Microphotometric quantitation of succinic dehydrogenase in whole isolated osteoclasts.

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Abstract

The microphotometric quantitation of succinic dehydrogenase (SDH) in whole isolated osteoclasts was performed. Osteoclasts were obtained from imprints of the external metaphyseal area of tibia, a zone of physiological bone resorption. SDH was demonstrated histochemically and the reaction product was measured in a Zeiss Cytoscan microphotometer in arbitrary units (AU) of activity from 0 to 500. Total enzyme activity (TEA) was 10 to 50 AU in 78% of the cells. The enzyme concentration (MEC), taken as the activity per unit volume was 0.10 to 0.40 AU in 94% of the cells. Values of reactive cytoplasm area (RCA) were high for most of the cells, but lower for a few, probably mononuclear, osteoclasts. The correlation index for TEA vs RCA was significant. The study of enzyme activity in whole osteoclasts by microphotometric quantitation has been proven possible. This technique could be employed in further studies of enzyme activity of osteoclasts in other physiological or pathological states of bone resorption.

Key words: osteoclast - microphotometry - enzymes - succinic dehydrogenase.

Cuantitación microfotométrica de succino-deshidrogenasa en osteoclastos aislados enteros

Resumen

Se presenta la evaluación cuantitativa del contenido de succino-deshidrogenasa (SDH) en osteoclastos. Las células se obtuvieron por improntas de una zona de reabsorción ósea fisiológica, como es la zona metafisiaria externa de la tibia. La SDH fue demostrada histoquímicamente, siendo el producto final de reacción medido en un microfotómetro Cytoscan Zeiss y expresado entre 0 y 500 unidades arbitrarias (UA) de actividad. La actividad enzimática total (AET) se encontraba en el 78% de las células entre 10 y 50 AU. La concentración enzimática media (CEM), tomada como la actividad por unidad de volumen fue encontrada en el 94% de las células entre 0,10 y 0,40 AU. El área citosplasmática reaccionante (ACR) fue en la mayoría de las células de gran tamaño, aunque también se encontraron algunas células cuya área reaccionante era de pequeño tamaño. Estas últimas podrían corresponder a osteoclastos mononucleares. El índice de correlación entre AET y ACR fue significativo. En este trabajo se demuestra la posibilidad de estudiar actividades enzimáticas en osteoclastos enteros por cuantitación fotométrica. Esta técnica puede ser aplicada al estudio de la actividad enzimática de osteoclastos bajo otras condiciones de reabsorción ósea, ya sea patológicas o fisiológicas.

Palabras clave: osteoclastos - microfotometría - enzimas - succino-deshidrogenasa.

Introduction

While the connection between osteoclasts and bone resorption is well accepted, the former's precise role in this complex process has not yet been completely clarified (1-5). Histochemical studies have demonstrated that osteoclasts are very active cells with a rich supply of several enzymes. Acid phosphatase (1, 6-9), non-specific esterases (3, 10), succinate dehydrogenase (14), cytochrome oxidase (3, 7) and ATPase (15), among others, have been shown to be highly active in osteoclasts.

The intense reactivity of osteoclast cytoplasm to the histochemical demonstration of some of these enzymes, e.g. succinic dehydrogenase (SDH) or acid phosphatase, has allowed the use of these techniques as osteoclast markers to distinguish them from other cell types in histological sections or smears (16).

Some authors have claimed that osteoclasts have temporarily functional states, but it is not clear whether this is a synchronic or asynchronic effect (4, 17, 18). The quantitative determination of SDH activity in osteoclasts acting in a zone of physiological bone resorption could afford useful data.

To date the intensity of enzymatic activity in osteoclasts has been evaluated subjectively by conventional scales after a fixed reaction time (3). However, since the quantitative determination of several enzymatic activities in tissue sections by histophotometric methods has been proven possible, (19-21) the aim of this work was to quantitate succinic dehydrogenase (SDH) activity in whole osteoclasts by applying this technique.

