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EFFECT OF CYCLIC NUCLEOTIDES ON PROLIFERATIVE ACTIVITY OF PRIMARY CELL CULTURES FROM THE ATHEROSCLEROTIC HUMAN AORTA

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KEY WORDS: atherosclerosis; culture of aortic cells; proliferation; cyclic nucleotides.

Artificially induced atherosclerosis in animals is characterized by intensification of proliferative activity of the cells in the intimal layer of arteries [4, 9]. It has been suggested that intensification of proliferation also takes place in a region of spontaneous atherosclerosis of blood vessels in man. Cells isolated from zones of initial atherosclerotic lesions, in primary culture, have been shown to have greater proliferative activity than cells of the undamaged intima [1, 2]. Abundant facts have now been gathered to show that proliferation and differentiation of certain types of cells in eukaryotes are under the control of the cyclic nucleotide system and that elevation of the intracellular cAMP level inhibits cell division [11]. It has been suggested that cAMP derivatives and compounds which raise the cAMP concentration may be effective preparations inhibiting cell proliferation. However, there is evidence in the literature that cAMP may have the opposite action on proliferative activity of certain types of cells [6]. Hence the need to investigate the role of cyclic nucleotides in the processes of cell division and differentiation for each cellular system.

The object of this investigation was to study the effect of cyclic nucleotides on incorporation of [^{3}H]thymidine into cells of the intima from unaffected and atherosclerotic regions of the human aorta.

EXPERIMENTAL METHOD

A culture of intimal cells from atherosclerotic and nonatherosclerotic regions of the human aorta was obtained as described previously [7]. The cells were cultured in plastic Petri dishes 35 mm in diameter (Corning, USA) with seeding density of 10^4 cells/cm^2 (plating efficiency 50-60%) in 1.5 ml of medium 199 containing 10% embryonic calf serum, 2.5 μg/ml fungizone, 100 μg/ml kanamycin, and 2 mM glutamine (all reagents from Gibco, USA) at 37°C in an atmosphere of 5% CO_2 and 95% air, saturated with water vapor. Every 3 days the medium was changed. On the 10th day dibutyryl-cAMP (dbcAMP), dibutyryl cGMP (dbcGMP), or sodium butyrate (Sigma, USA) was added to the cultures. Next day the compounds were added again, together with 1 μCi/ml of [^{3}H]thymidine (21 Ci/mmole, from Amersham Corporation, England). After one day the cultures were washed twice with isotonic Dulbecco's phosphate buffer and the cells were suspended by 0.25% trypsin with 1 mM EDTA (Gibco, USA). To determine incorporation of labeled thymidine 2 ml of 15% TCA was added to 1 ml of the cell suspension. The residue after centrifugation (6000g, 20 min) was washed twice with 2 ml of 10% TCA and dissolved in 200 μl of 0.5 N NaOH. The solution was neutralized with 0.5 M HCl and radioactivity was measured on a 1215 RackBeta II liquid scintillation counter (LKB, Sweden), using Bray's scintillation solution [3]. Liposomes containing cAMP were prepared from ovolecithin, stearylamine, and cholesterol (Gibco, USA) in molar proportions of 5:1:3 by Papahadjopoulos' method [8]. The liposomes (0.2 mg/ml of total lipid and 10^{-5} M cAMP in the final volume) were added to the cells on the 10th day of culture. [^{3}H]Thymidine was added to the medium 24 h after the second addition of liposomes and its incorporation was determined as described above. The experimental results were subjected to statistical analysis. The significance of differences was estimated by Student's t test.

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TABLE 1. Effect of dbcAMP, dbcGMP, and Sodium Butyrate on Incorporation of [^{3}H]Thymidine into Intimal Cells Isolated from Unaffected and Atherosclerotic Regions of the Human Aorta (mean ± mathematical standard deviation)

