AN ULTRACYTOCHEMICAL STUDY OF CYTOCHROME OXIDASE ACTIVITY IN RAT METAPHYSEAL BONE CELLS

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Cytochrome oxidase activity in rat metaphyseal bone cells was determined by enzyme cytochemistry. Factors such as fixation, incubation time and permeability of diaminobenzidine and cytochrome c, which might affect cytochemical reactivity of the enzyme, were evaluated. Three levels of cytochrome oxidase reactivity were observed in the mitochondria of each type of bone cell. These were 1) staining of the entire membrane system of mitochondria (type I), 2) partial staining in which only cristae were stained (type II) and 3) no staining (type III). In most osteoclasts, type I mitochondria predominated, and types II and III were few in number. Osteoblasts possessed mixed populations of mitochondria consisting mainly of types I and II, but some osteoblasts appeared to be in a functional transition and contained mitochondria weak in activity. Osteocytes had largely type III and few type II mitochondria. The results indicate that heterogeneity of cytochrome oxidase activity exists in bone cell mitochondria and that osteoclasts have the most active aerobic metabolism among bone cells, whereas osteoblasts favor oxidative metabolism and osteocytes are anaerobic.

Bone includes three cell types, namely, osteoblasts, osteocytes, and osteoclasts. Osteoblasts are located on bone surfaces and primarily participate in bone formation. Osteocytes are buried in the bone matrix and are responsible for bone apposition and resorption under certain conditions. Osteoclasts are located on bone surfaces and resorb calcified bone (18). It is well known that osteoblasts (8, 15, 19, 20), osteocytes (15, 19, 20) and osteoclasts (14, 17, 20) take up and retain calcium in mitochondria. It is suggested that mitochondrial calcium plays a role in cell mediated calcium movement during the processes of bone formation and resorption (5, 12, 18). Calcium regulatory hormones such as calcitonin (CT) and parathyroid hormone have been reported to affect mitochondrial calcium content in osteoblasts and osteoclasts (17, 22). Calcium uptake and retention by mitochondria are generally accepted as energy dependant processes (16). More recently, a cytochemical study revealed Ca-ATPase activity in mitochondria of bone cells including osteoclasts, osteoblasts and osteocytes and suggested that the enzyme activity might relate to calcium movement in mitochondria

(2). These observations indicate the importance of mitochondria in bone cell function. Cytochrome oxidase, which participates in oxidative metabolism in mitochondria, is widely accepted as a useful marker enzyme to assess mitochondrial oxidative metabolism (3, 23, 24). Previously, Addison (1), Balogh (4), Burstone (6), and Yamamoto and Gay (27) observed cytochrome oxidase activity in bone tissues, and cultured osteoclasts by light microscopy. Noda (21) examined cytochrome oxidase activity in experimentally induced osteoclasts by an ultracytochemical method and showed that CT decreased the enzyme activity. However, no ultracytochemical study of cytochrome oxidase activity in osteoblasts and osteocytes has been reported. The purpose of the present study was to analyze, by ultracytochemical means, cytochrome oxidase activity in rat metaphyseal osteoblasts, osteocytes, and osteoclasts. Also a methodological study of the cytochemical reactivity of cytochrome oxidase was included since fixation, incubation time and permeability of reagents during incubation affect the reactivity of the enzyme (3, 7, 13, 23).

