Histochemistry (1983) 78:481–485

Histochemistry

High Active Isoenzyme of Carbonic Anhydrase in Rat Calvaria Osteoclasts

Immunohistochemical Study

H.K. Väänänen and E.-K. Parvinen
Department of Anatomy, University of Oulu, Kajaanintie 52 A, SF-90220 Oulu 22, Finland

Summary. Calvaria from newborn rats were used to localize carbonic anhydrase isoenzymes in bone tissue. Highly sensitive peroxidase-anti-peroxidase method revealed strong reaction of high-active C form of carbonic anhydrase exclusively in the osteoclasts. There was no reaction in osteoblasts or fibroblasts but some population of bone marrow cells was positive. Isoenzyme B was found only in bone marrow cells. On the basis of the present and previous studies it is highly probable that carbonic anhydrase may have some crucial role in osteoclast mediated bone resorption. Carbonic anhydrase C may also be a suitable probe for studies of osteoclast function and origin.

Introduction

In mammals there are at least three different isoenzymes of carbonic anhydrase. Two of these, isoenzyme B and isoenzyme C, are present in red blood cells and in some other tissues. Third isoenzyme (type III) is almost exclusively present in striated muscle.

Carbonic anhydrase has been connected to bone resorption since 1960 when Dulce et al. showed that inhibition of the enzyme inhibits parathyroid hormone induced resorption in chickens. Later Minkin and Jennings (1972) demonstrated inhibition of bone resorption in vitro by acetozolamide, an inhibitor of carbonic anhydrase. Neuman and Neuman (1958) suggested that secretion of organic acids from osteoclasts play a major role in bone resorption. Gay and Müller (1974) showed that chicken osteoclasts contain carbonic anhydrase and suggested its role in H^+ secretion. However, there is only one type of isoenzyme of carbonic anhydrase present in birds and this study was undertaken to find out if mammalian osteoclasts contain carbonic anhydrase and what is the type of the isoenzyme present.

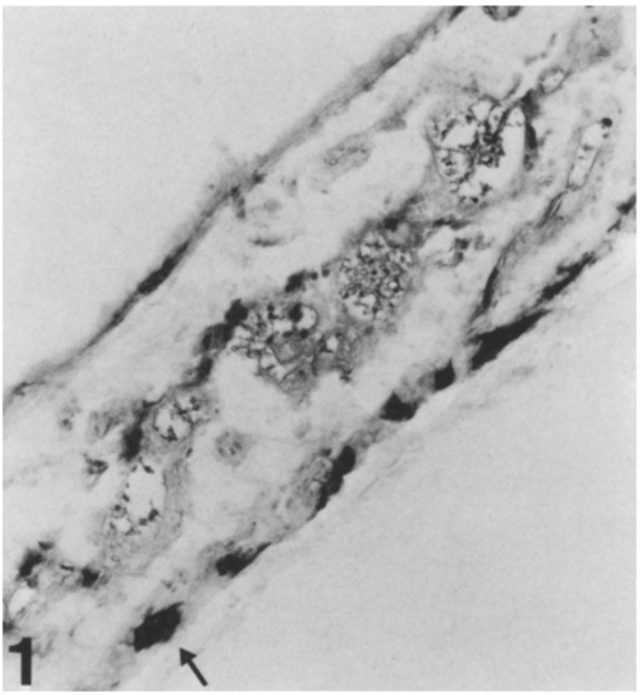


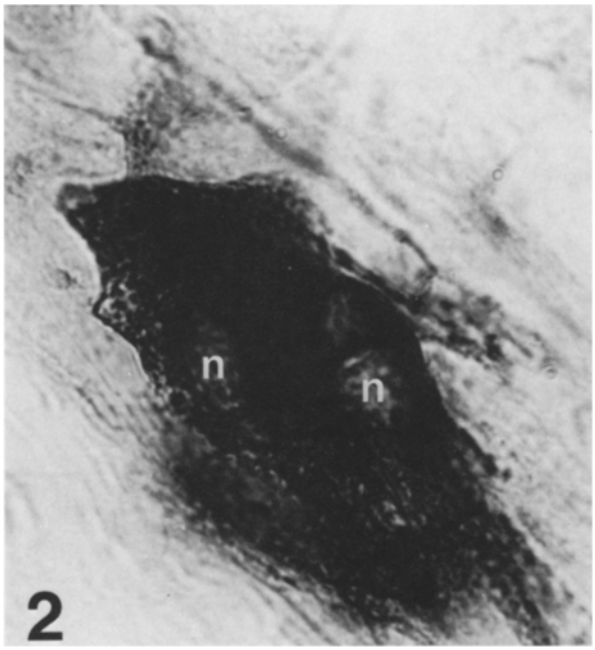
Fig. 1. Paraffin embedded section from rat calvaria stained with anti-rat carbonic anhydrase C. Most of the positive cells are situated on the endosteal surface of calvaria. Positive cells are large, often containing several nuclei. The cell marked with arrow is showed in Fig. 2 (×64)

