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MORPHOLOGICAL TRANSFORMATION OF SYRIAN HAMSTER EMBRYO CELLS BY LOW DOSES OF FISSION NEUTRONS DELIVERED AT DIFFERENT DOSE RATES

by

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Abstract

Both induction of cell transformation and killing were examined with Syrian hamster embryo (SHE) fibroblasts exposed to low doses of JANUS fission-spectrum neutrons delivered at high (10.3 cGy/min) and low (0.43 and 0.086 cGy/min) dose rates. Second-passage cells were irradiated in mass cultures, then cloned over feeder cells. Morphologically transformed colonies were identified 8-10 days later. Cell killing was independent of dose rate, but the yield of transformation was greater after low-dose-rate than high-dose-rate irradiations. Decreasing the neutron dose-rate from 10.3 to 0.086 cGy/min resulted in a two- to threefold increase in the yield of transformation for neutron exposures below 50 cGy, an enhancement which was consistently observed in repetitive experiments in different radiosensitive SHE cell preparations.

1. Introduction

During peacetime, persons at greatest risk from ionizing radiations are those exposed to radiation sources in an occupational setting, where the exposure is typically low level and long term, and the primary biological hazard is cancer induction (Sinclair and Fry, 1987). For example, approximately 100,000 workers in the United States are currently exposed to low-level neutron irradiation (Brackenbush et al. 1980), but for this particular type of radiation, little information is available from epidemiological studies to help determine the risk of cancer induction (Fry, 1986). It is necessary, therefore, to use relevant, reliable cellular models for risk evaluation. Cell transformation assays have proved useful both for evaluating the carcinogenic potency of different qualities of ionizing radiation and for studying the cellular processes involved (Little, 1979; Han et al. 1980; Borek, 1985). Temporal distribution of the radiation dose is an important determinant in the dose-response relationships for cell survival and transformation. In general, cell survival is increased and cell transformation is decreased when a specified dose of low- linear-energy-transfer (LET) radiation is delivered in a protracted or multifractionated mode instead of in a short exposure (Han and Elkind, 1979; Han et al. 1980; Terashima, et al. 1985; Balcer-Kubiczek et al. 1987). However, an increased yield of transformation was reported when the radiation doses were separated by a 5-h interval (Borek and Hall, 1974; Miller et al. 1979).

In contrast, for fission-spectrum neutrons, the yield of damage was not lessened when a particular dose was given at a lowered dose rate or in small repeated fractions. The degree of cell killing by neutrons remained the same after high- or low-dose-rate irradiations, indicating a lack of cellular repair capacity for this type of damage (Ngo et al. 1977; Han and Elkind, 1979; Hill et al. 1982; Han et al. 1984). The yield of cell transformation, however, increased when C3H 10T1/2 cells were exposed to neutrons at a lowered dose rate or in multiple fractions, compared to that observed after a single, high-dose-rate exposure (Hill et al. 1982, 1984, 1985; Miller et al. 1988). In studies by Hill et al. (1984), reduction of the dose rate from 10.3 cGy/min to 0.086 cGy/min for total neutron exposures below 100 cGy resulted in transformation enhancements up to 8-fold. Although cell transformation in vitro cannot be equated to uncontrolled, metastatic growth in vivo, these observations have important implications for radiation standards of protection. Further, in several, but not in all, studies the tumorigenic effectiveness of protracted exposures to neutrons was found to be increased. (Upton et al. 1970; Ullrich, 1984; Carnes et al. 1989).

We have examined with the Syrian hamster embryo (SHE) cell transformation assay, the action of low doses of fission-spectrum neutrons and ^{60}Co gamma rays delivered at different dose rates. Unlike the aneuploid C3H 10T1/2 cell line, SHE cells are normal, diploid fibroblasts with a limited lifespan in culture. Transformation of these cells by ionizing radiations can be sensitively and quantitatively determined by using a colony-formation assay wherein the fraction of cells undergoing

transformation can be identified directly by their clonal morphologies among those cells which survive and form colonies, (Berwald & Sachs, 1965; Borek and Sachs, 1967; Borek and Hall, 1973; Borek et al. 1978). We found that whereas protraction of a given exposure of ^{60}Co gamma rays resulted in a reduced yield of transformation, protraction of a low (<50 cGy) dose of neutrons lead to an enhanced yield of transformation.

