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SYNCHRONIZATION OF CELL PROLIFERATION IN THE ESOPHAGEAL EPITHELIUM OF MICE WITH TUMORS BY HYDROXYUREA

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The synchronizing action of hydroxyurea on the passage of esophageal epithelial cells through the S phase and mitosis was investigated in mice with tumors, making allowance for diurnal fluctuations in the number of these cells in particular phases under natural conditions. To evaluate synchronization *in vivo* two complementary criteria reflecting changes in the number of cells and the rate of change of synchronization are suggested. In artificial synchronization, two groups of cells passing synchronously through the S phase and mitosis were formed in the esophageal epithelium. The degree of artificial synchronization of the first group was less than natural. The number of cells in the second group in the period of DNA synthesis was twice the number of cells synthesizing DNA during natural synchronization, but the rate of change of synchronization was the same in the experimental and control groups.

KEY WORDS: index of labeled nuclei; mitotic index; synchronization; diurnal rhythm.

According to data in the literature, hydroxyurea, which synchronizes the passage of cells from the G_1 period into the S phase and DNA synthesis in experiments on animals *in vivo*, was sufficiently effective against both normal tissues [5, 8] and tumors [4, 7]. However, in the investigations cited no account was taken of diurnal rhythms of proliferative activity, the importance of which has been demonstrated several times when choosing the time of administration and analyzing the action of substances with an effect on particular phases of the mitotic cycle [1-3].

It was accordingly decided to investigate the synchronization of cell proliferation in the esophageal epithelium of mice inoculated with a tumor by means of hydroxyurea, allowing for diurnal fluctuations in mitotic activity and in the number of nuclei synthesizing DNA.

EXPERIMENTAL METHOD

Experiments were carried out on 120 male noninbred albino mice with a mean weight of 30 g. The animals were kept under natural conditions of illumination and with free access to food. Cell proliferation was studied in the basal layer of the epithelium of the lower third of the esophagus. For synchronization, the animals were given two intraperitoneal injections of hydroxyurea at 2 and 4 a.m., each in a dose of 100 mg/kg body weight. The compound was diluted before use in sterile isotonic sodium chloride solution. When given in this way, it was reckoned [6, 7] that the hydroxyurea would block the cells at the end of the G_1 period and in the S phase until 7-9 a.m.

On the second day after subcutaneous inoculation of all the mice with sarcoma 37, half of the animals (experimental group) were injected with hydroxyurea. The other half (control

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group) did not receive the compound. Five mice from each group were sacrificed at intervals of 2 h in the course of 24 h from 4 a.m. (first sacrifice) to 2 a.m. (12th sacrifice). In the experimental group the mice killed at 4 a.m. received one injection of hydroxyurea, whereas the rest each received two injections. For the autoradiographic investigation, all the animals received an intraperitoneal injection of thymidine-H^3 1 h before sacrifice in a dose of 30 μCi per mouse. The material was fixed in Carnoy's fluid. Sections 5 μ thick through the esophagus were coated with type M photographic emulsion, exposed to obtain the label, and then stained with Carazzi's hematoxylin. Nuclei were regarded as labeled if they had five or more grains of silver above them. The mitotic indices (MI) and indices of labeled nuclei (ILN) were determined by examining 9000-10,000 cells, and expressed in promille. Statistical analysis was carried out by the Fisher–Student method. Differences were taken as significant when $P \leq 0.05$.

The degree of synchronization was estimated by the use of two mutually complementary criteria, in the writer's modification.

1. The first criterion is the percentage increase in the number of cells in mitosis or in the S phase in the course of synchronization, relative to the diurnal number of cells in the same phases of the mitotic cycle under natural conditions.

This ratio was chosen because fluctuations in proliferative activity in the tissues have a diurnal rhythm and, consequently, the number of cells passing through any given phase of the mitotic cycle in 24 h is a stable characteristic within certain limits. The number of cells passing through a given phase of the cycle during a certain period can be expressed as the ratio between the area beneath this curve over the chosen time interval and the duration of the given phase. In that case the index of the degree of synchronization can be expressed in the general form of the equation

$$IS\Delta n = \frac{S\Delta n}{S_{24h}} \cdot 100\%,$$

where ISΔn is the index of synchronization reflecting the increase in the number of cells in the phase of the mitotic cycle investigated; SΔn the area beneath the curve of the cell indices during the phase of the cycle investigated and in the area of growth; S_{24h} the area beneath the curve of the same indices under natural conditions during a time interval of 24 h.

2. The second criterion is the rate of increase in the number of cells passing synchronously through the S phase or mitosis. For MI and ILN this rate was expressed in promille per hour.

By means of this criterion it is possible to compare the dynamics of the response of various tissues to the action of the same synchronizer and also the response of the same tissue to the action of different synchronizers.

EXPERIMENTAL RESULTS

Diurnal changes in MI in the esophageal epithelium (Table 1) in the mice of the control group followed a unimodal curve with a maximum at 6 a.m. and a minimum at midnight. The number of labeled nuclei was also maximal at 6 a.m. and minimal at 6 p.m.