Region of isolation of intimal cells	Incorporation of [^{3}H]thymidine, cpm $\cdot 10^{-3}/10^5$ cells			
	control	dbcAMP 10^{-4} M	dbcGMP 10^{-4} M	Sodium butyrate $2 \cdot 10^{-4}$ M
Aorta 1:				
Normal	19,5±1,7 (6)	9,4±1,0** (3)	26,3±2,1 (3)	—
Fatty infiltration	31,1±2,5*** (6)	16,6±2,3** (3)	26,2±4,4 (3)	—
Aorta 2:				
Normal	10,1±0,9 (6)	5,2±0,7** (3)	16,5±1,8** (3)	7,2±0,8* (3)
Lipid streak	18,9±1,3*** (6)	3,3±0,8** (3)	13,5±1,2* (3)	11,8±1,2* (3)
Aorta 3:				
Lipid streak	22,4±2,1 (4)	9,8±1,3* (3)	17,3±1,9 (3)	10,7±1,0* (3)
Plaque	20,7±1,5 (6)	12,7±1,3* (3)	14,3±1,2* (3)	14,1±1,2* (3)
Aorta 4:				
Normal	4,0±0,1 (3)	1,7±0,1** (3)	8,0±1,1* (3)	2,6±0,3* (3)
Fatty infiltration	4,7±0,2 (3)	2,1±0,6** (3)	5,0±0,2 (3)	4,0±0,1 (3)
Plaque	3,3±0,4 (3)	1,4±0,2** (3)	1,7±0,3* (3)	2,2±0,4 (3)

Legend. Number of determinations shown in parentheses. *$P < 0.05$, **$P < 0.01$ respectively compared with control; ***$P < 0.01$ compared with cultures obtained from unaffected regions of aorta.

EXPERIMENTAL RESULTS

Preliminary experiments showed that addition of cAMP or its enzymic degradation products (5'AMP and adenosine) did not affect incorporation of [^{3}H]thymidine by intimal cells of the human aorta. Later, therefore, we used the dibutyryl derivative of cAMP (dbcAMP), which is able to pass through the plasma membrane of cells. In a concentration of 10^{-4} M, dbcAMP lowered the proliferative activity of the intimal cells isolated from unaffected regions of the aorta (Table 1). An increase in the dbcAMP concentration increased the effect of this compound on incorporation of labeled thymidine by the cells (Fig. 1a).

Proliferative activity in cultures isolated from regions with fatty infiltration and lipid streaks was higher than in cells from unaffected regions (Table 1). dbcAMP inhibited incorporation of [^{3}H]thymidine by cells from regions with early atherosclerotic changes. When dbcAMP was used in a concentration of 10^{-3} M incorporation of [^{3}H]thymidine fell to a level close to the value for cells from unaffected parts of the aorta (Fig. 1a). Lowering of incorporation of labeled thymidine also was observed when the duration of action of dbcAMP was increased (Fig. 2). Prolonged exposure to dbcAMP prevented the rise in [^{3}H]thymidine incorporation by the cells for 4 days after the beginning of its action, i.e., blocking of the cells was observed at stages preceding the S phase. In these experiments dbcAMP also was shown to depress the proliferative activity of cells isolated from atherosclerotic plaques (Table 1).

There is evidence in the literature [10] of a fall in the proliferative activity of certain types of cells under the influence of the butyric acid residue which can be formed during degradation of dibutyryl derivatives of cyclic nucleotides. In the present experiments addition of sodium butyrate to the culture medium caused a decrease in incorporation of [^{3}H]-thymidine by cells of the human aorta, but less marked than when equimolar concentrations of dbcAMP were used (Table 1). Lowering of the proliferative activity of the cells under the influence of dbcAMP can thus be explained only partly by the effect of butyrate.

The cAMP content in the cells also was increased by introducing cyclic nucleotide by means of liposomes. Addition of liposomes containing cAMP to the cultures had an effect comparable with the action of dbcAMP (Fig. 3). Liposomes not containing cAMP did not affect proliferative activity. This, and also the fact that exogenous cAMP did not change the incorporation of [^{3}H]thymidine, indicates that in order to depress proliferative activity, the intracellular cAMP level must be raised.

Addition of the dibutyryl derivative of another intracellular regulator — cGMP — to intimal cells from unaffected regions of the aorta caused an increase in incorporation of [^{3}H]-thymidine (Table 1). These findings support Goldberg's hypothesis [5] that the cyclic nucleotides regulate cell processes in different directions. However, in the case when cells were isolated from regions with early atherosclerotic changes, dbcGMP depressed the proliferative

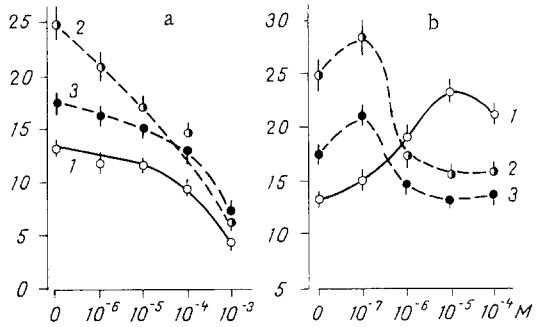

Fig. 1. Effect of dbcAMP (a) and dbcGMP (b) on incorporation of [^{3}H]thymidine by cells isolated from unaffected parts of the aorta (1), from regions with fatty infiltration (2), and from lipid streaks (3) as a function of concentration. Abscissa, concentration of dbcAMP and dbcGMP (in M); ordinate, incorporation of [^{3}H]thymidine (in cpm $\times 10^{-3}/10^5$ cells). Each point represents results of three to four determinations ± standard mathematical deviation.

