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MITOTIC BLOCKAGE FOLLOWING LASER MICRO-IRRADIATION OF PROPHASE CHROMOSOMES*

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Summary

An argon laser microbeam was used to irradiate prophase chromosomes of the salamander (Taricha) and the established cell of the rat kangaroo (PTK1, Potorous). In both cell types mitosis was significantly blocked when irradiation occurred in early prophase regardless of whether or not the irradiated chromosomes were nucleolar-associated. It was also determined that with identical irradiation conditions, the salamander cells were more susceptible to mitotic inhibition than the kangaroo cells. The results are compared with earlier studies performed with lower energy densities.

The use of microbeam irradiation to study the cell cycle, and in particular the mitotic process has been employed by several investigators (1, 2). Ultraviolet microbeam irradiation of nucleoli of mitotic cells resulted in a cessation of mitosis when the irradiation occurred before middle prophase. Irradiation after middle prophase did not affect mitosis. More recently (3) an argon laser microbeam of low intensity (about 10 μJ in the focal spot, 1 to 18 irradiations per cell) has been used to irradiate various regions of the mitotic nucleus of salamander lung cells vitally stained with acridine orange. It was found that irradiation of the chromosome regions immediately adjacent ("juxtanucleolar") to the nucleolus caused a cessation of mitosis when either both nucleoli had all their juxtanucleolar chromosome regions irradiated, or when the juxtanucleolar regions of the larger of the two nucleoli were

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irradiated. Irradiation of the juxtanucleolar regions of the smaller nucleolus did not inhibit mitosis. Similarly irradiation of random non-nucleolar chromosomes, and nucleoli directly, did not inhibit mitosis. A similar series of studies using a laser energy about twice as great gave results inconsistent with the lower energy irradiation. Irradiation of juxtanucleolar regions of the smaller nucleolus as well as direct irradiation of the nucleolus resulted in cessation of mitosis. In all experiments "cells in the middle or late stage of prophase were generally selected to be irradiated," (3). Since the ultraviolet microbeam experiments suggest that the timing of the irradiation within the prophase is critical, and the laser studies indicate that the energy is important, a series of laser microbeam studies was undertaken to determine more precisely the roles of both these factors in blockage of the mitotic process.

Materials and Methods

Experiments were performed on primary cultures of salamander (Taricha granulosa) lung cells and an established epithelial-like cell line (PTK1) of the rat kangaroo Potorous tridactylis (4). The salamander cultures were established by the standard procedures described by Seto and Rounds (5) using Eagle's medium fortified with antibiotics and 10% fetal calf serum (6). One to two weeks following establishment of the culture, mitotic cells in the tissue outgrowth were used for experimentation. Prior to irradiation the cells were sensitized to the laser light by five minutes treatment with acridine orange, 0.1 μg/ml of culture medium. The culture was then washed twice with fresh culture medium and a final change of medium was placed in the chamber. This is the same procedure reported by us earlier (6, 7), and similar to that used by Ohnuki et al. (3). Cells were maintained at room temperature,

18-22°C. The rat kangaroo cells were seeded into Rose chambers following trypsinization from a larger culture flask and resuspension in a modified Eagle's medium. Culture medium was Eagle's MEM with sodium pyruvate (0.11 gm/l), $NaHCO_3$ (0.85 gm/l), Earle's balanced salt solution, 10% fetal calf serum, and penicillin and streptomycin. pH was maintained at 7.2 with phenol red used as an indicator. Cells were incubated at 37°C for 1 to 5 days prior to use, and an air curtain incubator maintained the cells at 37°C during and after the irradiation. Acridine orange sensitization was used according to the procedure described for the salamander cells. Irradiation was conducted with a high power argon laser microbeam (7). Laser configuration was single mode, multiwavelength (514.5 nm, 50%, and 488.0 nm, 20%). Focal spot diameter was 0.5 - 1 micrometer. Energy density in the focal spot was 100 microjoules. Each irradiated cell received a total of between 10 and 16 irradiations (cumulative energy, 1000-1600 microjoules). This compares to a total cumulative imposed energy of 60 μJ in a 0.5 - 2 micrometer spot described by Ohnuki et al. (3) for their low level energy irradiation, and 120 μJ of cumulative energy for their higher energy system. Laser energy output was continually monitored with a calibrated vacuum photodiode.

