mg/ml of calcium chloride. The solution contained impurities consisting of the long-lived radionuclides ^{228}Th, ^{226}Ra, and ^{210}Pb whose total activities were not more than 5×10^{-6} Ci per Ci of ^{224}Ra (*10*). Before administration to the animals, the radium chloride solution was diluted about 1:100 with physiological saline to obtain the different amounts of ^{224}Ra. The maximal possible amount of calcium chloride which the mice could receive with the various dilutions of the radium chloride was approximately 0.8 mg/kg of body weight.

Four mice (group VI) which did not receive a ^{224}Ra injection served as controls. Eight mice, each of which received an injection of 1 mg/kg of body weight calcium chloride, were used as further controls (groups VII and VIII).

The mice were maintained on a standard pellet diet together with drinking water *ad libitum*. All animals were killed by inhalation of chloroform vapors. The mice of group VI were sacrificed at the beginning of the experiments. The animals of group III were sacrificed 2 hr after the incorporation of ^{224}Ra, groups I and IV were sacrificed 24 hr after, and groups II and V, 5 days after the injection of the radionuclide (Table I). The control groups VII and VIII were sacrificed 2 and 24 hr, respectively, following the injection of calcium chloride.

Immediately after sacrifice of the mice, the proximal part of the right tibia of each animal was rapidly removed. Two tibial specimens from each experimental group were placed in cold phosphate-buffered 1% osmium tetroxide (pH 7.3; *11*, *12*) and the other two specimens were placed in cold phosphate-buffered 6.25%

glutaraldehyde. While the specimens were immersed in the fixative solutions, the attached soft tissue was removed from the bones which were then split longitudinally. Both halves of each tibial metaphysis were cut into cubes of about 1 mm^3.

The tissue cubes were fixed either in the osmium tetroxide for 2 hr or in the glutaraldehyde for 4 hr followed by postfixation in cold phosphate-buffered 1% osmium tetroxide for 2 hr. Following dehydration through a graded series of alcohols to propylene oxide, the specimens were embedded in Epon 812. Sections were cut with a diamond knife on a Reichert Om U2 microtome. Semithin sections were stained with methylene blue and examined in the light microscope to select regions of the specimens with well-preserved trabecular bone tissue for further sectioning. Ultrathin sections were mounted on uncoated copper grids and stained with aqueous solutions of uranyl acetate and lead citrate. They were examined in an AEI EM6B electron microscope at 80 kV.

Several sections cut from at least three bone tissue blocks of each animal were studied. We examined all osteoblast-like osteocytes which we could randomly find in the ultrathin sections. The numbers of osteocytes occurring in each section varied greatly due to difficulties in sectioning the undecalcified tissue.

RESULTS

The relatively thin trabeculae from the proximal tibial metaphysis of the young untreated control mice contained mainly younger, osteoblast-like osteo-