Materials and Methods

Whole osteoclasts were isolated from the external surface of the methaphyseal area of the tibia of 15-day-old Wistar rats. After killing the rats by decapitation, tibiae were removed and surrounding tissues dissected away. Tibia surfaces were gently imprinted by stamping motions on a chemically clean coverslip, while the tibiae were rotated about their axis (22). This procedure frees tibia osteoclasts which in turn adhere to the coverslip. Imprints were allowed to dry for 20 minutes at room temperature and then processed for enzymatic SDH demonstration according to Nachlas et al (23), using Nitro Blue Tetrazolium as hydrogen acceptor.

Quantitation was performed in a Zeiss Microphotometer Cytoscan provided with an automatic bidirectional scanning stage. Measurements were made by scanning the cells with a 20 μm plug. Reliability and applicability of this methodology in affording relative measurements of enzymatic activity have been previously demonstrated (24). In order to scan only the reactant osteoclasts areas, blank measurements were performed on the zones surrounding the imprint near the coverslip border.

The optical densities afforded by single cell scanning and the number of spots used to scan the whole osteoclasts were automatically integrated.

These data allowed the analysis of the following parameters:

a) Total enzymatic activity (TEA) as the integrated value of single cell measurements;

b) mean enzymatic concentration (MEC) obtained by dividing TEA by the number of measurements, a value which represents the average cytoplasmic enzyme activity;

c) reactive cytoplasm area (RCA), calculated from the number of spots required to scan each cell and the spot diameter; assuming that all osteoclasts underwent a similar deformation during the imprint and histochemical procedure, RCA was taken as an index of cell volume.

Microphotometrically recorded optical densities were taken as arbitrary units (AU) of enzymatic activity ranging from 0 to 500, then plotted in frequency histograms, as is usual in this type of measurement (25).

Correlation indices were calculated for TEA vs MEC and for TEA vs RCA.

After photometric evaluation the coverslip was removed and the imprints counterstained with safranin to visualize the nuclei and facilitate morphological observations.

Results

Microscopic examination revealed that the cells were highly SDH-Positive. Most of them were multinucleated giant cells while a few were mononucleated smaller cells.

TEA histograms showed that 78% of the cell population contained 10 to 50 AU of enzyme activity, while a small number of cells exhibited greater activity (Fig. 1).

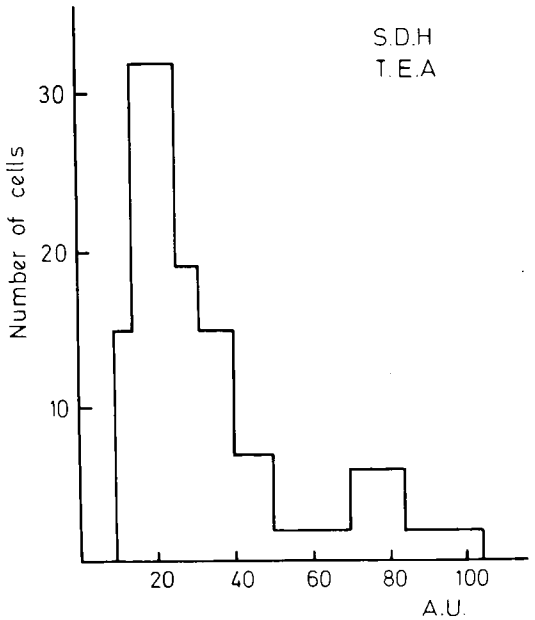

Fig. 1: Total enzyme activity (TEA) in a great number of cells (98) proved to be between 10 and 40 AU, while a small population exhibited greater activity. (Mean: 29.53; standard deviation: 21.03).

Fig. 2 depicts cell distribution according to enzyme density (MEC). The great majority of osteoclasts (94%) contained from 0.10 to 0.40 AU, with only a few cells showing a higher MEC.

