Vesicles with lactate dehydrogenase and without alkaline phosphatase present in the resting zone of epiphyseal cartilage

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Matrix vesicles are membrane-invested vesicles that initiate mineralization in the extracellular matrix of calcifying tissues. The epiphyseal cartilages of young-rat rib bones were divided into the growth zone and the resting zone, followed by the isolation of matrix vesicles after collagenase treatment. Matrix vesicles with both alkaline phosphatase and lactate dehydrogenase were detected in the growth cartilage found in the epiphyseal growth plates of young rabbits [Hosokawa, Uchida, Fujiwara & Noguchi (1988) *J. Biol. Chem.* **263**, 10045–10047], but were not detected in the resting zone. By contrast, and surprisingly, lactate dehydrogenase-containing vesicles without alkaline phosphatase were found in the resting zone, but not in the growth zone. In both the growth and resting zones, isoenzyme patterns of lactate dehydrogenase in the two different vesicles were identical with those of cytosolic lactate dehydrogenase of chondrocytes, suggesting the presence of a mechanism for specific uptake of cytosolic lactate dehydrogenase. The same results as for young-rat rib bones were obtained with the resting and growth cartilages of young-dog and monkey rib bones.

INTRODUCTION

Matrix vesicles are membrane-enclosed structures that initiate mineralization in the extracellular matrix of growth-plate cartilage [1,2] and other calcifying tissues [3–7]. Matrix vesicles isolated from mineralizing tissues contain alkaline phosphatase and related phosphatase activities associated with the plasma membrane of chondrocytes; this may be because matrix vesicles may be extracellular cell-membrane-derived microstructures [8,9].

Recently, lactate dehydrogenase isoenzymes were found to be located in matrix vesicles of the epiphyseal growth plate of young-rabbit leg bones [10]. In the cartilage, alkaline phosphatase is enriched in the growth zone, whereas it is rarely detected in the resting cartilage [11], suggesting that matrix vesicles with alkaline phosphatase are hardly ever present in the resting zone.

In the present study, the epiphyseal cartilages of young-rat rib bones were divided into the growth zone and the resting zone, followed by the isolation and identification of matrix vesicles. Matrix vesicles with both alkaline phosphatase and lactate dehydrogenase were also detected in the growth zone as well as in the epiphyseal growth plate of rabbit leg bones [10]. In contrast, lactate dehydrogenase-containing vesicles without alkaline phosphatase were found in the resting zone, but not in the growth zone.

MATERIALS AND METHODS

Enzyme assays

Lactate dehydrogenase was assayed by monitoring the rate of the oxidation of NADH at 340 nm with pyruvate as the substrate in 0.25 M-sucrose as described in [12]. Alkaline phosphatase was assayed with *p*-nitrophenyl phosphate as the substrate in 0.25 M-sucrose as described

in [13]. Aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1) [14], alanine:2-oxoglutarate aminotransferase (EC 2.6.1.2), [15], aldolase (EC 4.1.2.13) [16], glucose-6-phosphatase (EC 3.1.3.9) [17], glutamate dehydrogenase (EC 1.4.1.3) [18] and catalase (EC 1.11.1.6) [19] were assayed by methods described in the cited references.

Preparation of matrix-vesicle fraction and chondrocyte

The epiphyseal plates of rib bones of 40 20-day-old rats (Wistar strain) were exposed by fracturing, and the growth zone and the adjacent resting zone were obtained by sharp dissection [20]. Each zone of cartilage was sliced with a razor and incubated with shaking in 10 ml of the solution containing 1000 units of collagenase (Wako Pure Chemical Industries, Osaka, Japan)/ml, 1.5 mM-MgCl₂, 0.25 M-sucrose, 1000 units of penicillin/ml, 1 mg of streptomycin/ml and 50 mM-Tris buffer, pH 7.5, by a modification of the method of Ali *et al.* [8] at 37 °C for 4 h. The digested mixture was filtered through cheesecloth and centrifuged at 1000 *g* for 15 min to sediment chondrocytes. After centrifugation of the supernatant at 20000 *g* for 20 min, the resulting supernatant was centrifuged at 100000 *g* for 60 min to sediment the matrix vesicles. Matrix-vesicle and chondrocyte fractions were suspended in 4 ml of 20 mM-Tris/HCl, pH 7.5, containing 0.25 M-sucrose.

Rat (Wistar strain) liver (1 g wet wt.) was cut into small pieces with scissors and subjected to collagenase treatment and differential centrifugation as described above.