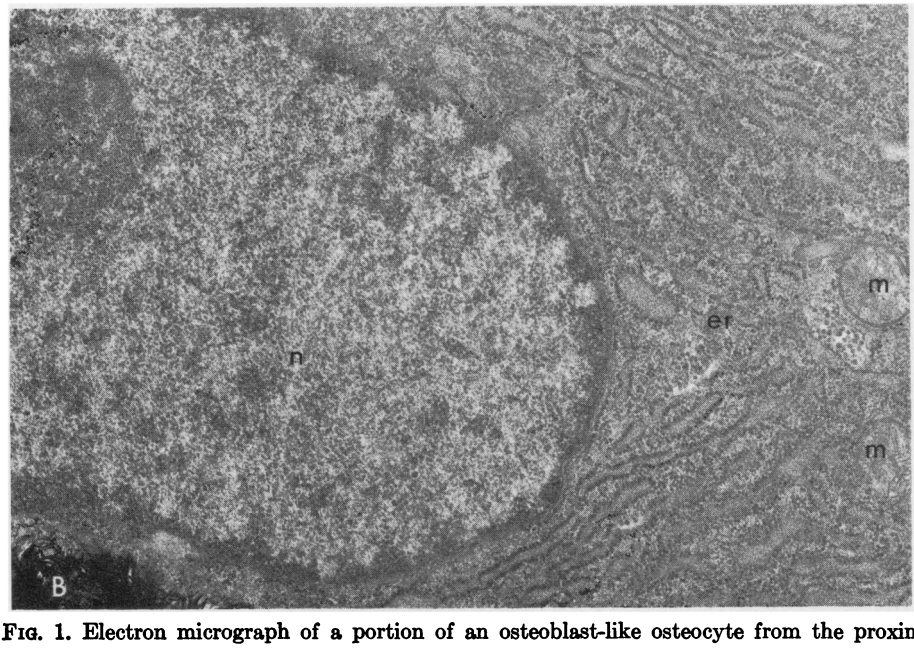

FIG. 1. Electron micrograph of a portion of an osteoblast-like osteocyte from the proximal tibial metaphysis of an untreated control mouse. The chromatin in the nucleus (n) is evenly dispersed. The membranes of the rough-surfaced endoplasmic reticulum (er) form narrow tubules. Two mitochondria (m) show a normal ultrastructure; B, mineralized bone matrix (glutaraldehyde–osmium fixation). ×22,500.

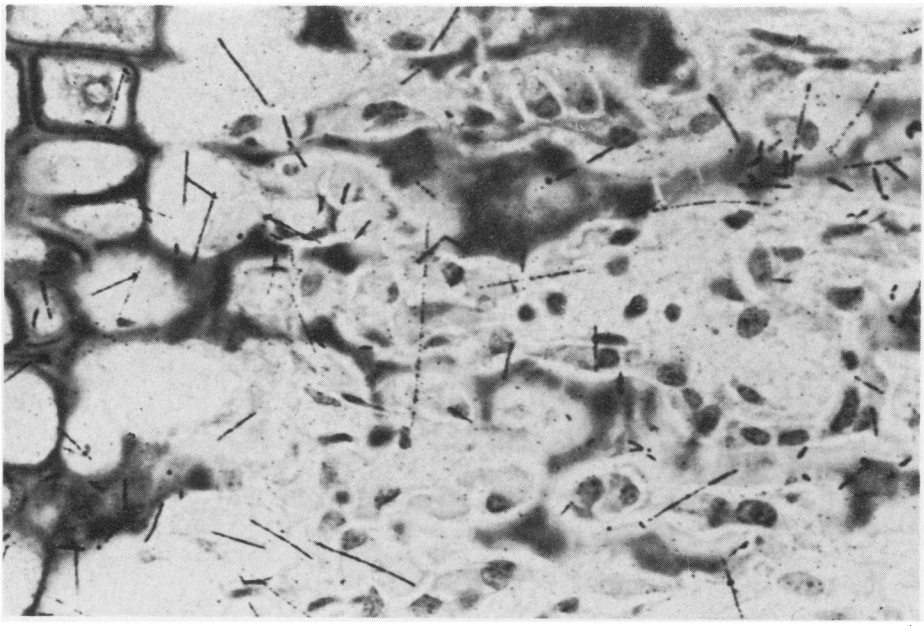

FIG. 2. Autoradiogram showing a portion of the proximal tibial metaphysis from a ^{224}Ra-treated mouse. The animal was sacrificed 2 hr after the incorporation of 1.5 μCi/kg of ^{224}Ra. The random distribution of the α-tracks is clearly visible (short fixation in ethyl-alcohol, methacrylate-embedding, stripping film technique, exposure time 6 days). $\times$500.

cytes. Their fine structure resembled that which several authors have observed in osteoblast-like osteocytes from normal bone tissue of different species (*5–9*). There were no significant differences in the quality of preservation of the osseous tissue between glutaraldehyde-osmium and primary osmium fixation. The osteoblast-like osteocytes examined in those control animals which had received calcium chloride had an ultrastructural appearance similar to that of the osteocytes from the untreated control mice (Fig. 1).

The autoradiographic studies revealed a random distribution of α-tracks in the proximal tibial metaphysis of all ^{224}Ra-treated mice (Fig. 2). No quantitative evaluations of the autoradiograms were made.

About half of the osteoblast-like osteocytes examined in specimens from animals which had received ^{224}Ra showed ultrastructural changes, as is described below.

Nucleus

Two hours after the incorporation of 1.5 μCi/kg and both 24 hr and 5 days following the incorporation of either 1 or 5 μCi/kg of ^{224}Ra, the chromatin in the nuclei of many osteocytes was partially condensed, mainly in the marginal zone (Figs. 3, 4, 10). It was only in the specimens from animals sacrificed both 24 hr and 5 days after the injection of either 1 or 5 μCi/kg of ^{224}Ra that some osteocytes

FIG. 3. Osteoblast-like osteocyte 24 hr after the incorporation of 1 μCi/kg of ^{224}Ra. The nucleus (n) shows an extensive, marginal area of condensed chromatin (arrows). The perinuclear space is partially enlarged (double arrow). The rough-surfaced endoplasmic reticulum (er) is dilated into cisternae (asterisks). Three mitochondria (m) show loss of cristae and condensations in the matrix; C, collagen fibrils; B, mineralized bone matrix (glutaraldehyde–osmium fixation). ×22,000.

with nearly homogeneous, dense nuclei were observed. Such nuclei were spherical in shape and considerably reduced in size (Fig. 4).