2. Materials and methods

2.1. Irradiation procedures

Fission-spectrum neutrons were generated by the JANUS Reactor at Argonne National Laboratory. The mean energy of the neutron spectrum was 0.85 MeV with a gamma contamination of less than 4 percent (Williamson and Frigerio, 1972). High-dose-rate irradiations were conducted at 10.3 cGy per min and low-dose-rate irradiations at 0.43 or 0.086 cGy per min. During all radiation exposures, the cells were maintained at 37°C in a styrofoam incubator placed inside the radiation chamber, (Hill et al., 1982, 1984). High-dose-rate exposures were always conducted immediately after low-dose-rate exposures. Gamma-rays from ^{60}Co sources at Argonne National Laboratory were administered to cells under the same conditions as for neutron irradiations. High and low dose rates of gamma-rays at 44 cGy per min and 1 cGy per min were used so that the exposure periods at the high dose rates and again at the low dose rates were approximately equivalent for both radiation qualities. Control

cultures were transported to the radiation facilities but maintained at 37°C in an incubator shielded from the radiation sources.

2.2. Preparation of SHE cells

Syrian golden hamsters (second pregnancy, 13 days gestation) from a viral-free (LVG) colony (Charles River, Wilmington, MA) were used as the cell source for these studies. Syrian hamster embryo (SHE) cells were prepared according to established procedures (Pienta et al. 1977; Tu et al. 1986; Jones et al. 1988). In brief, the minced embryo tissue was digested at room temperature in 0.1% trypsin. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum and 2 mM glutamine. After 3-4 days, the cells were disaggregated and either replated as required for a transformation assay or cryopreserved in liquid nitrogen (Tu et al. 1986) as a cell stock for repetitive studies.

2.3. Cell transformation and lethality assays

Throughout these studies, we used secondary or tertiary cultures of cells that had either been subcultured from primary cell preparations or recovered from cryopreserved stocks. In the latter case, cells that were cryopreserved after the first passage were quickly thawed and cultured to the required density for irradiation. Most experiments were conducted with cells from a cryopreserved pool designated SHE018; this pool is a

relatively homogeneous fibroblast population which exhibits no loss of growth efficiency, or sensitivity to radiation-induced cell transformation, after storage in liquid nitrogen.

For each experiment, SHE cells were seeded from the same cell preparation for both the high and low dose-rate irradiations to minimize effects of cell growth and passage number. T_{25}. Exponentially growing cultures (2 x 10^6 per T_{25} tissue culture flask) were irradiated with fission-spectrum neutrons in flasks filled with culture medium to minimize pH and temperature variations. The low dose-rate exposures were always conducted first and followed immediately by the high dose-rate exposures. All irradiations were conducted at 37°C. Nonirradiated (control) flasks were treated similarly except that they were not irradiated.

For the determination of the cytotoxicity of the radiation treatment, cells were dissociated with trypsin immediately after irradiation, resuspended in fresh culture medium, and counted. Cells were seeded into 100-mm dishes which, 24 h earlier had been inoculated with x-irradiated feeder SHE cells (50 Gy exposure, 10^5 cells/dish). The numbers of cells seeded ranged from 10^3 to 5 x 10^5, depending upon the expected survival; four dishes were seeded per dose point. After eight days, the dishes were rinsed with calcium-, magnesium-free phosphate buffered saline, fixed in methanol, and stained with 10% aqueous Giemsa. The numbers of surviving colonies were expressed relative to the number from control cells.

For the determination of the frequency of morphological transformation, fresh medium was added to the flasks after irradiation, and the cultures were incubated at 37°C. After 48 h, the cells were replated over feeder layers as already described. For each single experiment, the cells exposed to each dose of high-or low-dose-rate neutron irradiation were processed in parallel. Target-cell densities ranged from 1000 to 1500 cells per dish, 20 dishes per treatment. After seven days, cultures were washed, fixed, and stained as described. Each colony was examined microscopically, and the number of colonies exhibiting criss-crossed or piled-up growth was determined. The transformation frequency was expressed relative to the total number of surviving, scorable colonies per dose point.

3. Results

The survival curve for SHE cells exposed to fission-spectrum neutrons has a slight shoulder (D_q = 20 cGy), but thereafter cell killing is an exponential function of the dose (D_0 = 32 cGy), (figure 1). Killing by neutrons was not modified by decreasing the dose rate from 10.3 cGy/min, consistent with observations with C3H 10T1/2 cells (Hill et al. 1982). Different SHE-cell preparations, both fresh cells and those recovered from cryopreserved pools, exhibited similar sensitivities to killing by neutrons. At the 10% cell survival, the RBE was 3.8 relative to ^{60}Co gamma irradiation at a dose-rate of 44cGy/min.

Different cell pools displayed a range of sensitivities to the induction of morphological transformation by neutrons. A single, cryopreserved pool, designated SHE018, was found to be sensitive to ionizing radiation and was therefore used consistently for these studies. However, the data were confirmed with cells from three additional cryopreserved pools and from two freshly isolated cell pools.