MATERIALS AND METHODS

Three-week-old rats (Wistar strain) were used in the present study. The rats were perfused with saline via the left ventricle for a few min under anesthesia. Then the saline was replaced by a fixative composed of 1% glutaraldehyde-1% paraformaldehyde in 0.1 M Hepes buffer (pH 7.4). After 15 min fixation, metaphyses from tibiae were collected, cut longitudinally into small pieces (1–2 mm thick) and additionally fixed for 15 min in the same fixative at 4°C. The tissues were rinsed with 0.1 M Hepes buffer several times and decalcified in 5% ethylenediaminetetraacetic acid (EDTA)-0.1 M Hepes buffer (pH 7.4) containing 5% sucrose for 48 hr at 4°C. The decalcified metaphysis was sliced into 40 μ m thick sections with a Biomicroslicer DTK 1000 (Dosaka EM, Kyoto, Japan). The sections were washed with 0.1 M Hepes buffer (pH 7.4) containing 5% sucrose for 3 hr at 4°C with buffer changes at 1 hr intervals. Before incubation, some sections were frozen in liquid nitrogen. Specifically, each section was picked up with a pointed wooden rod and immersed in liquid nitrogen. The frozen sections were then thawed in 5% sucrose-0.1 M Hepes buffer in order to facilitate penetration of chemicals such as diaminobenzidine and cytochrome c into mitochondrial membranes, according to Kugler *et al.* (13). All sections were then incubated for 120 min or 180 min at 37°C. The incubation medium was renewed at 60 min intervals. The incubation medium consisted of 0.2% 3, 3'-diaminobenzidine (DAB), 2.5 ml; 0.2 M Hepes buffer (pH 7.4), 5 ml; 0.04% catalase, 2.5 ml; cytochrome c (type III), 10 mg, according to Seligman *et al.* (24). For a control, 10^{-3} M potassium cyanide was added to the incubation medium. After incubation, all sections were rinsed for 10 min with 0.1 M Hepes buffer (pH 7.4) and post-fixed with 0.1 M Hepes-buffered 1% osmium tetroxide (pH 7.4) for 1 hr. Sections were dehydrated with a graded acetone series and embedded in Epon 812. For light microscopy, thick sections (1–2 μ m) cut from specimen blocks were usually observed without counter stain, although some were stained with 1% toluidine blue in order to clarify cell profiles. Thin sections for electron microscopy were stained with lead citrate for 10 min.

RESULTS

By light microscopy, DAB reactive deposits indicating sites of cytochrome oxidase activity were observed in metaphyseal bone cells (Fig. 1a). Osteoclasts had numerous deposits throughout the

cytoplasm. Osteoblasts generally showed a significant amount of reaction product (Fig. 1a; arrowheads), although some had fewer deposits (Fig. 1a; arrows). Osteocytes had markedly less reaction product than the two other cell types, i.e. few positive granules were in the cytoplasm (Fig. 1a). Control sections, incubated with potassium cyanide, lacked reaction product (Fig. 1b).

Electron microscopy revealed cytochrome oxidase activity in the mitochondrial membrane system of bone cells. However there was heterogeneity of mitochondrial staining in each cell type. In the present study, mitochondria could be classified into three types based on the location of reaction products within mitochondria. Mitochondria which had reaction product in the membrane spaces between the inner and outer membranes including cristae were termed as type I (Fig. 2a). Reaction product in type II mitochondria was principally located in cristae (Fig. 2b). Mitochondria which lacked enzyme activity were classified as type III (Fig. 2c).

To facilitate penetration of cytochrome c and DAB, some samples were frozen prior to incubation. The distribution pattern of reaction product from cell to cell or mitochondria to mitochondria was essentially the same as in specimens that had not been frozen (Fig. 3). There was no significant difference in the distribution pattern or amount of reaction product between 120 and 180 min incubations. Control sections incubated with potassium cyanide were devoid of reaction product (Fig. 2d).

In most osteoclasts, type I mitochondria were predominant (Fig. 4), type II mitochondria were few in number and type III were rare. Some osteoclasts contained largely type II mitochondria and type III mitochondria occurred in greater number in these cells (Fig. 5).

Osteoblasts basically contained these three types of mitochondria, but the proportion of these types was variable in each cell. Most osteoblasts tended to possess types I and II (type I > II) as shown in figure 6a, although, some osteoblasts contained mainly types II and III mitochondria (Fig. 6b). There were no obvious ultrastructural differences between these osteoblasts.