All cells were irradiated during prophase. The prophase of the salamander cells was divided into three phases: early, middle, and late. Early prophase was characterized by an intact nuclear membrane, large phase-dark nucleoli, and slight chromosome condensation. This was the earliest stage at which chromosome association with the nucleolus could be discerned. Middle prophase was also characterized by an intact nuclear membrane and phase dark nucleoli, but the chromosome condensation was considerably more pronounced, and the nucleolar association with the chromosomes was

very clear. Late prophase was characterized by a breakdown of the nuclear membrane, and a gradual dissolution of the nucleoli. Chromosome condensation was pronounced. Salamander cells were irradiated in all three prophase stages. Specific experiments within each stage involved irradiation of all the juxtanucleolar chromosome regions of (a) all nucleoli, (b) only one nucleolus, and irradiation of random non-juxtanucleolar chromosomes. In addition, acridine orange treated cells were exposed to the regular microscope illumination, and the percentage of cells continuing through mitosis was determined. (In Tables I and II these cells are designated "No irradiation control".)

For irradiation of the rat kangaroo cells, prophase was divided into early and late prophase. Early prophase was characterized by two phase dark nucleoli, an intact nuclear membrane, and slight chromosome condensation. It was possible to distinguish the juxtanucleolar chromosome regions. Late prophase was characterized by nuclear membrane breakdown, marked chromosome condensation, and gradual dissolution of the nucleoli. Irradiation experiments similar to those described for the salamander cells were performed. In all irradiation experiments (salamander and rat kangaroo) cells were observed post-irradiation until it became evident whether or not the cell was continuing through mitosis.

Results

The data on the salamander cells is summarized in Table I. It is evident that irradiation during early or middle prophase results in a high frequency of mitotic blockage. No cells that had either one or all of the juxtanucleolar chromosomes irradiated in early prophase continued through division. Only 21% of the cells that had the juxtanucleolar regions of one nucleolus irradiated in middle prophase, continued through mitosis. In addition, early

TABLE I

PROPHASE IRRADIATION: SALAMANDER

Prophase Stage	Type of Irradiation	Mitosis Blocked	Mitosis Continued	% Continued
Early	All juxtanucleolar regions	8	0	(0)
Early	Juxtanucleolar of 1 nucleolus	6	0	(0)
Early	Random chromosomes (non-juxtanucleolar)	9	2	(18)
Early	No irradiation-control	3	6	(67)
Middle	Juxtanucleolar of 1 nucleolus	15	4	(21)
Late	All juxtanucleolar regions	1	8	(89)
Late	Juxtanucleolar of 1 nucleolus	4	14	(78)
Late	Random non-juxtanucleolar chromosomes	1	4	(80)
Late	No irradiation-control	1	4	(80)

prophase cells with irradiated random non-juxtanucleolar chromosomes appeared to undergo mitotic blockage (18% continued through division). The only group with a high percentage of cells continuing through division was the non-irradiated control. A very high percentage of cells irradiated in late prophase completed mitosis (78-89%).

Data on the rat kangaroo cells are summarized in Table II. As with the salamander, irradiation in early prophase resulted in a higher percent of mitotic blockage when compared to late prophase irradiation. However, the percent of cells irradiated in early prophase that continued through division was significantly higher for the rat kangaroo cells. One cell that had all of its juxtanucleolar chromosomes irradiated continued through division