The space between the membranes of the nuclear envelope was enlarged in many osteocytes in which the nuclear chromatin showed stages of condensation (Fig. 3). Cells showing damages in their nuclei always showed alterations in other cellular organelles.

Mitochondria

The most frequently observed changes in osteocytes following ^{224}Ra incorporation occurred in the mitochondria. These were often the only cellular changes

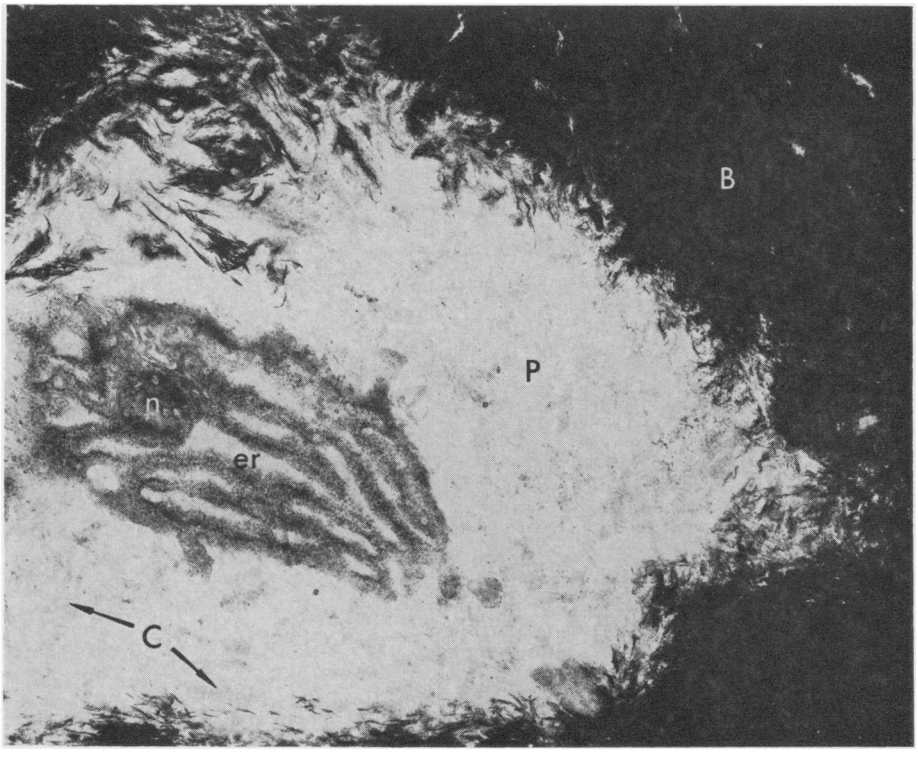

FIG. 4. An osteocyte, 24 hr after the administration of 5 μCi/kg of ^{224}Ra, is located in its rough-surfaced lacuna. The pericellular space (P) of the lacuna is grossly enlarged. The nucleus (n) is very dense and small. The rough-surfaced endoplasmic reticulum (er) is dilated; C, collagen fibrils; B, mineralized bone matrix (glutaraldehyde–osmium fixation). ×17,250.

