CHANGES IN GLYCOLYTIC ENZYME ACTIVITIES AND CYCLIC NUCLEOTIDE LEVELS OF CULTURED BOVINE VASCULAR CELLS IN HYPERLIPIDEMIC MEDIA

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Comparative studies on changes in glycolytic and hydrolytic enzyme activities and cyclic nucleotide contents in bovine vascular smooth muscle cells were carried out in an attempt to elucidate the metabolic changes which occur in vascular cells, under condition of hyperlipidemia. Decreased glycolytic enzymes and increased acid phosphatase activities were found in smooth muscle cells cultured with 5% hyperlipidemic LDL which contained many sudanophilic droplets in their cytoplasm. Statistically significant low levels of cAMP and high levels of cGMP were confirmed in these cells cultured in hyperlipidemic media, as compared with those in control cells. This data suggests that decrease in cyclic AMP content and glycolytic enzyme activities accelerates lipid phagocytosis and cell division, both important phenomena in the course of atherosclerosis.

Smooth muscle cells in vascular tissues with proliferate progression of atherosclerosis (20, 21) and recent studies have been focussed in factors stimulating and accelerating their proliferation. Since Ross found, for the first time, the factor derived from platelets has an effect to proliferate cultured vascular smooth muscle (19), many growth factors has been reported (7, 9). Hyperlipidemic LDL is one of such factors and participates in the process of hyperlipidemia which leads to an enhancement of the pathology of atherosclerosis(4).

Cyclic AMP (cAMP) is an intracellular regulator of cell proliferation. Studies have suggested that levels of intracellular cAMP decrease when the cell cycle proceeds from the resting state (G₀) to the dividing state (G₊) and that the addition of cAMP to the culture media when the cells are in the process of proliferation, inhibit DNA synthesis in various types of cells (5, 6, 11).

In the present work we studied changes in glycolytic and hydrolytic enzyme activities and cyclic nucleotides levels in bovine cultured smooth muscle cells in the hyperlipidemic media, in attempts to determine what metabolic changes occur in these cells and how these changes relate to the progression of atherosclerosis.

MATERIALS AND METHODS

TISSUE CULTURE:

Smooth muscle cells from bovine fetus aorta were cultured according to Ross (22). Bovine fetuses at 5-9 months of gestation were obtained from a local slaughter

house. Segments of the thoracic aorta were dissected and the inner one third of the media was stripped off under a dissecting microscope. After washing several times with fresh medium, these inner portions of the media were cut into about 1 mm squares and placed into a 25 cm² Falcon flask with 0.5 ml of Waymouth media (GIBCO, Cal. USA) with 15% fetal bovine serum (Flow Lab., Cal. U. S. A.). The cells which grew from the explants became confluent after 2-4 weeks. The cells were then trypsinized with 0.05% trypsin (Difco U. S. A.)—0.02% EDTA solution and centrifuged at 1200 r. p. m. for 5 min. The so-obtained pellets were resuspended in complete media and the preparations put into flasks. The 3rd-6th passages were used for experiment.

ENZYME ACTIVITIES:

Cells were seeded into 24 small Falcon dishes. These dishes were separated into two groups, one dish was cultured with Waymouth media and 1.6 mg/dl of BSA and others with Waymouth media and 5% LDL. LDL was prepared from three hyperlipidemic male rabbits fed 1% cholesterol containing diet for three months, according to the method of Havel *et al.* (8). Fresh media were added every other day. On the 14th day, activities of phosphorylase (PR: Takeuchi's method.), Glucose-6-phosphate dehydrogenase (G-6-PDH: Rudolph-Klim's method), phosphofructo-kinase (PFK: Sigel-Pette's method), lactate dehydrogenase (LDH: Nacklas-Seligman's method), succinic dehydrogenase (SDH: Nacklas-Seligman's method), acid phosphatase (Acid Pse: Burston's method), alkali-phosphatase (Alkali Pse: Burston's method), ATPase (Wachstein-Meisel's), and 5'-nucleotides (5'N: Wachstein-Meisel's method) were studied histochemically and compared between both groups. Sudan III staining was also performed in each dish of both groups to know how much lipid contained in cultured smooth muscle cells.

ASSAY OF CYCLIC NUCLEOTIDES:

After two weeks' culture, the cells were harvested from two dishes in each group with a rubber policeman, immediately lyophilized and contents cAMP and cGMP were measured according to the methods of Numano *et al.* (14).

RESULTS

Figs. 1a, b. shows the cultured cells stained with Sudan III. In the control group, several cultured cells whose cytoplasm contained fine sudanophilic substances were encountered (Fig. 1a). On the contrary, all smooth muscle cells cultured with hyperlipidemic LDL contained many sudanophilic droplets (Fig. 1b).