RCA was scanned with 35 to 115 spots in 61% of the cells (Fig. 3), which implies that in this population the reactive cytoplasm had a projection area ranging from 0.17 to 0.43 x 10^4 μm^2. Another significant population of bigger osteoclasts from 0.47 to 0.74 x 10^4 μm^2 was likewise defined. Isolated osteoclasts of 1.14 and 1.52 x 10^4 μm^2, as well as a group of smaller reactive cells from 0.7 to 0.17 x

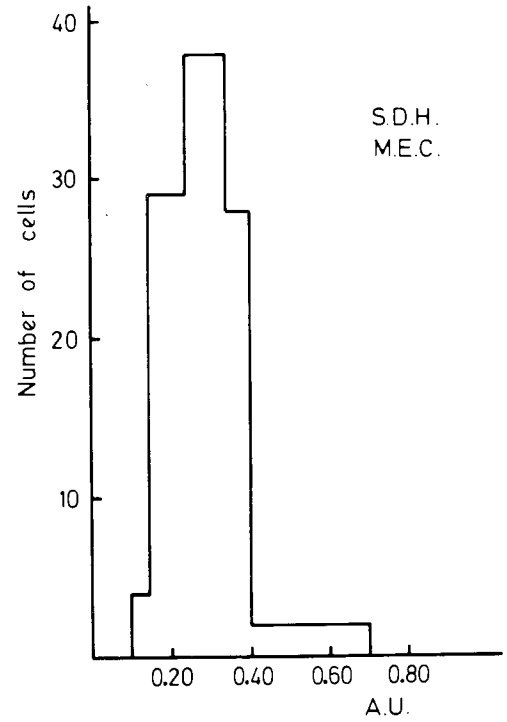

Fig. 2: Mean enzyme concentration (MEC) ranged from 0.10 to 0.40 AU in 94% of the total number of cells (98). (Mean: 0.25; standard deviation: 0.09).

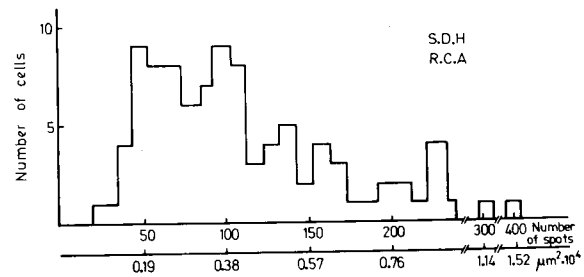

Fig. 3: The graph shows the reactive cytoplasm area (RCA) in μm^2 inferred from the number of spots necessary to scan each cell.

10^4 μm^2, were also present. The latter cells were found to be mononucleated following counterstaining of the imprints.

No correlation was found for TEA vs MEC, while a statistically significant correla-

tion was demonstrated for TEA vs PCA (Figs. 4 & 5).

Discussion

Although a great deal of knowledge of osteoclast metabolism has been obtained from qualitative descriptions of histoenzymatic reactions in tissue sections, this study shows that a more complete understanding of individual osteoclast behaviour arises with the introduction of objective measurements of enzyme activity. A significant trend towards quantitation in histochemistry has proven the reliability and accuracy of chemical and physical techniques applied to enzyme reaction evaluation (26). Among these methods, histophotometry has enabled relative variations of enzyme activity in diverse structures of tissue sections to be readily determined (24). It has also provided useful data when applied to a variety of enzymes in human and experimental research models (19, 20, 27, 28). Present results indicate that it could also be applied to the study of osteoclast behaviour in both normal and altered conditions of bone structure.

Since the osteoclast is a very large and irregular cell, orientation of tissue sections could account for considerable variations in size and shape, which in turn render quantitation hazardous or inaccurate. Thus, measurements on the whole cell, easily isolated by imprints of the external metaphyseal zone of tibiae (22, 29) afforded more reliable determinations.