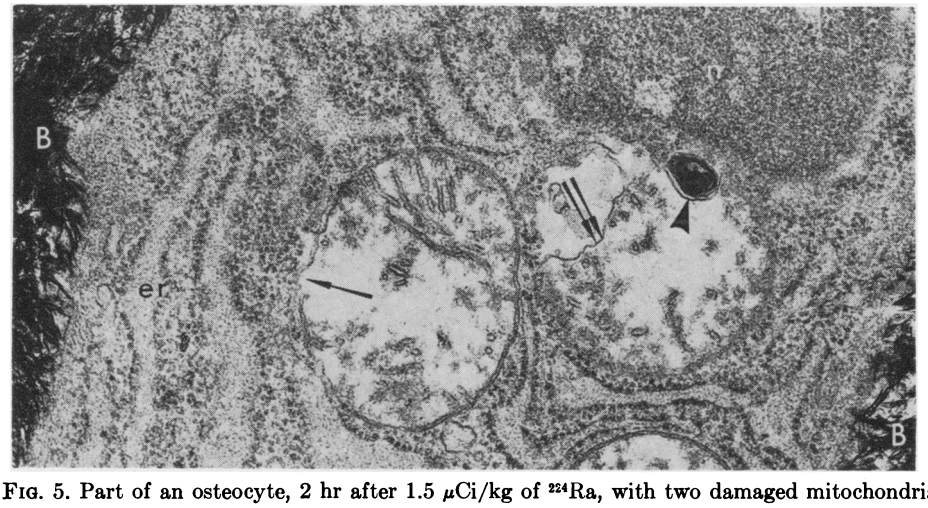

FIG. 5. Part of an osteocyte, 2 hr after 1.5 μCi/kg of ^{224}Ra, with two damaged mitochondria. Both are swollen, have an electron-lucent matrix, and lack cristae. The outer and inner limiting membranes of the mitochondrion to the left are ruptured (arrow), while those of the one to the right appear to be dissociated (the double arrow indicates the inner limiting membrane). This mitochondrion also contains a myelin-like figure (arrowhead). The nucleus (n) of the cell is condensed; er, rough-surfaced endoplasmic reticulum; B, mineralized bone matrix. ×31,500.

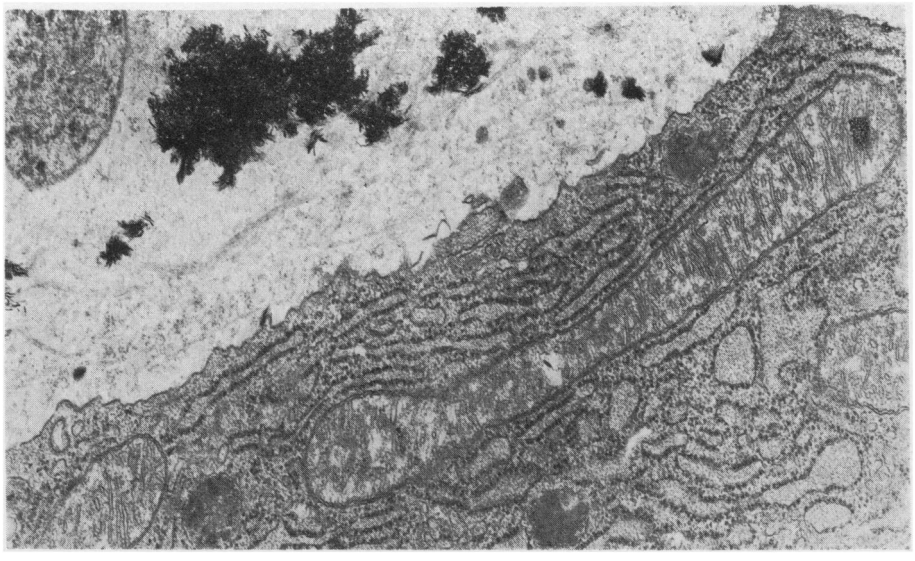

FIG. 6. An elongated giant mitochondrion, about four times the size of the oval-formed one in the lower left corner of the picture, in an osteoblast-like osteocyte 5 days after 5 μCi/kg of ^{224}Ra. ×24,000.

which were to be seen. Alterations in mitochondria consisted of partial to nearly complete loss of mitochondrial cristae, swelling, and dissociation or disruption of both limiting mitochondrial membranes.