Material and Methods

Purification of Human and Rat Carbonic Anhydrase Isoenzymes, Antibody Production and Specificity of Antisera: All used antisera were generous gifts from Dr. T. Kumpulainen, Department of Anatomy, University of Oulu, Finland.

Human carbonic anhydrases B and C and rat carbonic anhydrase C have been purified from human and rat erythrocytes respectively, and antisera (anti-HCAB, anti-HCAC and anti-RCAC) against these antigens were raised in rabbits as previously described (Kumpulainen and Korhonen 1978; Kumpulainen 1979). Specifities of the antisera were tested by immunodiffusion and immunoelectrophoresis. Anti-HCAB and anti-HCAC do not cross-react with each other (Kumpulainen and Korhonen 1978; Kumpulainen 1979). Cross-reaction between rat tissue CA and anti-human CA antibodies has been reported previously (Väänänen et al. 1982; Kumpulainen and Väänänen 1982; Spicer et al. 1979).

Immunohistochemical Demonstration of CA: Three-day-old Long Evans male rats were killed by decapitation and calvaria were removed immediately into Carnoy fluid (ethanol:cloroform:glacial acetic acid 6:3:1). The tissue was fixed 3 h in Carnoy fluid and embedded in low melting point paraffin. Deparaffinized and rehydrated sections were stained with the peroxidase-antiperoxidase (PAP) complex method as previously described in detail (Kumpulainen 1981). The principles of the method adapted for the present study were as follows: 1:12-1:200 dilutions of the anti-HCAB, anti-HCAC and anti-RCAC or control sera were used in the first step of staining. 1:20 diluted swine anti-rabbit serum immunoglobulin, 1:100 diluted PAP-complex (Dako Immunoglobulins Copenhagen, Denmark), and a solution of 3,3′-di-



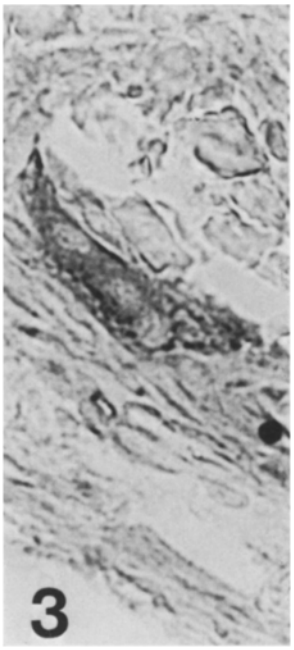


Fig. 2. Higher magnification of CA-C positive cell showing several nuclei (marked with *n*) and intense cytoplasmic staining. There is no clear evidence of extracellular staining around the cell. Osteoblasts are negative (× 640)

Fig. 3. Calvarial section stained with anti-CA-B serum showed strong staining of some cells in bone marrow but only occasionally very faint staining could be detected in osteoclasts (× 160)

aminobenzidine (Fluka AG, Switzerland) in the subsequent steps. In the control experiment, rabbit serum prepared before immunization with HCA C and rabbit anti-HCA C serum adsorbed with HCA C were used.

Some sections from each specimens were stained with haematoxyline and eosin for morphological orientation and cell identification.

Results

Staining pattern with anti-HCA C and anti-RCA C was identical. Higher dilutions of antiserum could be used with anti-RCAC than anti-HCA C in order to get equal intensity of staining reaction.

In calvaria sections most of the carbonic anhydrase containing cells are on endosteal surface (Fig. 1). They are large and often have more than one nuclei (Fig. 2). The other positive cell population is in bone marrow (Fig. 1). With anti-HCA B there was only very faint cytoplasmic staining reaction in multinucleated cells (Fig. 3), not very clearly distinct from controls. Fibroblasts as well as osteoblasts and osteocytes were negative but in the bone marrow there was a strongly positive cell population. Controls with pre-immune serum and with adsorbed anti-HCA C were all negative.

