α-L-FUCOSIDASE SECRETION BY HUMAN EMBRYONIC SKIN FIBROBLASTS

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The study of secretion of lysosomal enzymes is essential in connection with attempts to use subcutaneous transplantation of secreting cells in medical practice in order to correct hereditary enzymopathies associated with lysosomal glycosidase insufficiency [6, 9].

Many investigations of the secretion of lysosomal enzymes by macrophages, monocytes, and neutrophils in culture have been published [2, 14]. Less information on this process in normal skin fibroblasts has been given. Mainly secretion of N-acetyl-β-D-hexosaminidase (β-Hex) has been described in the literature [5, 8, 11], evidently because of its high relative activity in the cell and its high stability, on account of which the accumulation of this enzyme in the culture medium can be examined. Besides β-Hex, attempts have been made to find secretion of acid phosphatase, β-D-galactosidase, β-glucuronidase, and α-glucosidase by skin fibroblasts. According to some workers, these enzymes are almost undetectable in the culture medium, whereas according to others they are present in very small quantities [4, 7, 12]. This is evidently connected with the low level of their secretion or their instability in the medium. Meanwhile we could find only one reference in the literature with data on secretion of α-L-fucosidase (α-Fuc) by normal and pathological fibroblasts ("I-cell" disease); in that study most attention was paid to the effect of a disturbance of glycoprotein biogenesis on secretion of the enzyme [13].

This paper describes a study of α-Fuc secretion by human embryonic fibroblasts (dependence of the concentration of serum, an important component of the medium, on the stage of monolayer development) and secretion by different strains of cells was compared. In one series of experiments, for comparison, data were obtained on β-Hex secretion also.

EXPERIMENTAL METHOD

Experiments were carried out on three strains of embryonic skin fibroblasts: 845, 814, and 820 (from the bank of the Institute of Medical Genetics, Academy of Medical Sciences of the USSR). The cells were subcultured in Eagle's medium with 10% bovine serum (BS) with the addition of glutamine and kanamycin. The solution of BS was inactivated beforehand by heating for 45 min at 56°C, which led to total destruction of the α-Fuc contained in it. The culture medium in the experimental vessels was replaced by fresh medium 24 h after seeding of the cells (the volume of fresh medium added was accurately measured and, besides the above-mentioned comonents, it also contained 10 mM HEPES. The medium poured off after the experiment was

TABLE 1. Specific Activity of α-Fuc and β-Hex (in nmoles/h/10^6 cells, strain 820) Secreted Depending on Serum Concentration in Medium (M ± m)

Enzyme	Medium with 10% BS	Medium with 0.1% BS
α-Fuc	1,71±0,02	1,33±0,10
β-Hex	137±21	84±15

Legend. Here and in Tables 2 and 3 mean values for 3-5 culture vessels in an experiment are given.

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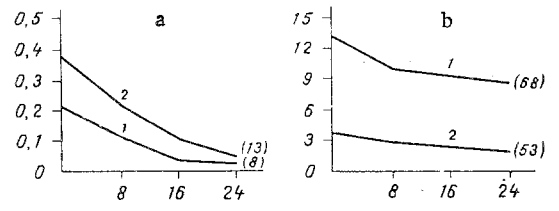

Fig. 1. Change in quantity of α-Fuc (a) and β-Hex (b) secreted in conditioned medium with 10% (1) and 0.1% (2) BS in early stages after incubation at 37°C. Abscissa, enzyme activity (in nmoles/h/150 μl medium); ordinate, incubation time (in h). Numbers in parentheses given quantity of enzyme preserved (in % of initial level).

centrifuged at 3500g for 30 min; cells were removed from the surface of the culture vessel with trypsin and counted in a Goryaev's chamber. Activity of the enzymes was determined according to their ability to release 4-methylumbelliferone (4-MUF) from the corresponding fluorogenic 4-MUF-glycosides. The incubation mixture consisted of 10 μl cell homogenate, 150 μl 0.05 M phosphate-citrate buffer (pH 4.5), and 100 μl substrate (diluted in the same buffer) or 150 μl of culture medium and 100 μl of substrate, made up in 0.2 M phosphate-citrate buffer (pH 4.5). The quantity of secretion was determined by measuring activity of the enzyme found in the culture medium and calculated per 10^6 cells. Details of the method of investigation were described by the writers previously [1].