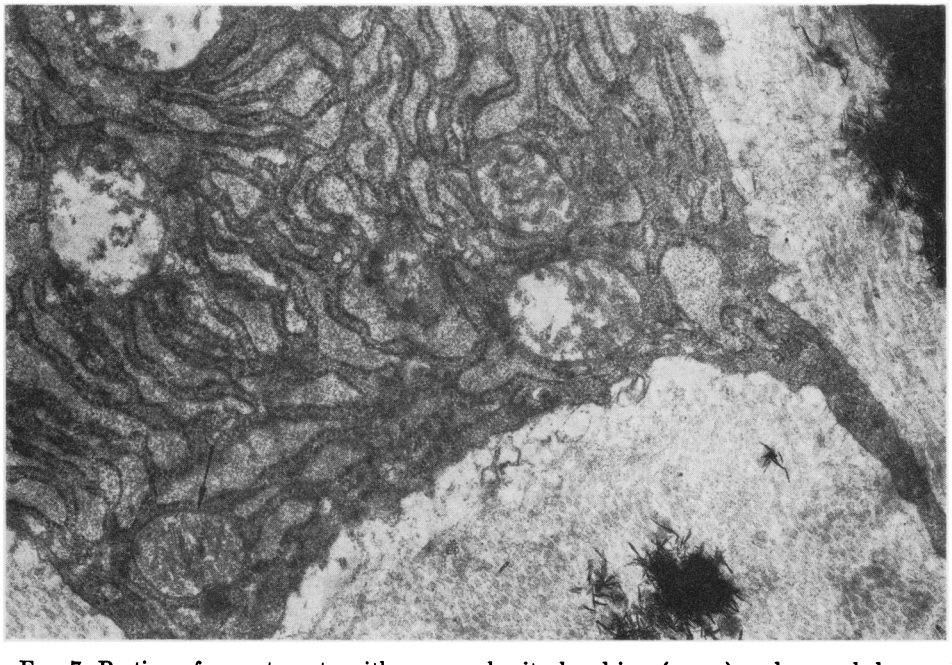

FIG. 7. Portion of an osteocyte with a normal mitochondrion (arrow) and several damaged ones 24 hr following the administration of 5 μCi/kg of ^{224}Ra. The RER is dilated. ×22,000.

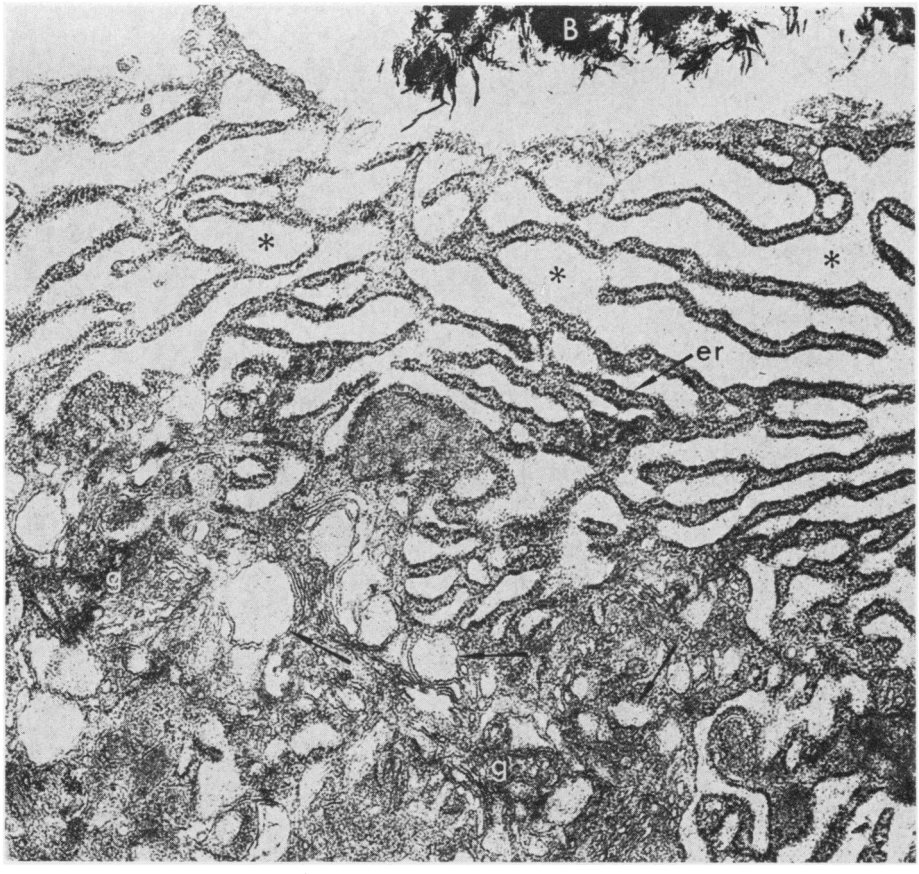

FIG. 8. Part of an osteoblast-like osteocyte 5 days after the incorporation of 5 μCi/kg of ^{224}Ra. The tubular elements of the rough-surfaced endoplasmic reticulum (er) are severely dilated and form large cisternae (asterisks), occasionally filled with a fine flocculent material. The tubular and vesicular elements of the Golgi complex (g) are also dilated and form vacuoles of various sizes (arrows); B, mineralized bone matrix. ×23,250.

