[CANCER RESEARCH 38, 4671-4675, December 1978]
0008-5472/78/0038-0000$02.00

Dependence on Cloning Method of Survival of Human Melanoma Cells after Ultraviolet and Ionizing Radiation[1]

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ABSTRACT

The resistance of a human melanoma cell line (MM96) to both ultraviolet and ionizing irradiation was compared by two different methods of cloning, on plates and in agar. A high level of resistance to both ultraviolet (D_0 = 320 ergs/sq mm) and ionizing irradiation (D_0 = 4300 rads) was observed when viability of cells was determined by cloning in agar. In contrast, melanoma cells were found to be as sensitive as were other cells when viability after irradiation was determined by cloning on plastic plates. The difference in sensitivity to radiation between the two methods of cloning can be explained in a model involving damage to membranes as well as to DNA. At least for ionizing radiation, this effect is not restricted to melanoma cells since a HeLa subline, HeLa-QB1, showed a similar response. In contrast, a human lymphoblastoid line (JHP) cloned in agar was sensitive under these conditions (D_0 = 120 rads).

INTRODUCTION

The correlation that has been demonstrated between the incidence of human malignant melanoma and exposure to UV (10, 19, 21, 28) poses the question of the mechanisms of this apparent etiological association. In view of the correlation in xeroderma pigmentosum between UV exposure, skin tumors (8, 12), and defective DNA repair systems (22, 35), a search for abnormal DNA repair underlying the occurrence of human melanomas could conceivably be fruitful. Examination of a variety of human melanoma cell lines has shown them to be resistant to UV (7) when cloned in agar, but thus far the cell lines themselves have not been shown to have major anomalies in the repair of UV damage in DNA (7, 20).

Malignant melanomas *in vivo* have been demonstrated to show poor response to radiotherapy or chemotherapy (6, 32). This resistance to radiation may be due to cell cycle changes or to the presence of an extremely efficient repair system (2). Barranco *et al.* (2) have reported that the response of melanoma cells *in vitro* to X-rays, when tested for viability by cloning on plates, does not correlate with the radioresistance observed under clinical conditions. Melanoma cells cloned in this way were found to be as sensitive to X-rays as were nontumor cells. Since it has been shown in this laboratory that melanoma cells are highly resistant to UV when cloned in agar (7), it was of interest to examine the resistance of these cells to ionizing radiation when cloned under the same conditions. We have compared survival of melanoma cells in response to both UV and ionizing radiation by cloning in agar and on plates. This comparison suggests an approach to interpretation of the effects of radiation on melanoma *in vivo*.

MATERIALS AND METHODS

Cell Culture. The melanoma cell line MM96 was derived from a lymph node metastasis in a 66-year-old female. At passage 65 the cells had a doubling time of 50 hr, were aneuploid with a mode of 49, and were pigmented (42). Examination by electron microscopy indicated that these cells contained up to 50 melanosomes and premelanosomes per cell. MM96 used in our experiments had passage numbers varying between 200 and 250, a doubling time of 32 hr, and a mode of 49. HeLa-QB1, a cloned subline aneuploid with a mode of 68 and a doubling time of 20 hr, was derived in this laboratory. A lymphoblastoid cell line (JHP) derived by transformation of human lymphocytes with Epstein-Barr virus was kindly provided by D. Moss, Queensland Institute of Medical Research.

Melanoma cells were grown in Medium H18 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 20% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). JHP cells were grown in suspension culture in the same media supplemented with 10% serum. HeLa-QB1 was grown in medium F15 (Grand Island Biological Co.) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. In all experiments asynchronous cells were used and were grown in an atmosphere of 5% CO_2.

UV Irradiation. Cells were grown in monolayers in 65-mm tissue culture plates prior to irradiation. When survival in agar was determined, cells were harvested with 0.02% trypsin and suspended in Hanks' balanced salt solution without colored indicator. A thin film of the cell suspension (1 ml) was added to a 52-mm bacterial plate and irradiated with a UV lamp (Mineralite UVS 12) at 254 nm, at a dose rate of 6 ergs/sq mm/sec. When survival after UV was determined on plates, harvested cells were added in appropriate numbers to 52-mm culture plates and were allowed to attach for 6 hr prior to washing with Hanks' solution and UV irradiation. Irradiation of cells in suspension prior to plating generally gave lower survival levels. The incident dose of UV was measured with a UV intensity meter (Ultra-Violet Products, Inc., San Gabriel, Calif.). Culture medium was returned to cultures immediately after irradiation.

Ionizing Irradiation. Cells were irradiated with either X-rays (General Electric SP-140) with a peak voltage of 100 kV filtered with a 1-mm thickness of aluminum in addition to

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Received December 6, 1976; accepted June 26, 1978.

the tube and tube housing at a dose rate of 260 rads/min or with γ-rays (Gamma Cell 220; Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada) at a dose rate of 280 rads/min. Calibration of the dose rate for X-rays was determined with a Queensland Standard Victoreen R-meter, and the output for γ-rays was determined by Fricke dosimetry. In both cases survival of cells was determined in agar and attached to plates. Irradiation of cells on plates was carried out as described for UV irradiation. When survival was assayed in agar, cells were irradiated when suspended in agar droplets.