EXPERIMENTAL RESULTS

Table 1 gives data on α-Fuc secretion in medium with 10 and 0.1% BS while the cells were kept for 24 h in these media (cells at the confluent monolayer stage were taken for the experiment). As Table 1 shows, levels of α-Fuc secretion were a little lower in medium with 0.1% BS. To compare the conditions of keeping (accumulation) of α-Fuc in the culture medium additional experiments were set up, with incubation of conditioned medium (containing the secreted enzyme) and with different BS concentrations. The results are given in Fig. 1a. In both media inactivation of the secreted α-Fuc took place at about the same rate throughout the 24 h, i.e., the stability of the enzyme was virtually identical in both media. It can therefore be concluded that secretion of α-Fuc in medium with 0.1% BS during the 24 h after the change of medium was in fact 30% less than in medium with 10% BS. Secretion of β-Hex also was determined in these experiments (by measuring the increase in its activity in the culture medium). Activity of β-Hex secreted by cells was 60% lower in medium with 0.1% BS than in medium with 10% BS. However, this difference in the level of secretion of β-Hex was evidently rather lower, in fact, because the conditions for its stabilization are better in medium with 10% BS than in medium with 0.1% BS (data on stability of secreted β-Hex in the medium with 0.1% BS may be the result of reduction of endocytosis, because culture medium with the minimum serum concentration is virtually free from macromolecules capable of inducing endocytosis [3].

In the next series of experiments we compared the level of α-Fuc secretion during development of the monolayer. For this purpose, it was determined in medium with 10% BS on the 4th and 7th days of growth of cells in it (on the 3rd and 6th days respectively after the change of medium). As already stated, the enzyme found in medium with 10% BS is part of that secreted mainly during the last day (because it did not accumulate). In the experiment described above we were therefore dealing with the level of secretion in the early and late stages of formation of a confluent monolayer. Table 2 shows that in the last case the level of α-Fuc secretion was higher than at the earlier stage of monolayer development. Table 2 also gives values of specific activity of secreted α-Fuc after the cells had been kept for 3 and 6 days in medium with 0.1% BS (as in the previous experiment, the cells were introduced into it on the day after seeding). They were found to be quite close in their values. Since in this particular case no monolayer was formed (because of the cessation of proliferation in the serum-free medium), elevation of the level of α-Fuc secretion which we observed on the 6th day of growth of the cells in medium with 10% BS, evidently characterizes this process in fact in cells at the late stages of the confluent monolayer. It is perhaps the result of the raised level of endocytosis under confluent monolayer conditions [15]. There are data in the literature showing increased β-Hex secretion in the stationary phase of growth of fibroblasts [10, 11].

Table 3 gives the results of determination of specific α-Fuc activity in the cells and culture medium with 0.1% BS after cells of various strains had been kept in it for 3 and 6 h (the medium was changed for medium containing 0.1% BS at the confluent monolayer stage). At each time of the experiment enzyme secreted mainly during the last day was found (see Fig.

TABLE 2. Specific Activity of Secreted α-Fuc (in nmoles/h/10^6 cells) after 3 and 6 Days of Growth in Medium with 10 and 0.1% BS, Strain 845 (M ± m)

Time of experiment, days	Medium with 10% BS	Medium with 0.1% BS
3	1,76±0,04	1,40±0,01
6	3,44±0,20	1,60±0,02

TABLE 3. Specific Activity (in nmoles/h/10^6 cells) of Intracellular and Secreted α-Fuc in Human Embryonic Fibroblasts of Various Strains in Medium with 0.1% BS (M ± m)

Strain	Specific activity		Ratio of quantity of secreted α-Fuc to its intracellular concentration, %
	intracellular α-Fuc	secreted α-Fuc	
845	5,2±0,2	2,6±0,1	50
	4,4±0,3	1,9±0,1	43
	6,6±0,5	2,4±0,2	42
			(on average 45)
814	7,2±0,2	1,9±0,15	27
	7,6±0,2	1,2±0,1	15
			(on average 20)
820	15,2±0,3	3,2±0,2	20
	16,3±0,5	2,6±0,2	16
	18,8±0,7	1,4±0,1	7
	15,7±0,6	2,0±0,3	13
			(on average 14)