In the animals of the experimental group the values of ILN were low from 4 to 8 a.m., rose until noon ($P_{4-12} = 0.0001$), fell until 2 p.m. ($P_{12-2} = 0.017$), and then rose considerably from 2 until 8-10 p.m. (the decrease from 10 p.m. to midnight was not significant). Values of MI were low until noon. At 2 p.m. and 2 a.m. there were peaks, separated by a plateau from 4 to 8 pm. [$P_{2-(4,6,8)} = 0.05$; $P_{(4,6,8)-2} = 0.01$]. The peak of ILN at noon and the increase in the values of MI at 2 p.m. in all probability characterized the same group of cells, for these peaks were the first after the end of the action of hydroxyurea. Since in the experimental group of mice the values of MI and ILN during the period of action of the compound were low, whereas in the mice of the control group they reached a maximum (6 a.m.), it must be assumed that the cells temporarily held up by hydroxyurea in the S phase and giving the first peaks of ILN and MI were cells corresponding to those responsible for the peak of ILN and MI at 6 a.m. in the animals of the control group. The coincidence between the times of the peaks of MI and ILN characterizing the same group of cells could occur if the cells were synchronized before the S phase (in the control animals this is shown by a sharp increase in ILN from 2 to 6 a.m.), and if during DNA synthesis by the group of synchronized cells, the number of cells continuing to enter the S phase was greater than the number of cell

TABLE 1. Diurnal Changes in MI and ILN in Esophageal Epithelium of Mice with Tumors, Intact Mice, and Mice Treated with Hydroxyurea

Time of day (24-h clock)	Control group		Experimental group	
	MI	ILN	MI	ILN
4	6,8	116,2	1,8	6,9
6	11,4	172,1	2,5	14,8
8	8,6	58,6	0,8	31,0
10	8,6	93,9	0,7	100,5
12	6,5	40,0	1,8	120,6
14	5,3	26,0	12,2	51,7
16	3,9	27,7	6,6	160,8
18	2,9	5,4	6,6	167,0
20	1,2	30,0	6,4	244,4
22	2,4	83,2	11,2	246,2
24	1,1	71,4	12,9	134,3
2	1,8	35,5	14,6	175,1
Mean diurnal values	5,0	63,3	6,5	121,1

completing it. In that case the maximum of the labeled nuclei would be recorded when thymidine was injected at the end of the period of DNA synthesis by the synchronized cells. Under those conditions, in the case of ideal synchronization, 1 h after administration of thymidine (when the animals were usually sacrificed) the group of labeled synchronized cells which had completed DNA synthesis would have advanced along the mitotic cycle by 1 h. Since these cells are responsible for the peak in the number of mitoses, if the duration of the G_2 phase was 1 h, the peaks of ILN and MI would coincide. In fact, considering the absence of ideal synchronization and the time intervals between sacrifices, measured in hours, it was not true maxima that were recorded simultaneously but values close to them. As regards the second peaks in the values of ILN (8-10 p.m.) and MI (2 a.m.) in the animals of the experimental group, these were not connected with cells piled up at the end of the G_1 period through the action of hydroxyurea. This conclusion is based on the fact that the increase in the ILN values began after noon-2 p.m., whereas the plasma hydroxyurea concentration falls to zero by 7-8 a.m. [6].

During artificial synchronization, the values of ISΔn reflecting the increase in the number of cells in the S phase in the period of the first peak of ILN (from 8 a.m. to noon) relative to the number of cells passing through this phase during the 24-h period in the control animals was 11.8%. The rate of change of synchronization from 8 a.m. to noon was 22.4 °/₀₀ per h. For the first peak of mitosis (noon-2 p.m.) ISΔn was 8.7%, and the rate of change of synchronization in this period was 5.2 °/₀₀ per h. At the second peak of ILN (2-8 p.m.), ISΔn was 38% and the rate of change of synchronization was 32.2 °/₀₀ per h, which did not differ significantly from the rate at the first maximum of ILN. Because of the plateau of MI observed from 4 to 8 p.m., the indices of synchronization at the second peak of MI values were not calculated.

In the animals of the control group at the peak of ILN from 2 to 6 a.m., ISΔn was 18% and the rate of change of synchronization was 34.1 °/₀₀ per h, significantly higher than the rate in the first peak of ILN in the animals of the experimental group ($P = 0.002$). In the period of increase in the values of MI from 2 to 6 a.m., ISΔn was 16% and the rate of change of synchronization was 2.4 °/₀₀ per h, not significantly different from the rate in the first peak of MI in the animals of the experimental group.

The higher values of ISΔn during natural synchronization compared with its value for the first peaks of ILN and MI during artificial synchronization was evidently connected with the toxic action of hydroxyurea on the cells naturally synchronized in the S phase. The same could also be said of the ratio between the rates of change of ILN in the animals of the control group and in the first peak in the animals of the experimental group.

In the animals of both groups mean diurnal values of MI were virtually identical, and the mean diurnal values of ILN differed almost by a factor of 2 (Table 1). Considering that the duration of the S phase was the same in the two groups, as also was the duration of mitosis, considering the higher value of the mean diurnal ILN in the animals of the experimental group it can be concluded that, during the period of the investigation, not all the cells embarked upon mitosis in the animals exposed to the action of the synchronizing agent.

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