Sucrose-density-gradient centrifugation of matrix-vesicle fraction and chondrocyte homogenate

The chondrocyte was homogenized with a micro-homogenizer (PHYSCOTRON Handy Micro Homogenizer; Niti-on, Tokyo, Japan) for 15 s. The chondro-

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Fig. 1. Sucrose-density-gradient centrifugation of matrix-vesicle fraction from growth-zone and resting-zone cartilages

Matrix-vesicle fractions were prepared from the growth and resting cartilages and separately subjected to sucrose-density-gradient centrifugation as described in the text. After fractions had been taken from the bottom of the tube, lactate dehydrogenase activity was determined in the absence (○) or presence (●) of Triton X-100 at a final concentration of 0.2%, and alkaline phosphatase activity in the absence (△) or presence (▲) of Triton X-100.

cyte homogenate (3 ml) was layered on to a 33 ml linear sucrose gradient (24–54%, w/v), and the matrix-vesicle fraction (3 ml) was layered on a similar sucrose gradient (20–54%, w/v) as described previously [21]. Each gradient was centrifuged at 132 400 *g* for 70 min in a vertical rotor (RPV 50T) on a Hitachi (Tokyo, Japan) 55P-72 centrifuge. After centrifugation, fractions (2.1 ml) were collected from the bottom of the tube.

Electrophoresis

Polyacrylamide-gel electrophoresis of lactate dehydrogenase isoenzymes was carried out as described by Fritz *et al.* [22].

RESULTS AND DISCUSSION

The matrix-vesicle fractions prepared from resting and growth cartilage were separately subjected to sucrose-density-gradient centrifugation as described in the Materials and methods section. After centrifugation, fractions were taken from the bottom of the tube; lactate dehydrogenase and alkaline phosphatase activities were then assayed in the presence or in the absence of Triton X-100 (Fig. 1). In the growth cartilage, the sedimentation profile of alkaline phosphatase activity in the absence of Triton X-100 was nearly identical with that in the

presence of Triton X-100. In contrast, lactate dehydrogenase activity was not detected in any fraction in the absence of Triton X-100. However, the addition of Triton X-100 to each fraction resulted in the appearance of lactate dehydrogenase activity. The sedimentation profile of alkaline phosphatase was similar to that of lactate dehydrogenase in the presence of Triton X-100. On the other hand, the two enzyme activities showed nearly identical sedimentation profiles when the matrix-vesicle fraction from the growth cartilage was subjected to metrizamide [23] and Percoll [24] gradient centrifugation performed as described in the cited references (results not shown). These results suggest that matrix vesicles with both alkaline phosphatase and lactate dehydrogenase are present in the growth cartilage.

On the other hand, in the resting cartilage, alkaline phosphatase activity was not detected in any fraction, either in the presence or in the absence of Triton X-100. However, lactate dehydrogenase activity was detected only in the presence, but not in the absence, of Triton X-100. Lactate dehydrogenase-containing vesicles without alkaline phosphatase in the resting cartilage were nearly identical in density with the matrix vesicles in the growth cartilage. These results suggest that lactate dehydrogenase-containing vesicles without alkaline phosphatase resemble matrix vesicles.

Fig. 2. Sucrose-density-gradient centrifugation of chondrocyte homogenates prepared from growth-zone and resting-zone cartilages

Chondrocytes prepared from growth and resting cartilages of young rats were homogenized with 0.25 M-sucrose in 20 mM-glycylglycine, pH 7.5, and then subjected to sucrose-density-gradient centrifugation. Lactate dehydrogenase activity (●) was determined in the presence of Triton X-100 at a final concentration of 0.2%, and alkaline phosphatase activity (△) in the absence of Triton X-100.

Fig. 3. Polyacrylamide-gel electrophoresis of lactate dehydrogenase isoenzymes from the matrix-vesicle and chondrocyte fractions

Cytosolic fractions of chondrocyte and matrix-vesicle fractions were prepared from growth and resting cartilages as described in Figs. 1 and 2. Matrix-vesicle fractions were solubilized with 0.2% Triton X-100 and centrifuged at 100000 *g* for 60 min, and the precipitate discarded. The resulting extracts of the matrix-vesicle fractions and cytosol of chondrocytes were separately subjected to polyacrylamide-gel electrophoresis as described in the text. A, chondrocyte from the growth cartilage; B, matrix-vesicle fraction from the growth cartilage; C, chondrocyte from the resting cartilage; D, matrix vesicle fraction from the resting cartilage.

Fig. 2 shows representative sedimentations for chondrocyte homogenates prepared from the growth and resting cartilages in sucrose density gradients. In each case, lactate dehydrogenase activity was recovered only in the soluble top fraction, but did not sediment, suggesting the presence of lactate dehydrogenase only in the cytosol of chondrocytes. The results show that lactate

dehydrogenase in the vesicles is not from the plasma membranes and the membranes of intracellular organelles.