Osteocytes observed in the metaphysis had the characteristics of young osteocytes, i.e. a moderately developed Golgi apparatus and rough endoplasmic reticulum. In these osteocytes, cytochrome oxidase activity was weak and mitochondria were mostly type III, although some type II were present (Fig. 7). Osteocytes with all type III mitochondria were also observed.

Figs. 1a, b. Cytochrome oxidase activity in metaphysis. Counterstained with toluidine blue. 1a. Reaction products are brown granular deposits. Osteoclasts (OCL) are filled with numerous granules throughout the cytoplasm. Most osteoblasts (OB) contain numerous positive granules (arrowheads), but some osteoblasts have few granules (arrows). Few positive granules are present in osteocytes (OC). $\times 700$ 1b. Control section incubated with 10^{-3} M KCN. Reaction product was absent in OCL, OB and OC. 120 min incubation. $\times 700$

DISCUSSION

Cytochrome oxidase, which participates in the electron transfer system in mitochondria, has been employed as a useful marker enzyme to assess mitochondrial oxidative metabolism (3, 23, 24). The cytochemical method used in the present study to detect cytochrome oxidase activity is based on the DAB reaction introduced by Seligman *et al.* (24). The method is widely accepted as a reliable tool to demonstrate cytochrome oxidase activity. In the present study, reaction product was localized on the mem-

brane system of mitochondria and was inhibited by 10^{-3} M potassium cyanide.

Cytochemical detection of cytochrome oxidase activity has been reported to be unaffected by EDTA decalcification (4, 21). In the present study, metaphyses were completely decalcified in 48 hr with EDTA. This is a much reduced exposure to EDTA than employed in previous studies (4, 21). Therefore, the inhibitory effect of EDTA decalcification can be considered negligible in this study.

Three types of mitochondria were classified on the basis of the localization of cytochrome oxidase activity,

FIGS. 2a-d. Three types of mitochondria in osteoclasts on the basis of cytochrome oxidase activity. 2a. Type I mitochondria showing reaction product as electron dense deposits in the intermembrane spaces, including cristae. 2b. In type II mitochondria, reaction product was located on the cristae. 2c. Type III mitochondria lack reaction product (arrow). A positive mitochondrion can also be seen (arrowhead). 2d. Control section for cytochrome oxidase detection. Frozen specimen. The reaction product is absent from mitochondria. Osteoclasts. 120 min incubation. $\times 30,000$

FIG. 3. Electron micrograph of an osteoblast from specimen that was frozen and thawed before incubation. Heterogeneously stained mitochondria are present. BM: bone matrix. 120 min incubation. $\times 9,200$

FIG. 4. Electron micrograph of a typical osteoclast stained for cytochrome oxidase activity. Numerous type I mitochondria are present. Most osteoclasts were of this type. $\times 9,600$ RB: ruffled border, N: nucleus, BM: bone matrix. 120 min incubation.

FIG. 5. Osteoclast showing mostly type II mitochondria. Number of positive cristae among mitochondria was variable. Arrows indicate type II mitochondria which had very small amount of reaction product. N: nucleus. 180 min incubation. $\times 9,500$

FIGS. 6a, b. Cytochrome oxidase activity in osteoblasts. 4a. Most osteoblasts contain type I mitochondria. Type II mitochondria also exist in these osteoblasts (arrows). $\times 9,200$ 4b. Osteoblast which has low enzyme activity; mitochondria are mainly types II and III. $\times 8,600$ BM: bone matrix. 180 min incubation.

FIG. 7. In osteocytes, cytochrome oxidase activity is markedly decreased, and most mitochondria are type III (arrows); the other mitochondria are type II. L: lacuna. 180 min incubation. $\times 13,500$

namely, type I mitochondria were positive along the entire membrane system, whereas in type II mitochondria only cristae stained, and type III mitochondria lacked reaction product. Heterogenous staining patterns of cytochrome oxidase activity have also been reported among mitochondria of liver (11), spinal neurons (26), alveolar epithelial cells in lung (9, 10), chondrocytes (28) and osteoclasts (21).