Table 1 gives a comparison of glycolytic and hydrolytic enzymes activities in the two groups. Almost all enzyme activities in smooth muscle cells cultured with

Figs. 1a,b. a. Smooth muscle cells cultured with control media stained with Sudan III. b. Smooth muscle cells cultured with 5% LDL stained with Sudan. a, b, $\times 400$

FIG. 2. Phosphorylase activity in smooth muscle cells cultured in control media. $\times 100$

FIG. 3. Acid phosphatase activity in smooth muscle cells cultured in hyperlipidemic LDL. $\times 400$

FIG. 4. G-6-PDH activities in smooth muscle cells cultured in control media (a) and hyperlipidemic LDL (b). $\times 400$

FIG. 5. LDH activities in smooth muscle cells cultured in control media (a) and hyperlipidemic LDL (b). $\times 400$

FIG. 1a.

FIG. 1b.

FIG. 2.

FIG. 3.

FIG. 4a.

FIG. 4b.

FIG. 5a.

FIG. 5b.

TABLE 1. *Enzyme activities in bovine aortic smooth muscle cells cultured in control media and 5% hyperlipidemic LDL*

	Control	Hyperlipidemic LDL
PR	++	—
G-6 PDH	++	+
PFK	++	+
LDH	++	+
SDH	++	±
Acid Pase	+	++
Alkali Pase	—	—
ATPase	++	+
5'-N	++	+

TABLE 2. *Contents of cyclic nucleotides in cultured bovine smooth muscle on the 14th cultured day*

Cyclic Nucleotides Group	cAMP		cGMP	
	pmoles/mg D.W.	pmoles/mg protein	pmoles/mg D.W.	pmoles/mg protein
Control	0.42±0.07	4.84±0.78	0.13±0.01	1.50±0.10
5% LDL	0.28±0.05**	2.12±0.79**	0.18±0.01	2.71±0.21**

** P<0.01 Control vs. 5% LDL

hyperlipidemic LDL revealed a weak reaction as compared with those in control cells. Especially phosphorylase activities shown in Fig. 2 were negative in hyperlipidemic group.

Figs. 4 and 5 show the activities of G-6-PDH and LDH in both group. Remarkably decreased activities in both enzymes were noticed in hyperlipidemic cultured smooth muscle cells.

On the other hand, shown in Fig. 3, acid phosphatase in smooth muscle cells cultured in hyperlipidemic media revealed an increased activity as compared with that of control cells.

Table 2 shows changes in the contents of cAMP and cGMP in both groups on the 14th culture day. A statistically significant decreased content of cAMP in smooth muscle cells cultured with LDL was confirmed, as compared with that in cells cultured with control media. On the contrary, the content of cGMP increased in cells cultured with LDL and a statistically significant increase was obtained when the contents of cGMP were expressed as picomoles per mg protein of tissues.

DISCUSSION

Since the discovery of LDL receptors in tissue cells by Goldstein and Brown, lipid metabolism in vascular cells has been intensively studied in relation to the progression and or regression of atherosclerosis (2, 3, 24). Furthermore hyperlipidemic LDL has been reported its promoting effect in the proliferation of cultured smooth muscle cells and normolipemic LDL and other liprotein fractions were less effective

(12, 26), which should be naturally followed that it may play a role in developing atherosclerosis.

On the other hand, cyclic nucleotides are important modulators of cell proliferation and cell differentiation(1, 18) and intracellular levels of cAMP were inversely proportional to the rate of DNA synthesis(17). Tadoro reported, when quiescent cells were exposed to fresh serum, the level of cAMP was reduced(25). This data makes us suppose that our present data regarding the decrease of cAMP content and increased cGMP suggest the proliferative phase in hyperlipidemic LDL cultured smooth muscle cells and that the proliferative effect of LDL may lead a decrease in the contents of cAMP.

Histochemical studies on glycolytic enzyme also revealed the decreased activities in the hyperlipidemic LDL cultured cells. Numano *et al.* also reported that glycolytic enzyme activities and contents of cAMP in the aortic wall of rabbits gradually decreased with the progression of atherosclerotic changes, under conditions of cholesterol feeding (13, 15).

Numano *et al.* (16) also observed the decreased content of cAMP in endothelial line in the aorta of animals when vascular permeability increased, They suppose the decrease in the content of cAMP enhanced the contraction of endothelial cells and exerted an increase in plasmal infiltration into the aortic wall(16). Our present histochemical studies in tissue culture suggest the decrease in cAMP contents causes the decreased glycolytic enzyme activities, lipid phagocytosis and acceleration of cell division findings comparable with the results observed in the course of progression of atherosclerosis.

The mechanism of action of cAMP on the suppressive effect on cell proliferation remains unknown. Roles of Ca, phosphorylation of chromatin or two types of protein kinase have been postulated(10, 23). On this subject further studies will be followed in connection with prevention and regression of atherosclerosis.

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