la for this fraction of it). The strains of human embryonic skin fibroblasts which we studied evidently differed (two-threefold) from one another in the specific activity of the intracellular enzyme. Meanwhile absolute values of activity of α-Fuc secreted by cells of the three strains tested were quite close, including in strains 845 and 820, which differ the most in specific activity of the intracellular enzyme. The similar values of absolute quantities of α-Fuc secreted by different strains may perhaps be evidence that the level of secretion is a sufficiently constant characteristic of cells of this type. Hence it can perhaps be concluded that when the choice of donor cells is made as depots of the enzyme, there is absolutely no need to pick strains of fibroblasts with a high level of intracellular α-Fuc activity. The disparity between the quantity of the enzyme secreted by the cells and its intracellular activity leads to considerable differences in the ratio between these values (the relative level of secretion); the lower the specific activity of the intracellular enzyme, the higher the relative level. There is evidence in the literature that the level of β-Hex secretion is not determined by its intracellular concentration [12]. At the same time, there are also indications that an identical fractional rate of enzyme secretion was found in two lines of human skin fibroblasts, which is possible only if correlation is present between these values [11].

The strains of human embryonic skin fibroblasts studied in the present experiments thus actively secrete α-Fuc, which can be found in comparatively large quantities in the culture medium. One of the factors regulating its secretion is evidently the level of endocytosis in the cell, whereas the intracellular enzyme concentration has no significant effect on the rate of secretion. The relatively less variability of α-Fuc secretion between different strains of fibroblasts, compared with differences in the intracellular activity of the enzyme in these same strains probably reflects the particular features of the secretion process itself, in which an essential role belongs to the plasma membrane.

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REVERSIBLE ACCUMULATION OF DOUBLE- AND SINGLE-STRANDED DNA BREAKS IN DNA IN GROWTH-ARRESTED CELLS

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The formation of breaks in DNA and their repair play an important role in the regulation of many processes taking place in the cell. We know that DNA breaks accumulate during cell differentiation [10, 15], aging of cellular structures and organisms [1, 4, 14], and transition of cells into the resting state [5-7, 9, 11-13]. Stimulation of cell proliferation, on the other hand, is accompanied by repair of DNA breaks [7, 11-13]. As a rule, it is single-stranded breaks that are referred to in the publications cited above. However, detachment of DNA from the nuclear matrix in growth-arrested cells, recently discovered by the present writers, can be explained only by the formation of double-stranded DNA breaks [5].

The aim of this investigation was to study the possibility of formation and repair of double-stranded breaks in DNA during a change in the proliferative status of the cell.

EXPERIMENTAL METHOD

Jungarian hamster fibroblasts, transformed by SV-40 virus were cultured in Carrel's flasks in a nutrient mixture containing Eagle's medium, lactalbumin hydrolysate, and bovine serum in the ratio of 4.5:4.5:1. DNA was labeled by the addition of 0.4 MBq/ml of ^{3}H-thymidine to the incubation medium for 2-7 days. To arrest cell growth, the monolayer formed (7th day after seeding) was placed in medium with the serum concentration reduced to 1% and incubated under these conditions for 7-9 days. The cells were restimulated for division with fresh complete medium. The mitotic index (MI) in the exponentially growing cultures was 20‰ in resting cultures 1-2‰, and 2 days after stimulation it was 16‰. Double-stranded DNA breaks were determined by neutral elution of DNA [8] and single-stranded breaks by the alkaline DNA uncoiling method with fixation of hydroxyapatite [2].

EXPERIMENTAL RESULTS

Typical neutral elution curves of DNA of 4/21 cells, with different proliferative status, are given in Fig. 1. DNA of actively proliferating cells has high molecular weight. During elution not more than 15% of the DNA passes through the filter (Fig. 1, 1). The character of the curves changed sharply after transition of the cells into the resting state. In some preparations the curves sloped steeply in the first fractions, but later tended asymptotically toward zero (Fig. 1, 2). Curves of the other type sloped less steeply, and about 60% of the DNA passed through the filter (Fig. 2, 1). Despite the differences between these curves, their shape is evidence of accumulation of many double breaks of DNA in growth-arrested cells. The two types of curves reflect differences in the degree of DNA fragmentation. To make sure that the accumulation of double-stranded DNA breaks is connected with a change in the proliferative status of the cell, and not with cell death, it was necessary to show that DNA fragmentation was reversible. To do this, the cells were stimulated with fresh complete medium. The neutral elution curves 48 h after stimulation were either indistinguishable from the elution curves of the exponentially growing cells (Fig. 2, 2) or sloped a little more steeply, when 20-30% of the DNA was eluted (Fig. 1, 3). Since the view was held until recently that double-

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