After irradiation of the SHE018 cells at a high dose rate (10.3 cGy/min), the yield of transformation increased with increasing neutron dose up to approximately 70 cGy, (figure 3). Thereafter, the dose-response curve plateaued, and after exposures above 100 cGy, it declined. A neutron dose of 70 cGy reduced cell survival to approximately 15%. Under these conditions of relatively high toxicity, the developing colonies tended to be smaller with sparse morphologies compared to those formed at higher survivals; hence it was difficult to identify the transformed phenotypes. Most assays were conducted, therefore, by using radiation doses not exceeding 50 cGy, which did not decrease cell survival below 30%.

Reduction of the neutron dose rate from 10.3 to 0.43 cGy/min produced an increased transformation response, which was demonstrated particularly by an increased steepness of the initial slope of the dose-response curve, (figure 2). For total exposures above 30 cGy, the transformation response declined to the level observed after high-dose-rate exposures. When the dose rate was lowered to 0.086 cGy/min, it became possible to expose the cells accurately to doses of neutrons as

low as 0.1 cGy. ~~Significant induction of morphological transformation could be measured following neutron exposures in this range, (figure 3).~~

As was mentioned previously, individual cell pools exhibited different sensitivities to neutron-induced transformation; the six cell pools used in this study were selected specifically for their sensitivity to the effect of neutron dose-rate protraction as well as for their overall responsiveness to radiation-induced transformation. With several cell pools, however, the transformation response in single assays was not modulated by decreasing the neutron dose-rate. These assays have not been repeated as yet but the cells were cryopreserved for future studies. The pooled data for the responsive cells are shown in Figure 4 and Table 1. Parts of this data have been reported earlier in Jones et al. 1989. Although initial slopes are difficult to estimate with confidence for the dose rate 10.3 cGy/min we estimate the slope to lie between 0.63×10^{-4}/cGy and 1.09×10^{-4}, and for 0.086 cGy/min, to be 5.1×10^{-4}/cGy. Thus, the range of the ratio of initial slopes we estimate to be from four to eight.

The data for γ-rays are shown in figure 5. At 44.0 cGy/min, the initial results can be fitted by a straight line of slope approximately 3.68×10^{-5} /cGy, but beyond 150 cGy the curve plateaued. At 1.0 cGy/min, the slope of the line which fits the data is 0.80×10^{-5} suggesting a five-fold reduction in the initial slope at this deceased dose rate.

Discussion

Our results show that reducing the dose rate of fission-spectrum results in a higher yield of morphogical transformation than is achieved by high-dose-rate exposures. These results are in qualitative agreement with the findings of Hill et al. (1982, 1984) for the aneuploid, immortalized C3H 10T1/2 cell line and demonstrate, for the first time, that this neutron effect can occur in normal, diploid cells. For a dose of about 20 cGy, the degree of enhancement with SHE cells is approximately threefold, which is less than that observed by Hill et al. (1982, 1984) but is in close agreement with the enhancements reported by Miller et al. (1988) for monoenergetic neutrons. Quantitative differences in the magnitude of enhancements between different studies may be attributable to variations intrinsic to the cells and/or the cell culture conditions, in particular the lot of fetal calf serum which was used (Hsiao et al. 1987). This latter factor might be the reason for the total absence of a low-dose-rate enhancement in the experiments with C3H 10T1/2 cells reported recently by Balcer-Kubiczek et al. (1988). In addition, the recent study of Belli et al. (1989) suggests the possibility that the particular spectrum of fission-spectrum neutrons could be important. These authors reported important variations in the relative biological effectiveness of protons in the energy region in which significant proportions of proton secondaries are produced by a beam of fission-spectrum neutrons. Hence, the results of Balcer-Kubiczek

obtained using a TRIGA reactor, and those of Hill et al. (1982, 19

et al. (1988) (1982, 1984), who used the JANUS Reactor, may reflect the actual spectrum of neutrons in each case.

Miller et al. (1988) reported that the enhancement produced by fractionated, low doses of neutrons is dependent upon the neutron energy. Low-energy neutrons (<5.9 MeV), which are effective in causing various kinds of biological damage, were found to produce the low-dose-rate enhancement. Consistent with the results following exposures to JANUS fission neutrons (65 KeV/μ), low dose-rate exposures to argon (120 KeV/μ) and iron (200 KeV/μ) particles also produced an enhanced transformation response but neon ions and α-particles (147 KeV/μ) did not (Yang et al., 1986; Hieber et al., 1987). Therefore, there does not appear to be a straightforward relationship between radiation quality, as expressed by LET values, and the enhancement of the transformation response under low-dose-rate irradiation conditions.

