Effect of Irradiation on DNA Synthetic Period of the Mitotic Cycle in Cells

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Kinetics of DNA synthesis in mitotic cycle of mouse corneal epithelial cells after single γ-irradiation (4 Gy) at the end of S period was studied by the method of radioautography. Normally, S period of corneal epithelial cells consists of several stages separated by intervals without DNA synthesis. The estimated mean duration of the first (S_1) and second (S_2) phases of S period was 16 and 10 h, respectively, and the interval between them was 7 h. Single irradiation at the end of S period changed the duration of mitotic cycle periods: S_2 phase became 2.2-longer than S_1 phase and the duration of g_1 period decreased, because the time for reparation in irradiated cell increases at the expense of g_1 period. Shortening of g_1 period is a factor promoting the appearance of transformed cells.

Key Words: *cell; DNA synthesis; mitotic cycle; irradiation; reparation*

Increasing incidence of industrial disasters necessitates elucidation of the mechanisms of action of various factors, *e.g.* ionizing radiation, on cell population of the body. Of particular interest is the effect of ionizing radiation on DNA-synthetic period as the most vulnerable phase of the cell cycle.

Here we studied parameters of mitotic cycle in mouse corneal epithelial cells after irradiation at the end of DNA synthetic period and evaluated radioresistance of S-phase cells.

MATERIALS AND METHODS

Autoradiography experiments were carried out on male C57Bl/6 mice weighing 18-20 g; 3-4 animals per experimental point. Taking into account some peculiarities of the studied tissue (all metabolites are delivered to the cornea with fluids washing its surface) and the effect of reutilization of ^{3}H-thymidine, for maximum reduction of the concentration of "secondary" ^{3}H-thymidine the isotope was applied in drops (7 μCi per eye) during morning hours. The first experimental series (control) was carried out for evaluation of temporal regularities of DNA synthesis and evaluation of the duration of mitotic cycle periods. In experimental series 2 we studied the effect of single γ-irradiation in a dose of 4 Gy on mouse corneal epithelial cells at the end of S period. Irradiation was performed in 100 min after application of ^{3}H-thymidine. For preparing photomicrographs of ^{3}H-thymidine-labelled cells, the cornea was embedded into paraffin and 4-μ sections were prepared. After standard histological processing, the samples were coated with nuclear emulsion and exposed for 8 days. It is known that DNA replicated at the beginning of the synthetic period is enriched with G-C base pairs, while DNA replicated at the end of S period is enriched with A-T base pairs; therefore, when evaluating the kinetics of DNA synthesis we often see weakly labeled cells among intensively labeled ones on photomicrographs. These cells are usually not counted, which can distort the information on the duration of S period. This necessitates the development of simple formulae allowing calculation of both the background and weakly labeled cells. Labeled cells were counted visually as cells with 1, 2, 3, 4, and more silver grains of a certain diameter above the nucleus.

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Assuming that silver grains can be located not above the cell nucleus, but at a certain distance from it, we ascribed each silver grain in the analyzed field of view to the closest nucleus. The density of silver grains in sites located at a considerable distance from the analyzed objects was determined as the background density. Single silver grains as well as pairs and groups of 3 grains located at a distance not exceeding nucleus diameter were also counted. Mathematical analysis of weakly labeled cells with correction for the background was performed by balance formulae:

$$\tilde{N}_i=\tilde{N}_i+(M-N)\times n_i$$
or
$$N_i=\tilde{N}_i+(M-N)\times n_i;$$
$$N_1=\tilde{N}_1-(M-N)\times n_1 \textit{ etc.},$$

where $\tilde{N}_i$ is the number of labeled interphase nuclei with *i* grains (*i*=1, 2, 3), including cells labeled due to the presence of background grains; M is the total number of labeled and unlabeled cells counted in a certain number of fields of view; N_i is the number of true labeled cells with *i* silver grains (*i.e.* with correction for the background); $N=N_1+N_2+N_3+N_4$ (and more) is the total number of true labeled cells including cells with 4 and more silver grains per nucleus; $n_1=n_1+n_2+\ldots$ is the background density calculated for the area consisting of m fields of view and including the density of single (n_1), double (n_2) background grains, *etc.* For convenience, the area of a cell was taken as a unit and the background grain density was expressed as the number of grains per cell.