Fig. 3 shows electrophoretic profiles of lactate dehydrogenase isoenzymes from vesicles and from the cytosol of chondrocytes isolated from the growth and resting cartilages. Isoenzyme patterns of lactate dehydrogenase were identical in each case (vesicles from growth and resting cartilages and chondrocyte cytosols from the growth and resting cartilages). Other cytosolic enzymes such as aldolase, aspartate:2-oxoglutarate aminotransferase and alanine:2-oxoglutarate aminotransferase were not detected in the two different vesicles from the growth and resting cartilages, suggesting the presence of a mechanism for the specific uptake of cytosolic lactate dehydrogenase.

On the other hand, the same results were obtained with the growth and resting cartilages of young-dog and young-monkey rib bones, suggesting that lactate dehydrogenase-containing vesicles without alkaline phosphatase are also present in other animals, including humans.

When the slices of the growth and resting cartilages were separately subjected to differential centrifugation after the collagenase treatment described in the Materials and methods section, 5.6% of the total lactate dehydrogenase activity was recovered in the matrix-vesicle fraction of the growth cartilage and 5.2% in that of the resting cartilage (Table 1). In each case, lactate dehydrogenase activity was detected only in the presence of Triton X-100, suggesting that lactate dehydrogenase is present in latent form in the vesicles. On the other hand, about 50% of the total lactate dehydrogenase activity was recovered in the soluble fraction of the growth cartilage and about 70% in that of the resting cartilage, suggesting the possibility that lactate dehydrogenase in the matrix-vesicle fraction results from cell lysis and vesicle capture during the matrix-vesicle preparation (Table 1).

When 2 ml of the chondrocyte fraction was homogenized and then subjected to the differential centrifugation described above, lactate dehydrogenase activity

Table 1. Distribution of lactate dehydrogenase activity in the centrifugal fractions obtained from collagenase digestion of growth and resting cartilages

Details of differential centrifugation and assay methods are given in the text. Lactate dehydrogenase activity was determined in the presence of Triton X-100.

Fraction	Growth				Resting			
	Protein (mg/g of tissue wt.)	Total activity (units)	Sp. activity (units/mg)	Recovery (%)	Protein (mg/g of tissue wt.)	Total activity (units)	Sp. activity (units/mg)	Recovery (%)
Cell suspension after digestion	21.17	17.70	0.84	(100)	25.90	6.10	0.24	(100)
Chondrocyte fraction	0.43	2.36	5.49	13.3	0.34	0.16	0.47	2.6
20000 <i>g</i> /20 min pellet	0.08	0.32	4.00	1.8	0.19	0.15	0.79	2.5
Matrix-vesicle fraction	0.16	1.00	6.25	5.6	0.19	0.32	1.68	5.2
Soluble fraction	19.18	9.20	0.48	51.9	22.66	4.08	0.19	70.5

was not detected in the microsomal fraction (20000 *g* for 20 min and 100000 *g* for 60 min) having a density identical with that of the matrix vesicle in the presence of Triton X-100. When 2 ml of the chondrocyte fraction was homogenized, sonicated at 10 kHz at 0 °C for 20 s and subjected to differential centrifugation, about 2.2 % of the lactate dehydrogenase activity of the chondrocyte homogenate was detected in the microsomal fraction (20000 *g* for 20 min and 100000 *g* for 60 min), in both the presence or absence of Triton X-100, suggesting that lactate dehydrogenase activity in the microsomal fraction binds to the outer surface of the vesicles.

On the other hand, when rat liver slices were digested with collagenase and a matrix-vesicle fraction (20000 *g* for 20 min and 100000 *g* for 60 min) was prepared by differential centrifugation (see the Materials and methods section), little or no lactate dehydrogenase activity was detected in the matrix-vesicle fraction, suggesting that lactate dehydrogenase-containing vesicles are not present in tissues other than cartilage.

These results show that, in the cartilage, lactate dehydrogenase in the vesicle does not result from cell lysis and vesicle capture during the matrix-vesicle preparation.

Why are the matrix vesicles with both alkaline phosphatase and lactate dehydrogenase present in the growth, but not in the resting, cartilage? Why are lactate dehydrogenase-containing vesicles lacking alkaline phosphatase in the resting cartilage, but not in the growth cartilage? These questions are reasonable, because (1) matrix vesicles may originate from the plasma membrane of chondrocytes and (2) alkaline phosphatase is enriched in the plasma membrane of chondrocytes in the growth cartilage and is almost never located in chondrocytes in the resting cartilage [25].

Matrix vesicles with alkaline phosphatase are known to be associated with the initial mineralization of cartilage. However, the function of lactate dehydrogenase containing-vesicles without alkaline phosphatase is unknown. It would be of interest to determine whether these vesicles are also associated with biological calcification or have other functions.

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