A great percentage of isolated cells (64%) had similar total SDH activities (TEA), but a small number of cells exhibited strikingly greater enzymatic activity. These data suggest the existence of two distinct cellular populations, as far as their total SDH activity is concerned.

Although values for the reactive cytoplasm area (RCA) were more disperse than those for TEA, they may also be grouped into two distinct populations with a statistically significant correlation index for TEA vs RCA.

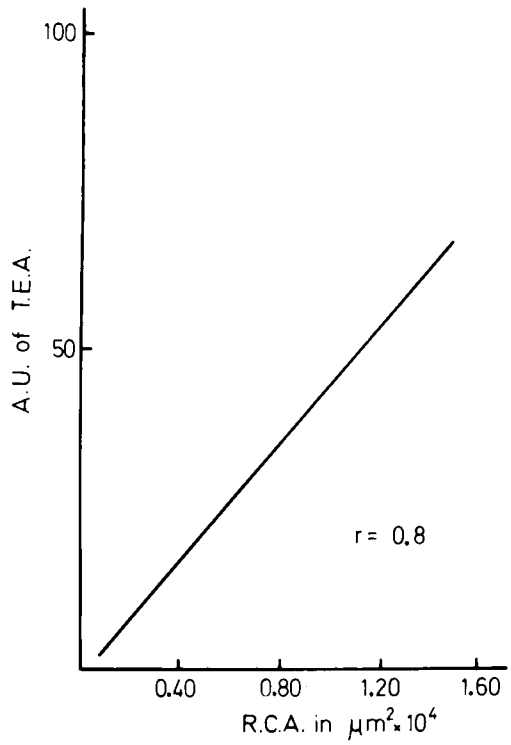

Fig. 4: Correlation between total enzyme activity (TEA) and mean enzyme activity (MEA).

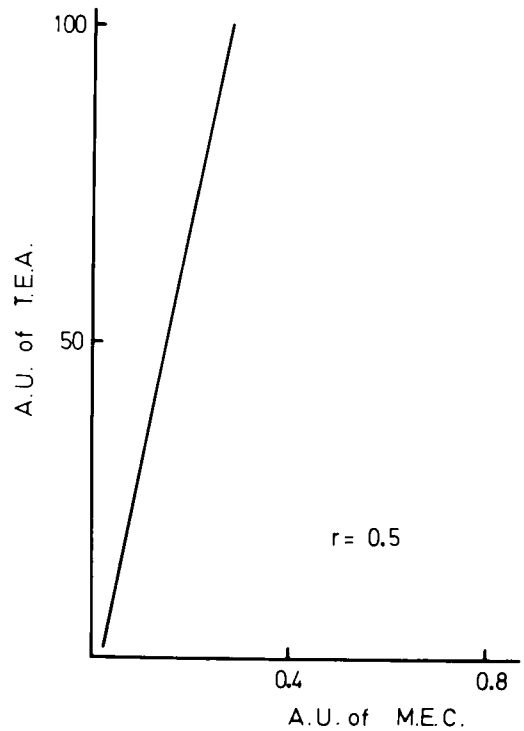

Fig. 5: Correlation between total enzyme activity (TEA) and reactive cytoplasm area (RCA).

On the other hand, most cells had a uniform enzyme concentration, as shown by similar MEC values for 94% of osteoclasts measured.

An interesting finding was the presence of rather smaller mononuclear cells with positive reaction to a similar degree as that of the bigger multinucleated osteoclasts, which supports the existence of mononuclear osteoclasts, as advanced by several authors (2, 30, 32).

Since most of the osteoclast cytoplasm is occupied by densely packed mitochondria, the variations detected in SDH activity would depend more on osteoclast size rather than on its specific mitochondrial activity.

It would be worthwhile to investigate whether the behaviour of the osteoclasts under study, isolated from an area of intense normal remodelling, is common to other physiological or pathological states of bone resorption.

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