TABLE II

PROPHASE IRRADIATION: RAT KANGAROO

Prophase Stage	Type of Irradiation	Mitosis Blocked	Mitosis Continued	% Continued
Early	All juxtanucleolar regions	7	1	(12)
Early	Juxtanucleolar of 1 nucleolus	12	10	(45)
Early	Random non-juxtanucleolar	7	13	(65)
Early	No irradiation control	5	25	(84)
Late	Juxtanucleolar 1 nucleolus	1	16	(94)
Late	All juxtanucleolar regions	2	7	(77)
Late	Random non-juxtanucleolar	2	8	(80)
Late	No irradiation control	0	5	(100)

and 10/22 (45%) of the cells that had the juxtanucleolar sites of one nucleolus irradiated, continued through division. None of the salamander cells in either of these groups progressed through mitosis. Irradiation of random chromosomes resulted in 65% successful mitosis as compared to 18% in the salamander cells. The successful mitosis of non-irradiation controls was 84%. Irradiation of juxtanucleolar regions of late prophase cells resulted in a high percent of successful mitosis (77-94%).

Discussion and Conclusions

The data clearly indicate that the time of irradiation during prophase is a critical factor in determining whether or not the cell continues through division. In both the salamander and the rat kangaroo cells, mitotic blockage was significant when the irradiation occurred in early prophase (before nuclear membrane break-

down begins, and initiation of the dissolution of nucleoli). In the case of the salamander cells, irradiation in middle prophase resulted in a slightly higher successful mitosis rate than irradiation in early prophase (0% for early prophase, and 21% for middle prophase).

The data also suggest that the site of irradiation may not be as critical a factor as initially thought. In both the salamander and rat kangaroo cells irradiation of random chromosomes in early prophase resulted in a significant inhibition of mitosis (18% successful mitosis for the salamander and 65% for the rat kangaroo). This contrasts to 100% successful mitosis with similar irradiation using less energy in the earlier studies. Ohnuki et al. (3) irradiated random chromosomes with a cumulative imposed energy in a 0.5 - 1 micrometer spot of 60 - 120 μJ (1/10 the energy we are using). Mitotic blockage by irradiation of random chromosomes may be a result of using an imposed energy 10 times greater than was used previously (3). However, the fact that 21% of the salamander cells irradiated around one nucleolus in middle prophase, and 45% of the rat kangaroo cells similarly irradiated in early prophase, did continue through mitosis, would suggest that the amount of energy used is not always critical. In some of these cells a total of 1600 microjoules of laser energy did not inhibit the mitotic process. In previous studies (3) it had been suggested that the juxtanucleolar region was especially sensitive to laser microirradiation, resulting in an inhibition of the mitotic process.

Perhaps one of the most interesting findings is the differential sensitivity of the two cell types used. The percent of cells continuing through mitosis was higher in all early prophase categories for the rat kangaroo cells. This differential sensiti-

vity of the salamander and rat kangaroo cells is most evident in the cells subjected to chromosome irradiation around one early prophase nucleolus (45% continued for rat kangaroo, 0% for salamander), and early prophase random chromosome irradiation (65% continued for rat kangaroo, and 18% for salamander).

In summary, the data suggest that there is differential sensitivity to microirradiation (1) during the stages of prophase, and (2) between cell types. The first observation agrees with the earlier findings of Gaulden and Perry (1) who used an ultraviolet microbeam to irradiate nucleoli directly. However, in our investigations mitosis was inhibited by juxtanucleolar chromosome and random chromosome irradiation. This is in contrast to the selective inhibition of mitosis by nucleolar irradiation, described for the ultraviolet microbeam. Gaulden and Perry (1) suggest that the synthetic capabilities of the nucleolus through middle prophase are prerequisites for successful mitosis. In the case of laser microirradiation it is possible that juxtanucleolar chromosome irradiation actually affects the nucleolus directly (3), thus precipitating a response similar to the ultraviolet studies. However, Ohnuki et al. (3) have already demonstrated that low intensity laser microirradiation of the nucleolus does not affect mitosis, whereas similar irradiation of juxtanucleolar chromosomes does inhibit mitosis. Our finding of mitotic inhibition by random chromosome irradiation might suggest a more general, non-site specific radiosensitivity of the mitotic cell. It is also entirely possible that the nature of the differences between ultraviolet and intense visible laser irradiation, precipitate very different responses in the cell.

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