Labeled mitoses were counted using the following formulae:

$$Z_i=\frac{M\times z_i-X\times n_i}{M-n};$$

$$Z_1=\frac{M\times z_i-X\times n_i}{M-n},$$

where X is the total number (per M examined cells) of labeled and unlabeled mitoses, including background and mitoses containing not less than 4 silver grains per nucleus; Z_i is the total number of labeled mitoses including mitoses labeled due to background silver grains; and Z_1 is the number of true labeled mitoses with 1 silver grain.

Radiosensitivity of cells in different periods of the cell cycle in the irradiated population was evaluated by the index of labeled cells (ILC), taking into account the percent of cells with different silver grain density per true labeled cell of the total number of analyzed cells. Parameters of the mitotic cycle were determined graphically by the percent of true labeled mitoses (Z) of the total number of mitoses (X).

RESULTS

Parameters of mitotic cycle of mouse corneal epithelial cells were determined using the above formulae (series I). We found that the curve describing the number of labeled mitoses has several rises and driops (Fig. 1). For instance, in the interval from 3 to 36 ч from the time of ^{3}H-thymidine addition (Fig. 1, *a*) the maximum percent of labeled mitoses (PLM) was observed at 4 (58.5%), 6 (54.0%), 12 (87.6%), 22 (63.0%), 24 (61.0%), 28 (80.0%), and 36 h (57.6%). Drops were observed at 7 (9.6%), 16 (23.3%), 20 (20.3%), 26 (53.0%), 32 (22.3%), 38 (13.5%), 45 (12.4%), 56 (10.0%), 70 (9.2%), 86 (17.0%), 91 h (15.3%). Starting from 94 h (20.2%), PLM steadily increased and peaked at 102 h (40.4%), thereafter the percent of labeled mitoses decreased at 106 to 19.6%. This wavy pattern of the curve is probably related to the phenomenon of DNA synthesis discontinuity in eukaryotic cells [3,4,6,7]. The fact that PLM was minimum from 38 to 94 h (on average, it did not exceed 10%) attests to the beginning of a new wave of PLM related to S period of the second mitotic cycle. This is why the mean time of generation (T) is 90 h (94-4=90). If the mean duration of g_2 period is 3 h and the mean duration of the entire S period, including pauses in DNA synthesis, is 33 h (37-4=33), the duration of g_1 period can be determined by the formula:

$$t_{g1}=T-(t_s+t_{g2}+t_m)_3,$$

where t_m is the duration of mitosis that in mouse corneal epithelial cells can vary from 1.1 to 4.1 h over the day [1,4]. Under these conditions, g_1 period varies on average from 55 to 58 h. Experimental data suggest that normally, S period of corneal epithelial cells includes several stages separated by intervals without DNA synthesis. The mean duration of the first phase of S period (S_1) between PLM minima at 20 and 38 h is 16 h. The second phase of S period (S_2) between 20 and 38 h is 10 h and the pause between S_1 and S_2 phases is 7 h. Both S_1 and S_2 phases had minima and maxima on PLM curve. For instance, S_1 phase has two clear-cut waves of labeled mitoses. If we measure the duration of the first S_1 subphase (S_1^1) by the period between minima of labeled mitoses at 20-32 h, the maximum duration of (S_1^1) subphase is 12 h. Hence, the duration of the second S_1 subphase (S_1^2) is 5 h (time interval between 32 and 38 h). Similar calculations for S_2 phase yields maximum duration of S_2^1 and S_2^2 subphases: S_2^1=9 h (16-7=9) and S_2^2=4 h (7-3=4). Thus we can conclude that phases, similarly to subphases of S period, differ by the time of DNA synthesis and non-synthesis periods. Exposure to physical factors on the organism disturbs evolutionary determined regimen of

