Effect of Low Oxygen Tensions on Glucose-Metabolizing Enzymes in Cultured Articular Chondrocytes (37349)

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A variety of evidence suggests that low oxygen tension is a major factor in the differentiation of cartilage from precursor cells (1). Articular cartilage has a high glycolytic activity (2, 3) presumably as an adaptation to its avascularity. Its lactate:malate dehydrogenase ratio is higher than any other studied (4). Furthermore, the ratio increases greatly during the chondrogenic phase of the response of subcutaneous tissue to instillation of powdered bone. The purpose of the following study was to see whether articular chondrocytes in monolayer culture express this phenotype. They were compared for this purpose with skin-derived fibrocytes under aerobic conditions and when challenged by low O_2 concentrations.

Technic and design of culture. The chondrocytes and fibrocytes were isolated from 6–8 week old New Zealand rabbits by methods described previously (5, 6). The cells studied were in secondary culture. They were grown in glass petri dishes containing Dulbecco–Vogt medium supplemented with 10% fetal calf serum and pencillin–streptomycin (10 μg/ml and 10 units/ml, respectively). Three gas mixtures containing 10% CO_2 were employed to provide final O_2 concentrations of 19, 6.8 and 0.6% (6). On Day 7 the cells were scraped from the dishes, washed twice with Gey's solution, and centrifuged. In most experiments, the pellets were stored frozen for 3 days; in others, assays were carried out immediately on unfrozen cells. The cells were disrupted hypotonically by immersion in 4–6 ml cold 0.01 *M* Tris buffer. This method resulted in higher specific activities of the enzymes than did two other methods of disruption: glycerol treatment or repeated freeze–thawing. The extract was centrifuged in the cold for 30 min at 13,200*g*. The supernatant fluid was divided into aliquots, some of which were frozen until used, generally within 60 hr. With the exception of the two enzymes indicated in Table II, freezing did not significantly affect the values obtained.

Enzyme assays and chemicals. Assays for 12 glucose-metabolizing enzymes were carried out on the extracts (Table I). The rate of oxidation or reduction of the nucleotide coenzymes was measured spectrophotometrically for 5 min upon adding appropriate substrates and enzymes (7–11). Absorption changes were measured using silica cuvettes having a one-cm light path in a Beckman DU spectrophotometer. The net reaction rate was calculated as the difference between the values before and after addition of the extract, divided by 5. All assays were performed in a total volume of 3 ml at 33°. The concentration of substrates and added enzymes employed were found in preliminary experiments to give maximal reaction rates for each enzyme. Each reaction mixture contained 200 μmoles Tris–HCl pH 7.6. The enzyme reagents were purchased from Sigma Chemical Company, St. Louis, MO, as were sodium fructose-6-phosphate, sodium fructose diphosphate and glyceraldehyde-3-phosphoric acid. Tris base was obtained from Schwarz/Mann, Orangeburg, NY, and all other reagents from Nutritional Biochemicals Corporation, Cleveland, OH. Protein content of the cell extract was determined by the method of Lowry, *et al.* (12).

Results. The specific activities of the

TABLE I. Methods of Enzyme Assay.

Enzyme	E.C. number	Abbreviation	Reaction mixture (3.0 ml)[a]	Reference
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6PDH	$MgCl_2$, 10; G6P, 5; NADP, 0.24	7
Hexokinase	2.7.1.1.	HK	$MgCl_2$, 10; ATP, 10; Glucose, 4; NADP, 0.24; G6PDH, 2 U	8
Phosphoglucomutase	2.7.5.1.	PGM	$MgCl_2$, 10; Cysteine, 20; Glucose-1-phosphate, 20; NADP, 0.24; G6PDH, 4 U	9
Phosphoglucose isomerase	5.3.1.9	PGI	$MgCl_2$, 10; Fructose-6-phosphate, 10; NADP, 0.24; G6PDH, 2 U	7
Phosphofructokinase	2.7.1.11	PFK	$MgCl_2$, 10; Fructose-6-phosphate, 10; ATP, 10; $NADH_2$, 0.24; Aldolase, 0.6 U; Triosephosphate isomerase (TPI), 17 U; Glycerophosphate dehydrogenase (GDH), 2 U	10
Aldolase	4.1.2.13	—	Fructose-1, 6-diphosphate, 20; $NADH_2$, 0.24; TPI, 17 U; GDH, 2 U	11
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	GAPDH	Glyceraldehyde-3-phosphate, 5; Cysteine, 15; Arsenate, 17; NAD, 0.3	7
Enolase	4.2.1.11	—	$MgCl_2$, 10; 2-phosphoglycerate, 5	7
Pyruvate kinase	2.7.1.40	PK	$MgCl_2$, 10; Phosphoenolpyruvate, 5; ADP, 10; KCl, 100; $NADH_2$, 0.24; LDH, 4 U	7
Lactate dehydrogenase	1.1.1.27	LDH	Pyruvate, 5; KCl, 100; $NADH_2$, 0.24	7
Malate dehydrogenase	1.1.1.37	MDH	$MgCl_2$, 10; Oxaloacetate, 10; $NADH_2$, 0.24	7
Isocitrate dehydrogenase	1.1.1.41	IDH	$MgCl_2$, 10; isocitrate, 10; NADP, 0.24	7

[a] Substrate and cofactor values, μmole; U, enzyme units.

enzymes were generaly higher in the chondrocytes than in the fibrocytes (Table II). Statistical significance was calculated by the Student *t* test. Because of the limited number of experiments, only conservative estimates of significance are presented. The *p* values for PK, enolase and PGM at 19% O_2 were < 0.01. Reducing the oxygen concentration in the chondrocyte and fibrocyte cultures from 19 to 0.6% resulted in an increased activity of LDH ($\Delta = 88$ and 58%, respectively), MDH (30 and 42), PGM (67 and 141), aldolase (151 and 23), enolase (59 and 80), PFK (43 and 406), and PGI (120 and 164). Chondrocyte HK and fibrocyte PK demonstrated a slightly increased activity. An inhibitory or no stimulatory effect was demonstrated by IDH, GAPDH, and G6PDH.