Some mitochondria showed partial cristolysis with the development of clear areas or cloudy condensations in the matrix (Figs. 3, 5, 7, 9, 10). Other mitochondria were swollen, had an electron-lucent matrix and almost completely lacked cristae (Figs. 5, 7, 10). Dissociation or rupture of the outer and inner limiting mitochondrial membranes, which appeared in swollen mitochondria, were observed only in a small number of cells (Fig. 5).

Additionally, elongated giant mitochondria, up to three to four times the size of normal oval-formed ones, were sometimes found in osteoblast-like osteocytes after ^{224}Ra administration (Fig. 6). Such mitochondria, which were not noted in osteocytes from control animals, displayed a normal array of cristae.

Occasionally, altered as well as normal mitochondria were seen in the same cell (Figs. 6, 7). The mitochondrial changes were observed in osteocytes from all animals which were sacrificed at various time intervals after the injection of differing amounts of ^{224}Ra.

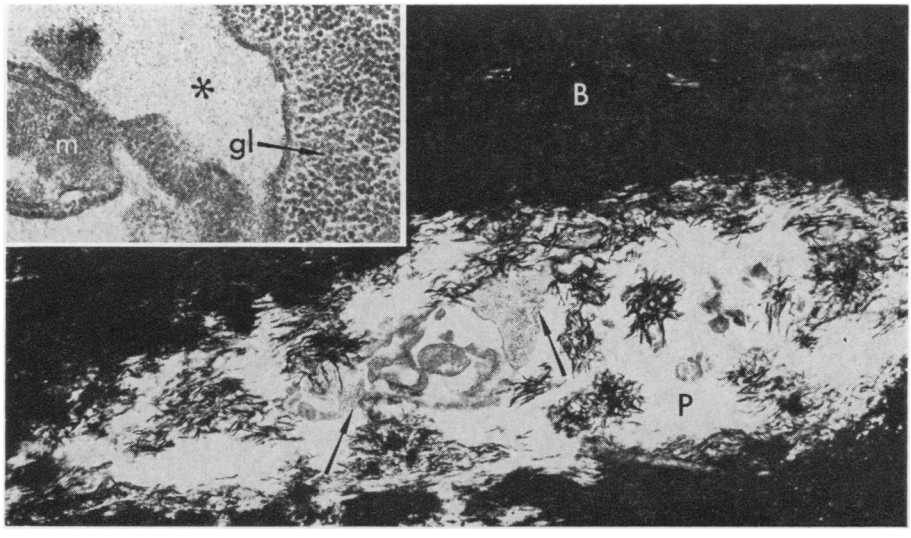

FIG. 9. A severely damaged osteocyte in its lacuna 24 hr after the incorporation of 1 μCi/kg of ^{224}Ra. There are nodules of calcification in the grossly enlarged pericellular space (P) of the lacuna. Accumulations of glycogen granules (arrows) appear in the osteocytic cytoplasm; B, mineralized bone matrix. ×9000. The inset shows a portion of the cytoplasm at a higher magnification; m, mitochondrion with partial condensation of the matrix; gl, glycogen granules; the asterisk indicates a large cisterna of the RER filled with a fine flocculent material. ×40,000.

Rough-Surfaced Endoplasmic Reticulum

Normally, the membranes of the rough-surfaced endoplasmic reticulum (RER) were arranged in almost parallel rows (Fig. 1). In many osteocytes, following the incorporation of ^{224}Ra, the tubular elements of the RER were dilated and formed cisternae of various sizes (Figs. 3, 4, 7–9). The cisternae were filled with a fine flocculent material.

These alterations in the appearance of the RER were found only in osteocytes showing changes in mitochondrial and nuclear structure.

Smooth-Surfaced Endoplasmic Reticulum (Golgi Complex)

Occasionally, the tubular and vesicular elements of the osteocytic Golgi complex were dilated after the incorporation of ^{224}Ra, forming smooth-surfaced vacuoles of various sizes (Fig. 8). These changes in the smooth-surfaced endoplasmic reticulum were always observed together with alterations in the RER.

Osteocytes displaying changes in mitochondrial and nuclear structure, together with alterations of the RER and Golgi complex, occasionally showed accumulations of glycogen granules in the cytoplasm (Fig. 9). The pericellular spaces of lacunae containing such severely damaged osteocytes were enlarged (Figs. 4, 9). In several of these lacunae nodular calcification was seen (Fig. 9).