Discussion

Thus the present study gives the firm evidence of the presence of high-activity CA-C in osteoclasts. Previous studies have suggested the active role of carbonic anhydrase in osteoclasts mediated bone resorption (Minkin and Jennings 1972). The occurrence of high-activity isoenzyme form is in accordance with this. Both parathyroid hormone as well as calcitonin probably regulate the synthesis and secretion of CA-C in osteoclasts (Minkin and Jennings 1972; Anderson et al. 1982).

The reliability of immunohistochemical staining method is critically based on the specifity of used antiserum and thus primarily on the purity of antigen used for immunization. The purity of used antigen as well as monospecifity of antiserum both has been extensively studied previously (Kumpulainen and Korhonen 1978; Kumpulainen and Väänänen 1982). The use of pre-immune serum, other high-titer antisera and absorbed anti-CA-C further confirms the specifity of staining reaction.

The final role of carbonic anhydrase in osteoclasts remains still without firm explanation. It is interesting that recently the presence of extracellular carbonic anhydrase in calcified epiphyseal growth plate was shown in rat (Kumpulainen and Väänänen 1982) and also in chicken (Gay et al. 1982). Also in epiphyseal growth plate the high activity isoenzyme C is present (Kumpulainen and Väänänen 1982). There the role of CA-C has been suggested to facilitate the diffusion of CO_2 from extracellular fluid of cartilage matrix into circulation and thus to maintain the high pH of cartilage fluid (Kumpulainen and Väänänen 1982; Gay et al. 1982). In osteoclasts carbonic anhydrase could have the similar functions facilitating the diffusion of CO_2 from the cell or some cellular organelles. The other possible role, secretion of hydrogen ions into surrounding bone matrix, could be more directly connected to mineral resorption.

The lack of a suitable osteoclasts marker has been partly preventing the efforts in bone resorption studies as well as in studies concerning the origin of osteoclasts. Recently tartrate resistant acid phosphatase has been suggested into this role by Minkin (1982). In our opinion high activity carbonic anhydrase C may also be beneficial in these studies especially when highly sensitive and specific radioimmunoassay and immunohistochemical methods are already in use.

References

Anderson RE, Schraer H, Gay CV (1982) Ultrastructural immunocytochemical localization of carbonic anhydrase in normal and calcitonin-treated chick osteoclasts. Anat Rec 204:9–20

Dulce HJ, Siegmünd P, Körber F, Schüffe E (1960) Zur Biochemie der Knochenauflösung, II. Über das Vorkommen Carboanhydratase im Knochen. Hoppe-Seylers Z Physiol Chem 320:163–167

Gay CV, Müller WJ (1974) Carbonic anhydrase and osteoclasts: Localization by labelled inhibitor autoradiography. Science 183:432–434

Gay CV, Anderson RE, Schraer H, Howell DS (1982) Identification of carbonic anhydrase in chick growth plate cartilage. J Histochem Cytochem 30:391–394

Kumpulainen T (1979) Immunohistochemical localization of human carbonic anhydrase isoenzyme C. Histochemistry 62:271–280

Kumpulainen T (1981) Human carbonic anhydrase isoenzyme C. Effects of some fixatives on the antigenicity and improvements in the method of localization. Histochemistry 72:425–431

Kumpulainen T, Korhonen LK (1978) Immunohistochemical demonstration of carbonic anhydrase. Histochemistry 58:183–192

Kumpulainen T, Väänänen HK (1982) Immunohistochemical demonstration of extracellular carbonic anhydrase in epiphyseal growth cartilage. Calcif Tissue Int 34:428–430

Minkin C (1982) Bone acid phosphatase: Tartrate-resistant acid phosphatase as a marker of osteoclast function. Calcif Tissue Int 34:285–290

Minkin C, Jennings JM (1972) Carbonic anhydrase and bone remodelling: Sulfonamide inhibition of bone resorption in organ culture. Science 176:1031–1033

Neuman WF, Neuman MW (1958) The chemical dynamics of bone mineral. University of Chicago Press, Chicago

Spicer SS, Stoward PJ, Tashian RE (1979) The immunohistolocalization of carbonic anhydrase in rodent tissues. J Histochem Cytochem 27:820–831

Väänänen HK, Kumpulainen T, Korhonen LK (1982) Carbonic anhydrase in the type I skeletal muscle fibers of the rat. J Histochem Cytochem 30:1109–1113

Accepted June 1, 1983