An increased neutron effectiveness with exposure protraction, or fractionation, has also been observed for mutation induction in several cellular systems (Grahn et al., 1986: Kubota et al., 1989; Kronenberg and Little, 1989) and for life shortening, (Upton et al., 1970; Thomson et al., 1981, 1985). Recent analysis of data from mice irradiated with JANUS neutrons showed that dose protraction produced an increase in the yield of certain epithelial tumors (Carnes et al., 1989). In view of the significant implications for human health protection, it is important to elucidate the critical cellular events that are differentially modulated by acute or fractionated/protracted neutron exposures. Ullrich (1984) observed that the rate of neoplastic

development was increased under low-dose-rate irradiation conditions. Others have postulated that cell cycling or DNA repair/misrepair process could play an important role (Elkind and Hill, 1986 a&b; Burch and Chesters, 1986; Rossi and Kellerer, 1986). Because SHE cells are normal cells in which the cellular and molecular events that underlie each stage of the process of neoplastic transformation can be examined, the study of neutron action upon these cells may provide important insights into the critical events that are differentially modulated by low- and high-dose-rate exposure conditions.

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Table 1. Morphological Transformation of Syrian Hamster Embryo Cells by Fission Neutrons Delivered at Different Dose Rates - Compilation of Results from Five Cell Preparations.

Dose Rate (cGy/min)	Dose (cGy)	No. of Experiments	Relative Cloning Efficiency[a] ± SE	Colonies Counted	No. of Transformed Colonies	Transformation Frequency (%)[b]
	0	12	1.00	19,724	11	0.06[c]
	5	10	0.73 ± 0.06	15,294	26	0.17
	10	7	0.70 ± 0.17	17,324	50	0.29
	21	9	0.60 ± 0.06	19,007	38	0.20
	31	6	0.47 ± 0.04	10,400	26	0.25
10.3	41	8	0.48 ± 0.04	14,747	47	0.32
	61	2	0.29 ± 0.07	3,918	15	0.38
	83	8	0.26 ± 0.04	16,024	77	0.48
	103	2	0.08 ± 0.06	1,861	11	0.58
	5	5	0.60 ± 0.28	8,916	32	0.36
	10	3	0.51 ± 0.16	4,290	21	0.49
	21	6	0.56 ± 0.13	10,315	50	0.48
0.43	31	4	0.25 ± 0.09	8,019	45	0.56
	41	6	0.29 ± 0.05	11,771	59	0.50
	61	2	0.26 ± 0.10	5,009	28	0.56
	83	2	0.25 ± 0.00	5,755	33	0.57
	103	2	0.14	2,762	15	0.55
	0.9	3	1.10 ± 0.03	9,142	37	0.40
0.086	5	5	0.95 ± 0.10	9,623	44	0.46
	10	5	0.67 ± 0.12	9,891	63	0.64
	21	4	0.56 ± 0.05	7,549	42	0.55
	31	4	0.53 ± 0.16	7,103	42	0.59
	41	5	0.23 ± 0.08	9,916	61	0.62

[a]Cloning efficiency of irradiated cells/cloning efficiency of parallel controls.
[b]Transformation frequencies are expressed as the percent of transformed colonies among the total number of colonies scored in all experiments.
[c]Spontaneous frequency of combined cell populations, experimental range 0.04 to 0.11%.

Figure Legends

Fig. 1 Cell survival following irradiation by fission neutrons at 10.3 cGy/min (•) and 0.43 cGy/min (○) or ^{60}Co gamma irradiation at 44 cGy/min (■). Standard errors are smaller than points.

Fig. 2 Morphological transformation of SHE018 cells by fission neutrons at 10.3 cGy/min (■) and 0.43 cGy/min (•). For each experiment, high dose-rate exposures were performed immediately following low dose-rate exposures using cells from a single seeding. Spontaneous transformation frequency = 0.07%, i.e. 4 transformed colonies identified in total population of 5967 colonies. Uncertainities are standard errors.

Fig. 3 Morphological transformation of SHE018 cells by fission neutrons at 10.3 cGy/min (■) and 0.086 cGy/min (•). For each experiment, high dose-rate exposures were performed immediately following low dose-rate exposures using cells from a single seeding. For this series, no transformed colonies were identified out of a total of 6647 colonies in the unirradiated controls, but historical value for this pool is 0.07%. Uncertainities are standard errors.

Fig. 4 Morphological transformation of five SHE cell preparations (data pooled) by fission neutrons at different dose rates. Mean spontaneous frequency of combined cell populations was 0.06% (range 0.04-0.11%).

Fig. 5 Morphological transformation of SHE cells by ^{60}Co gamma irradiation at 44.0 cGy/min (■) and 1.0 cGy/min (•). Uncertainities are standard errors.