Often both normal and damaged osteoblast-like osteocytes were observed in the same trabecula near each other in specimens from animals which had been

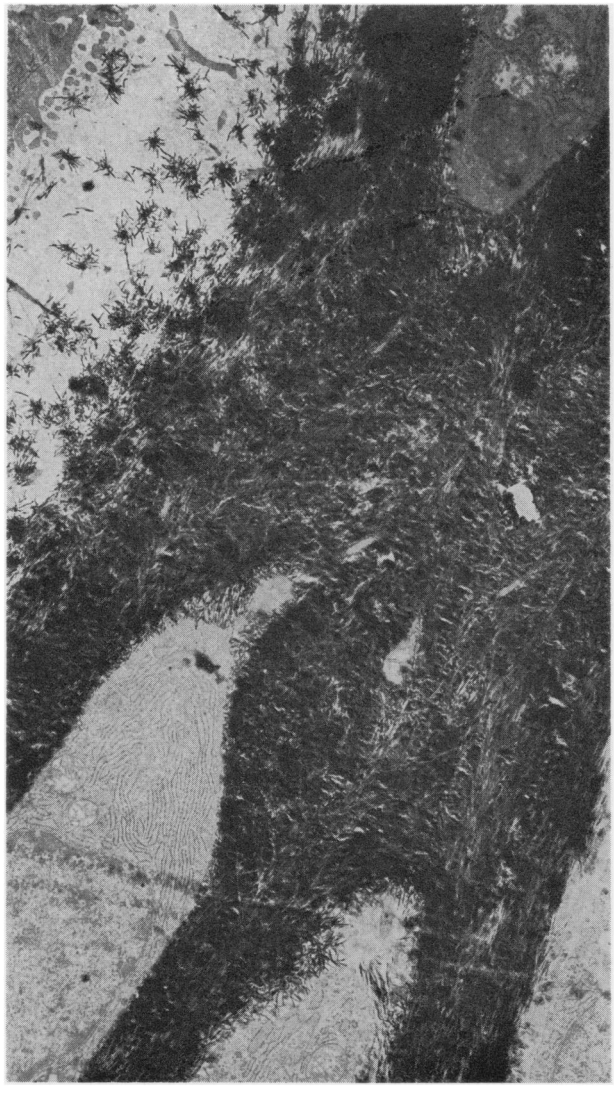

FIG. 10. Part of a trabecula containing portions of three osteoblast-like osteocytes 2 hr following the treatment with 1.5 μCi/kg of ^{224}Ra. The cell in the upper right corner of the picture has a condensed nucleus and swollen, electron-lucent mitochondria which lack cristae. A part of this osteocyte is to be seen in Fig. 5 at a higher magnification. The organelles of the two cells in the lower part of the picture show a normal morphology. ×6000.

treated with ^{224}Ra (Fig. 10). The minimal cell-to-cell distance in these cases was about 10 μm.

DISCUSSION

Various ultrastructural changes were observed in osteoblast-like osteocytes from the proximal tibial metaphysis of mice 2 hr after the incorporation of

1.5 μCi/kg and both 24 hr and 5 days after either 1 or 5 μCi/kg of body weight ^{224}Ra. About half of the cells were damaged. As already mentioned, it was impossible to find differences in the numbers of cells showing lesions between specimens from animals sacrificed at various time intervals after the incorporation of differing amounts of activity.

There were differences in the nuclear changes between specimens from animals sacrificed at various time intervals after the injection of ^{224}Ra. Two hours after 1.5 μCi/kg of ^{224}Ra nuclei with partially condensed chromatin were observed. They were obviously undergoing pyknosis. Osteocytes with very small and dense, i.e., completely pyknotic nuclei appeared at a period later than 2 hr following ^{224}Ra administration. Such nuclei were found both 24 hr and 5 days after either 1 or 5 μCi/kg of ^{224}Ra. These differences in nuclear changes, therefore, do not seem to be related to the amounts of administered ^{224}Ra but to the time intervals after administration.

It is almost certain that the early changes observed in osteoblast-like osteocytes, after the incorporation of ^{224}Ra, are degenerative, leading to necrosis of those cells which were irreversibly damaged. According to Jee (*13*), the damage of the bone cells was probably caused by the α radiation emitted from the^{224} Ra which was deposited in the trabecular mineralized bone matrix surrounding the cells. The ultrastructural alterations in the bone cells are similar to those reported in cells from various tissues of mice or other species after local or total-body X irradiation (*14–16*). The observed giant mitochondria were presumably the morphological expression of a cellular process trying to compensate for damaged mitochondria by the hypertrophy of others. Cottier (*14*) reported the occurrence of similarly elongated mitochondria in lymphoblasts of mice following total-body X irradiation.