Cell Cloning. Cloning in agar was carried out according to the procedure of Patuleia and Friend (31). As described previously a thin film of cell suspension (10^6 cells/ml) was UV irradiated at room temperature. A 1-ml sample of the irradiated culture was immediately mixed with an equal volume of culture medium containing 1% agar that had been kept at approximately 40°. This resulted in an almost immediate reduction in temperature to a level that was not damaging to the cells. On mixing, 0.3-ml samples were immediately pipeted onto 52-mm bacterial plates and allowed to solidify. Bacterial plates were used since the agar drops adhere thereto more readily than to tissue culture plates. When the agar drops were semisolid, 5 ml of growth medium were added carefully to each plate. Cloning was carried out for approximately 3 weeks with a change of medium every 6 days. In the case of ionizing radiation, cell suspensions were mixed with agar-containing medium as described previously. Medium was added to the 0.2-ml agar droplets, and irradiation was carried out within 30 min. Irradiation of cells attached to plates prior to trypsinization and suspension in agar did not appreciably affect survival. The number of cells per droplet varied from 200 to 500 in the case of unirradiated cells up to about 5×10^4 at the higher radiation doses. Individual colonies were scored in each droplet. Cloning of cells on plates was carried out as described previously for 10 to 12 days with one change of medium.

Colonies on plates (50 cells or more) were fixed with methanol, stained with 0.5% crystal violet for 5 min, and counted. For colony counting in agar, medium was removed and the agar droplets were washed successively with 0.18 M sodium chloride and water and allowed to dry onto the surface of the plate. Staining was carried out as for colonies on plates prior to counting. Precise counting of cells in colonies in agar was difficult, but an approximation indicated that the colonies contained more than 50 cells. The viability of cells in agar colonies after high radiation dose was established after cell isolation followed by cloning on plates. Cells were recovered by removing an agar droplet and gently breaking to facilitate removal of colonies with a Pasteur pipet. Colonies were then disrupted by incubation in a drop of 0.025% trypsin for 5 min in a 36-mm tissue culture dish. Growth medium (1 ml) was added, and cells were seen to attach to the plate in a few hr. After 1 week a large number of colonies was obtained, indicating that the cells isolated from the agar clone were viable.

RESULTS

Survival after UV Irradiation. The cloning efficiency of unirradiated MM96 cells was about 25% in agar and about 10% on plates. For HeLa-QB1 the corresponding values were 48 and 15%, respectively. The survival of MM96 in response to UV irradiation in agar and on plates is shown in Chart 1. In general agreement with previous data (D_0 = 400 ergs/sq mm; Ref. 7), MM96 exhibited a high level of resistance to UV (D_0 = 320 ergs/sq mm) when cloned in agar. However, when cloning was carried out on plates, a marked increase in sensitivity to UV was observed. The D_0 was reduced by approximately an order of magnitude to 30 ergs/sq mm. Similar experiments with HeLa-QB1 did not reveal this discrepancy between cloning in agar and on plates after UV irradiation (Chart 1); the D_0's after UV were the same (25 ergs/sq mm) when cloning was carried out in agar or on plates.

Survival after Ionizing Irradiation. The X-ray survival curve for MM96 when cloned in agar (Chart 2) demonstrates the resistance of these cells to ionizing radiation. The survival curve indicates a D_0 of 4300 rads. Markedly increased sensitivity was observed, as in the case of UV irradiation, when survival was determined on plates (D_0 = 180 rads). In contrast to the UV data, the response of HeLa-QB1 cells to X-radiation was analogous to that of melanoma. The sensitivity of HeLa-QB1 cells on plates (D_0 = 190 rads) was approximately the same as that obtained for MM96 on plates (Chart 2), but they showed increased resistance when survival was determined in agar. Here the D_0 (4300 rads) was approximately the same as that obtained for MM96 in agar.

The survival data obtained with γ-irradiation appear in Chart 3. Again melanoma cells showed a greater sensitivity when cloned on plates (D_0 = 150 rads) than when cloned in agar (D_0 = 3700 rads). Analogous behavior was observed in the case of HeLa-QB1 cells after γ-irradiation; cloning on plates gave a D_0 of 160 rads while the value in agar was 3300 rads. After exposure of MM96 to both forms of ionizing radiation, a shoulder was observed in the survival curves at lower doses of radiation when cloning was carried out in