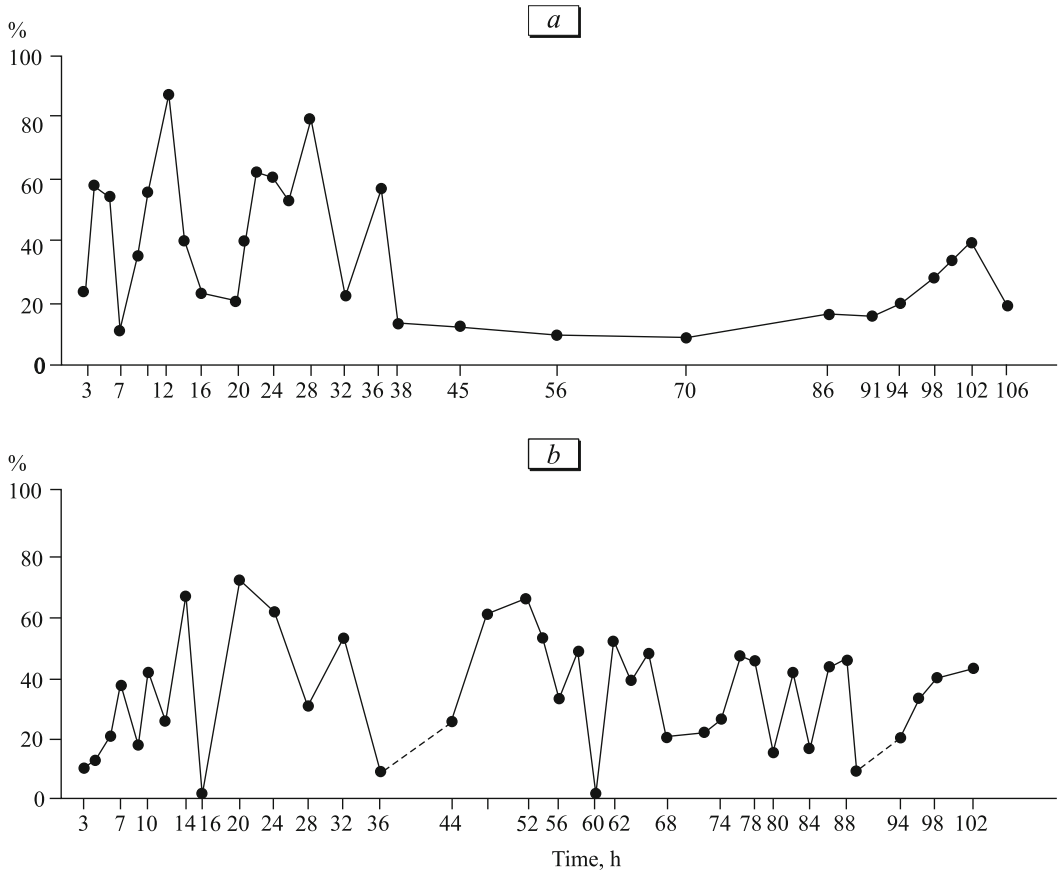

Fig. 1. Changes in percentage of labeled mitoses in mouse corneal epithelium after single Irradiation in a dose of 4 Gy. *a*) control; *b*) experiment. Abscissa: time after ^{3}H-thymidine administration; ordinate: percent of true labeled mitoses.

functioning of various processes in both the cell and the whole organism. In light of this let us consider the effects of single irradiation of S-phase epithelial cells in a dose of 4 Gy (Fig. 1, *b*). First, blocks at the boundaries of g_1-S and S-g_2 periods appear, which led to shrinkage of S period and disappearance of pauses (7 and 32 h) between subphases of S_1 and S_2 phases. In parallel, S_1^1 subphase was shifted by 4 h towards S_2 phase.

This resulted in a shift of the peak of labeled mitoses in S_1^1 subphase to 20 h and the peak of S_1^2 subphase to 32 h. Changes in S_2 phase after irradiation were more pronounced than in S_1 phase: in the interval from 3 to 16 h, 3 waves of labeled mitoses with clear-cut PLM drops at 9 h (18%) and 12 h (26%) appeared. Hence, irradiation affected, though to a different degree, both phases of DNA synthesis. For instance, ILC in time points corresponding to S_2 phase was on average 5.1%, while in time points corresponding to S_1 phase ILC attained 25.5%. This 5-fold decrease in ILC in S_2 phase in comparison with that in S_1 phase is directly related to disappearance of weakly labeled cells during S_2 phase due to reparation occurring in the irradiated population after termination of radiation exposure.