Often both normal and damaged osteoblast-like osteocytes were observed in the same trabecula near each other in specimens from animals treated with ^{224}Ra. It is probable that those osteocytes which were damaged were situated within the range of the α radiation of the randomly distributed ^{224}Ra, whereas the normal ones were not (see Fig. 2). Damaged and undamaged cells had often the same topographical location and sometimes almost the same distances to the borders of the trabeculae within which they were located (see Fig. 10). Therefore, it seems unlikely that ultrastructural differences in these cells were due to variations in penetration of the fixatives during immersion. These facts also suggest that ^{224}Ra-induced lesions in osteocytes from trabecular bone result from direct irradiation rather than from radiation-induced vascular damage, i.e., ischemia. Ischemia would not have caused damage in single cells but in most cells from a more or less large area of bone tissue. This assumption is supported by the fact that the ultrastructure of the capillaries in the proximal tibial metaphysis of mice did not show severe damage following ^{224}Ra incorporation (*4*). Jee (*13*) found evidence that ^{239}Pu-induced osteocyte death in trabecular bone from dogs was from the α radiation and not from ischemia.

Often the only ultrastructural alterations in osteocytes observed after the incorporation of ^{224}Ra occurred in the mitochondria, while the rest of the cell

appeared to be normal. This finding may indicate that the mitochondrion of the osteoblast-like osteocyte is the cellular organelle most sensitive to the effects of ^{224}Ra administration.

The accumulation of glycogen granules, sometimes observed in the cytoplasm of osteocytes undergoing degeneration, corresponds to a similar observation made during naturally occurring osteocyte degeneration (*5*). The enlargement of the lacunar pericellular spaces of severely damaged osteoblast-like osteocytes was presumably due to shrinkage of the cells and demineralization along the lacunar borders. A similar finding has been reported in older osteocytes under normal conditions. In the phases of both resorption and degeneration, osteocytes are normally associated with osteolysis resulting in the formation of large acellular lacunae (*5*, *6*, *8*).

The ^{224}Ra-induced degenerative changes observed in osteoblast-like osteocytes from trabecular bone are somewhat similar to the morphological changes occurring naturally in aging osteocytes. Thus, compared with the normally slow involutional process, the incorporation of ^{224}Ra induces a hyperinvolution of osteocytes.

No disintegration of osteocytes was observed after the incorporation of ^{224}Ra in any of the material examined. There was no evidence of either lacunae filled with cellular debris or empty lacunae, such as have been observed by other authors, in light (*13*, *17*) and electron microscopic studies[4] of various osseous tissues after the incorporation of α- or β-emitting bone-seeking radionuclides. These different results are probably due to the fact that, in the present study, osseous tissue was examined at very short time intervals after the incorporation of small amounts of ^{224}Ra, in order to discover early ultrastructural alterations in osteoblast-like osteocytes resulting from the administration of the short-lived radionuclide.

The significance of ^{224}Ra-induced early ultrastructural changes in osteoblast-like osteocytes as well as in other cell types from murine trabecular bone tissue (*4*) remains to be clarified, especially in relation to late effects of ^{224}Ra. In this respect, it is interesting to note that the osteosarcoma incidence in NMRI mice having received 1 or 5 μCi/kg of body weight ^{224}Ra at the age of three to four weeks is 7 and 10–22%, respectively (*18*). At present, little information is available about the initial events preceding the development of radionuclide-induced osteosarcomas.

Early biological effects due to low-level α radiation, as they are reported in the present study, may be surprising. But one must consider that the short-lived ^{224}Ra delivers its energy and that of its α-emitting daughters for the most part at the sites where it is initially deposited. Thus, cells may receive doses that may differ considerably from the calculated average skeletal dose (*10*). Additionally, it has been found that the relative biological effectiveness of the α radiation emitted from ^{224}Ra increases at low dose levels resulting in a higher tumor yield per dose unit (*18*). It is further well known that α radiation is more effective than β or γ radiation in producing various biological effects.

[4] W. S. Whitson and W. S. S. Jee, Electron microscopy of radiation damage in bone. In *Research in Radiobiology* (T. F. Dougherty, Ed.), COO-119-244, pp. 250–269. Radiobiology Division of the Department of Anatomy, University of Utah, College of Medicine, 1971.

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