Several reports indicate that cytochemical reaction of cytochrome oxidase is affected by composition of the fixative (3), fixation time (23), incubation time (23) and permeability of DAB and cytochrome c (13). To minimize the inhibitory effects of fixation and to produce as consistent ultracytochemical detection of cytochrome oxidase as possible, weak glutaraldehyde-paraformaldehyde was employed as a fixative for 30 min fixation, according to Anderson *et al.* (3). This was done to avoid variable fixation effects which might cause heterogenous staining patterns of the enzyme (7). Perotti *et al.* (23), Kugler *et al.* (13) and Hirai *et al.* (10) employed prolonged incubation times to obtain sufficient reaction product. We incubated the specimen for 120 min or 180 min according to the previous reports (10, 13, 23) and found no further change at the longer incubation time. Moreover, some frozen specimens were also employed in the present study in order to facilitate penetration of DAB and cytochrome c into mitochondria during incubation according to Kugler *et al.* (13).

After consideration of various factors such as decalcification, composition of fixative, fixation time, incubation time and permeability of DAB and

cytochrome c which might affect the reactivity of cytochrome oxidase activity, our results showed that heterogeneously stained mitochondria were still present in each cell type. Also no differences were observed between frozen and non-frozen specimens. Therefore we conclude that the results provide cytochemical confirmation for the existence of mitochondrial heterogeneity with respect to oxidative metabolism in bone cells as reported in other cells and tissues (9–11, 26, 28). In addition, it is suggested that both DAB as capture agent and cytochrome c as substrate penetrated mitochondrial membranes in non-frozen specimens during incubation as well as frozen specimens, and that specimens that were not frozen are usable for cytochemical detection of cytochrome oxidase activity. It should be pointed out that negative mitochondria, classified as type III, may merely reflect mitochondria containing low levels of cytochrome oxidase rather than mitochondria that totally lack the enzyme.

One of the most notable morphologic features in osteoclasts is their abundant mitochondria (12). Most osteoclasts in metaphyses observed in the present study contained cytochrome oxidase enriched mitochondria. This finding is in concert with that of experimentally induced osteoclasts in alveolar bones (21), in which cytochrome oxidase levels were high.

Other studies have reported calcium retention in osteoclast mitochondria (14, 17, 20), and a cytochemical study has demonstrated Ca-ATPase activity in mitochondria (2). These findings suggest that calcium movement across mitochondria depends on

energy generated by Ca-ATPase (2). In addition, mitochondrial oxidative metabolism participates in H^+ production, which is essential for bone resorption, by CO_2 generation in the oxidative process (18, 25). The present study provides cytochemical evidence that such energy-requiring metabolic processes occur in osteoclast mitochondria. Osteoclasts having moderate or weak mitochondrial cytochrome oxidase, classified as type II mitochondria, may be in transition to a more active phase or to a resting phase. It has been shown that cytochrome oxidase activity diminishes markedly in osteoclasts that have been inactivated by CT treatment (21).

The present study is the first ultracytochemical study of cytochrome oxidase in osteoblasts. Osteoblast mitochondria have been suggested to play a role in calcium translocation for bone calcification (5, 8, 18, 20) and mitochondrial Ca-ATPase is presumed to support the calcium movement in osteoblasts as well as in osteoclasts (2). It is postulated that osteoblasts which contain many cytochrome oxidase enriched mitochondria are active in calcium transport, while osteoblasts having relatively low mitochondrial cytochrome oxidase activity support only low levels of calcium translocation. It is worth mentioning that the osteoblasts which have relatively low cytochrome oxidase activity, appear to be still active in organic matrix production as well as in high cytochrome oxidase osteoblasts because of their well-developed Golgi apparatus and rough endoplasmic reticulum.

The present study is also the first report on cytochrome oxidase activity in osteocytes. The decreased number of mitochondria and cytochrome oxidase activity in osteocytes indicates that osteocytes, which are believed to have differentiated from osteoblasts, lose their capacity for oxidative metabolism.

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