The effect of reparation in S-phase cells is related to the following processes. During reparation, two parallel DNA synthesis processes occur in the damaged cells: 1) *de novo* synthesis at the site of injury (reparative synthesis) and 2) supplementary synthesis during the period of reparation in the remaining undamaged genome competent for replication. Let us suppose that by the moment of irradiation S-phase cell contains four fragments (1, 2, 3, 4) of the same matrix DNA strand and daughter fragment are already synthesized on the first three of them 1^1, 2^1, 3^1 (Fig. 2). The first daughter fragment (1^1) was more resistant to irradiation, because it was bound with proteins and the more radiosensitive elements of this fragment were protected from radiation. This fragment was practically ready to transition to g_2 period. In the fourth fragment, DNA synthesis was not started yet, but it was competent for replication. After single irradiation (Fig. 2, stage I), daughter fragments 2^1 and 3^1 are most damaged. However, before starting reparative processes the cell should complete the synthesis of the daughter fragment (4^1) on the matrix fragment 4 (stage II), because the cell cannot start the next process without completing the previous one. After completion of replication, processes aimed at reparation of

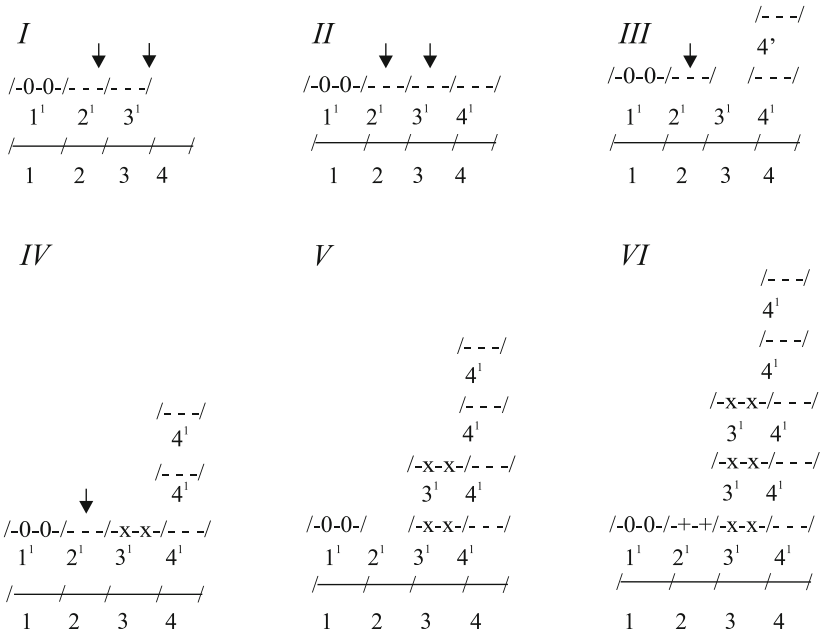

Fig. 2. Scheme of reparation processes in the cell after irradiation. I-VI stages of reparative processes; 1, 2, 3, 4: fragments of matrix DNA in S period, ($1+1^1$): DNA fragment consisting of matrix fragment (1) with replicated daughter fragment (1^1) before irradiation; 2^1, 3^1: radiation-damaged daughter fragments (↓); 4: fragment of matrix DNA competent to the synthesis of daughter fragment (4^1).