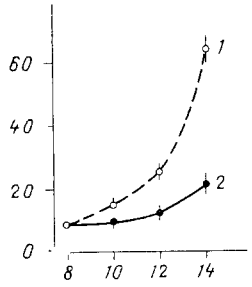

Fig. 2

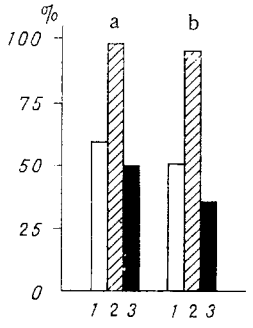

Fig. 3

Fig. 2. Effect of dbcAMP on incorporation of [^{3}H]thymidine into intimal cells isolated from lipid streaks as a function of time. Abscissa, duration of action of dbcAMP (in days); ordinate, incorporation of [^{3}H]thymidine (in cmp·$10^{-3}/10^5$ cells). 1) Control, 2) 10^{-4} M dbcAMP. dbcAMP was added daily to the cells starting on the 8th day of culture. Incubation with [^{3}H]thymidine for 24 h.

Fig. 3. Effect of 0.1 mM dbcAMP (1), liposomes (2), and liposomes containing cAMP (3) on proliferative activity of cells obtained from unaffected parts of aorta (a) and from lipid streaks (b).

activity of the cells (Fig. 1b). The cause of this effect is not clear, but it can be postulated that normal functioning of the cyclic nucleotide system is disturbed in cells from atherosclerotic regions.

This investigation thus showed that an increase in the intracellular cAMP concentration lowers the proliferative activity of intimal cells of the human aorta. Incorporation of [^{3}H]-thymidine into cells isolated from early atherosclerotic lesions is lowered to the level observed in cells from unaffected regions. It can be tentatively suggested that cAMP derivatives and compounds capable of increasing the intracellular cAMP concentration can be used to depress cell division in a region of atherosclerosis.

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ACTION OF THIOTEPA ON THE DIFFERENTIATING OOCYTE POPULATION OF CBA, 101/H, AND AKR MICE

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KEY WORDS: thiotepa; oocytes.

A previous study of the action of the trifunctional alkylating agent thiotepa on mouse somatic embryonic cells showed that this mutagen, like certain other alkylating agents, induces the formation of cross-bridges in the DNA molecule, the number of which rises slowly with time [8]. This injury may be very important for determination of the cytotoxic, cytostatic, and mutagenic action of these compounds on mammlian cells. The mutagenic action of alkylating agents on somatic cell chromosomes has been studied sufficiently well [3, 8]. It has also been shown [6] that if thiotepa is given to males it induces dominant lethal mutations in mature spermatozoa and spermatids of mice which are realized in progeny obtained from males treated with this mutagen. The action of alkylating agents on differentiating female sex cells has been inadequately studied although we know that extremal influences acting on female sex cells in the period of antenatal oogenesis may be the cause of development of partial or total sterility of individuals in the postnatal period [13, 15].

This paper gives the results of a quantitative cytological assessment of the progress of oogenesis in mice after injection of thiotepa into females on the 12th day of pregnancy. According to data in the literature [9] the population of sex cells in embryonic mouse ovaries on the 12th day of development consists of proliferating oogonia and preleptotene oocytes.

EXPERIMENTAL METHOD

Pregnant CBA, 101/H, and AKR mice kept under standard conditions in the vivarium of the Institute of Medical Genetics, Academy of Medical Sciences of the USSR, were used. The females were mated with males (two females to one male) on one night. The first day of pregnancy was taken to be the day of removal of the females from the males. On the 12th day of pregnancy the mice were given an intraperitoneal injection of thiotepa in physiological saline in a dose of 5 mg/kg body weight and in a volume of 0.5 ml/20 g body weight [2]. The animals were killed on the 19th day of pregnancy. During this period of development the majority of oocytes in the embryonic ovaries were in the pachytene stage. Fetuses at the same time of development taken from intact females, served as the control.

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