The LDH:MDH ratios increased progressively as the oxygen tensions were lowered (Table III). At each specific oxygen concentration the ratio for the chondrocytes was greater than that for the fibrocytes.

Discussion. When rabbit articular chondrocytes are cultured under monolayer conditions, several cartilaginous properties are expressed while apparent metaplasia to fibrocytic patterns is found in other respects (13). In this study, chondrocytes were compared with cutaneous fibrocytes because they are kindred mesenchymal cells but the parent tissue of the former has a disproportionally higher glycolytic metabolism as manifested by the LDH:MDH ratio (4). Glycolytic activity characteristically is higher in cultured cells than *in vivo* (14). It is thus not surprising that the enzyme pattern of the cultured fibrocytes, as well as the chondrocytes, resembled intact articular cartilage (15). Nevertheless, the significantly higher LDH:MDH ratio of the cultured chondrocytes is consistent with preservation of a chondroid phenotype in monolayer culture. It parallels a higher rate of glycolysis (6) and several other cultural, morphological and chemical characteristics of these cells (13).

TABLE II. Enzyme Activities (nmoles/min/mg protein).[a]

	Oxygen concentration					
	Chondrocytes			Fibrocytes		
Enzyme	0.6	6.8	19	0.6	6.8	19
LDH	5997 ± 258 (5)	4290 ± 535 (5)	3190 ± 299[d] (5)	4902 ± 608 (4)	4082 ± 331 (4)	3100 ± 245[d] (4)
MDH	4702 ± 483 (5)	4242 ± 476 (4)	3629 ± 581 (5)	4685 ± 274 (4)	3963 ± 131 (5)	3290 ± 346 (6)
PGI	4436 ± 588 (2)	2968 ± 76 (2)	2016 ± 297 (2)	3656 ± 400 (2)	1672 ± 115 (2)	1382 ± 203 (2)
PK[b]	870 ± 278 (3)	1226 ± 292 (3)	948 ± 171 (4)	143 ± 45 (2)	136 ± 10 (3)	91 ± 19 (5)
Enolase	834 ± 73 (4)	713 ± 100 (5)	526 ± 34[c] (5)	537 ± 55 (5)	365 ± 47 (6)	299 ± 27[c] (6)
GAPDH	402 ± 23 (4)	874 ± 75 (3)	891 ± 137[c] (2)	457 ± 278 (3)	232 ± 69 (3)	193 ± 105 (3)
PGM	358 ± 33 (4)	249 ± 19 (5)	215 ± 25[c] (6)	263 ± 43 (4)	138 ± 13 (5)	109 ± 14[c] (5)
PFK[b]	318 (1)	267 (1)	224 (1)	71 ± 20 (2)	37 ± 11 (2)	13 ± 6 (2)
Aldolase	168 ± 28 (4)	108 ± 14 (4)	67 ± 10[c] (5)	134 ± 34 (5)	118 ± 19 (4)	109 ± 20 (4)
IDH	60 ± 10 (4)	92 ± 17 (4)	116 ± 17 (4)	24 ± 3 (3)	38 ± 3 (3)	42 ± 6 (3)
HK	29 ± 2 (5)	30 ± 2 (5)	25 ± 3 (5)	21 ± 4 (5)	19 ± 4 (6)	20 ± 4 (6)
G6PDH	20 ± 5 (4)	19 ± 3 (4)	25 ± 3 (4)	23 ± 8 (3)	19 ± 4 (3)	20 ± 4 (3)

[a] Mean ± standard error; the values in parentheses are the number of experiments.
[b] Performed on fresh extracts.
[c] $p < 0.01$ (0.6 vs 19% O_2).
[d] $p < 0.0011$ (0.6 vs 19% O_2).

Low O_2 concentrations increased the specific activities of several glycolytic and one Krebs cycle enzyme. Similar findings have been reported for other cells *in vitro* (16, 17). A Pasteur effect was found in the chondrocyte cultures under reduced oxygen concentrations (6). Although the anaerobic stimulation of PFK may explain this effect as hypothesized by others (18), it was not accompanied by a comparable increase in HK, the apparent rate-limiting enzyme in this system. The higher IDH activity under normal O_2 concentrations favors oxidative metabolism through the Krebs cycle.

TABLE III. Lactate/Malate Dehydrogenase Quotients.[a]

	Oxygen concentration		
Cell type	0.6	6.8	19
Chondrocytes	1.61 ± 0.11 (4)	1.41 ± 0.23 (5)	1.21 ± 0.17 (5)
Fibrocytes	1.28 ± 0.19 (5)	1.18 ± 0.14 (4)	0.84 ± 0.13 (3)

[a] Mean ± standard error; the figures in parentheses are the numbers of values tested.

Summary. The specific activities of 12 glucose-metabolizing enzymes of rabbit articular chondrocytes in monolayer culture were generally higher than those of cutaneous fibrocytes. A greater LDH:MDH ratio in the former suggests preservation of a chondroid phenotype *in vitro*. At reduced oxygen concentrations, levels of LDH, MDH, PGM, aldolase, enolase, PFK and PGI increased. IDH and GAPDH decreased under these conditions while HK and G6DPH were unaffected.

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Received Nov. 27, 1972. P.S.E.B.M., 1973, Vol. 143.