daughter fragments 2^1 and 3^1 are started. We believe that in the proposed scheme of reparation the radiation damages only the daughter fragments without affecting the matrix structures. Thus, damaged daughter fragment 3^1 is most likely eliminated at stage III and a gap appears at the site of damage (opposite to matrix fragment 3). Simultaneously, DNA synthesis is going on in another daughter fragment (4^1) as on the matrix. The duration of this "supplementary" DNA synthesis is determined by the time spent for cutting out the damaged daughter fragment 3^1. Stage IV consists of filling the formed gap with nucleotides identical to that in matrix fragment 3. This reparative synthesis proper results in the formation of a new (undamaged) daughter fragment 3^1. Simultaneously, supplementary DNA synthesis is going on in the other daughter fragment (4^1) as on the matrix, this leads to multiplication of copies consisting of nucleotides identical to matrix fragment 4. Stage V consists in cutting out the damaged daughter fragment 2^1 paralleled by supplementary DNA synthesis on daughter fragments 3^1 and 4^1, which leads to multiplication of copies identical to matrix fragments 3 and 4. During stage VI reparation is eventuated in the formation of undamaged daughter fragment 2^1, a result of reparative synthesis, and formation of new copies of DNA corresponding to matrix fragments 3 and 4. After completion of reparation processes, the content of genetic material in the cell will surpass the normal and hence, period of DNA synthesis will be longer that normal S period of non-irradiated cells. One more remark: in a multicellular organism, each cell belongs to a certain group of cells with peculiar evolutionary determined timing of metabolic processes. In other words, activity of reparation systems is an element of the integral chain of metabolic processes and hence, is accomplished during a certain time interval. Under these circumstances, the cell exposed to damaging factors after reparation can considerably lag behind other cells of its group in its passage through the metabolic cycle. To eliminate this lagging, another cell according to non-equivalency principle [5] should inhibit though its own regulatory system all or some functionally active genes, except the genes essential for normal passing of $S\text{-}G_2$ periods and mitosis, the phases of the mitotic cycle responsible for reproduction, the main goal of the cell. In this case, the cell (that performs now only two functions – reduplication and division) will belong to a new type of cells, transformed cells. It can be concluded that reparation after irradiation in the cell is performed only at the expense of g_1 period. However, g_1 period is responsible for the growth and development in the organism; therefore; shortening of the time required for regulation of cell differentiation can be a factor promoting induction of pathological processes in the organism (due to changed duration of certain periods of mitotic cycle in cells). These assumptions were experimentally proven. Thus, repeated rises and drops of PLM curve were observed in the interval of 44-89 h (normally corresponding to g_1 period, Fig. 1, *b*). This is believed to be a new S period in cells (exposed to radiation during DNA synthesis period) passing g_2 period after completion of reparation and entering mitosis. In this case, the point 44 h is analogous to the point 3 h on PLM curve, while the interval 44-89 h corresponds to the second S period in which the maximum duration of S_2 phase is 24 h (68-44=24) and S_1 phase 17 h (89-72=17).

TABLE 1. Changes in ILC in Mouse Corneal Epithelial Cells in Different Points of S Periods after Single Irradiation

Interval*, h	Control						Experiment					
	AC	TLC				IMC, %	AC	TLC				IMC, %
		1	2	3	≥4			1	2	3	≥4	
3	1786	168	96	30	8	16.9	3018	107	-	-	57	5.4
4	1673	200	66	25	27	19.0	3012	-	-	-	56	1.9
6	2068	287	103	5	20	20.1	3050	-	-	-	42	1.4
7	928	9	5	5	1	2.2	2997	36	-	-	33	2.3
9	1052	42	39	22	44	14.0	2800	216	-	-	30	8.8
10	1802	45	10	12	20	4.8	2935	209	68	13	33	11.0
20	1626	183	53	13	47	12.1	899	33	72	68	98	30.1
24	3157	234	35	13	27	10.0	1138	12	33	16	47	9.5
28	1423	287	108	27	46	32.8	1119	99	56	56	89	29.4
32	1502	167	55	5	13	16.0	1000	75	36	40	117	28.7
36	480	40	42	34	55	35.6	1020	86	97	84	38	29.9

Note. *Time interval between isotope administration and sacrifice of experimental animals. AC: analyzed cells; TLC: true labeled cells with silver grains.

The maximum duration of the second S period (total, 45 h) surpasses that of the first S period (33 h) due to lengthening of S_2 phase. This can be explained by the fact that reparative processes in S_2 are paralleled by supplementary DNA synthesis. This process involved not only S_2 phase, because the duration of S_1 phase did not increase, but even decrease in comparison with that in the first S period in irradiated cells. Thus, the maximum duration of S_1 phase of the first S period was 20 h (36-16=20) and S_1 phase of the second S period 17 h (89-72=17). The duration of S_1 phase of the second S period became close to that of S_1 phase of the control (18 h). The pause in DNA synthesis between S_1-S_2 phases of the second S period increases to 7 h (74-67=7) most likely at the expense of shortening of S_1 phase of the second S period.

Thus, single irradiation of the cell population at the end of S period affects primarily S_2 phase of DNA synthesis without considerably changing S_1 phase. A general feature of reparative processes in cells irradiated during their passage through the mitotic cycle is reparation of damages during time interval corresponding to g_1 period. This leads to changes in the duration of g_1 period of the mitotic cycle.

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