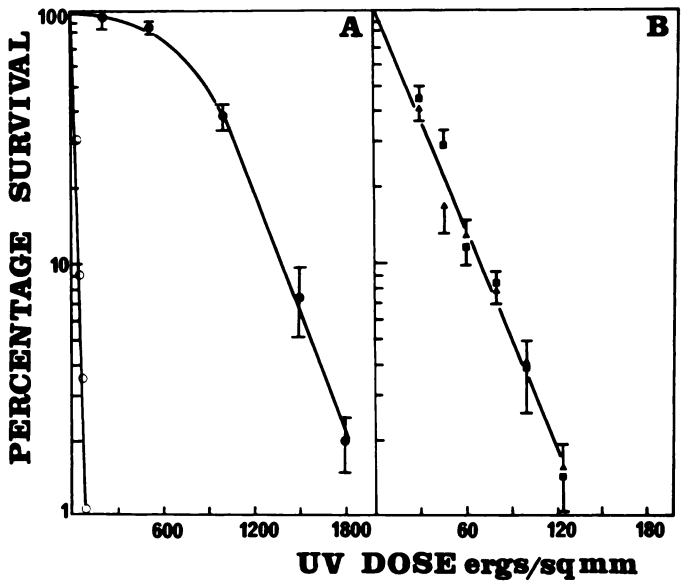

Chart 1. Cell survival in response to increasing UV dose as determined by cloning efficiency in agar and on plates. *A*, MM96 cloned in agar (●); MM96 cloned on plates (○); *B*, HeLa-QB1 cloned in agar (▲); HeLa-QB1 cloned on plates (■). *Bar*, S.E.

agar. However, in the case of HeLa-QB1 a linear dose-response curve was obtained, indicating that these cells were unable to sustain sublethal damage (14). By the criterion of agar cloning then, HeLa-QB1 cells are resistant to ionizing radiation but sensitive to UV radiation. The basis of the latter sensitivity has been discussed previously (7). The increased resistance to ionizing radiation of melanoma and HeLa-QB1 cells cloned in agar did not appear to be an artifact of the cloning technique since the human lymphoblastoid line JHP was highly sensitive to ionizing radiation (D_0 = 120 rads) when cloned in the same manner (Chart 3C). It seems unlikely that the high levels of survival in agar are due to multiplicity effects since it has been reported that use of greater numbers of cells at higher radiation doses decreases survival (3, 4, 27) or that survival is independent of cell numbers used (27).

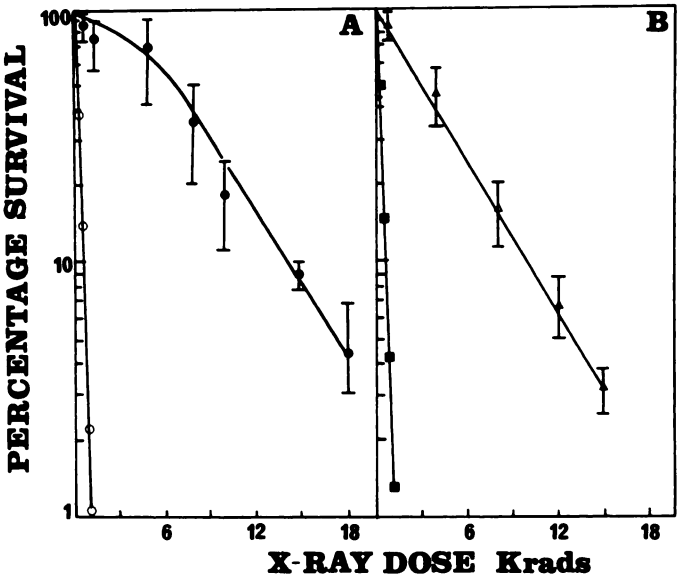

Chart 2. Cell survival after exposure to X-rays as determined by cloning efficiency in agar and on plates. *A*, MM96 cloned in agar (●); MM96 cloned on plates (○); *B*, HeLa-QB1 cloned in agar (△); HeLa-QB1 cloned on plates (■).

DISCUSSION

The data obtained demonstrate that in certain cases survival of cells both after UV irradiation and ionizing irradiation is highly dependent upon the method of cloning used. Previous results indicated that a series of human melanoma cell lines was highly resistant to UV irradiation when cloned in agar (7). However, when one of these cell lines, MM96, was cloned attached to plates, it no longer exhibited resistance, giving a D_0 of 30 ergs/sq mm, comparable to that of the least pigmented and most sensitive melanoma cell line described in another investigation (2). Furthermore, this low D_0 is in keeping with data obtained for a variety of other mammalian cells (34). After exposure to ionizing radiation, both MM96 and HeLa-QB1 showed high D_0's when cloned in agar. The ionizing radioresistance demonstrated by this method appears exceptionally high. However, as mentioned previously it does not appear to be artifactual, since the lymphoblastoid line JHP was very sensitive when assayed in the same manner.

Ionizing radiation gives rise to single and double strand breaks in DNA (23), base damage, and alkali-labile sites (25), while UV radiation damage includes the formation of pyrimidine dimers in DNA (36) and cross-linking of protein with DNA (16). In addition, there is evidence that radiation damages a number of cell structures and organelles (19) including the plasma membrane (40, 43), the endoplasmic reticulum (45), mitochondria (43), lysosomes (11, 41, 44), and microtubules (30, 33). Bacq and Alexander (1) suggested that critical biochemical lesions after irradiation *in vivo* may be the result of membrane damage that could give rise to lack of control of permeability and loss of surface proteins. Several reports do indeed support this sugges-

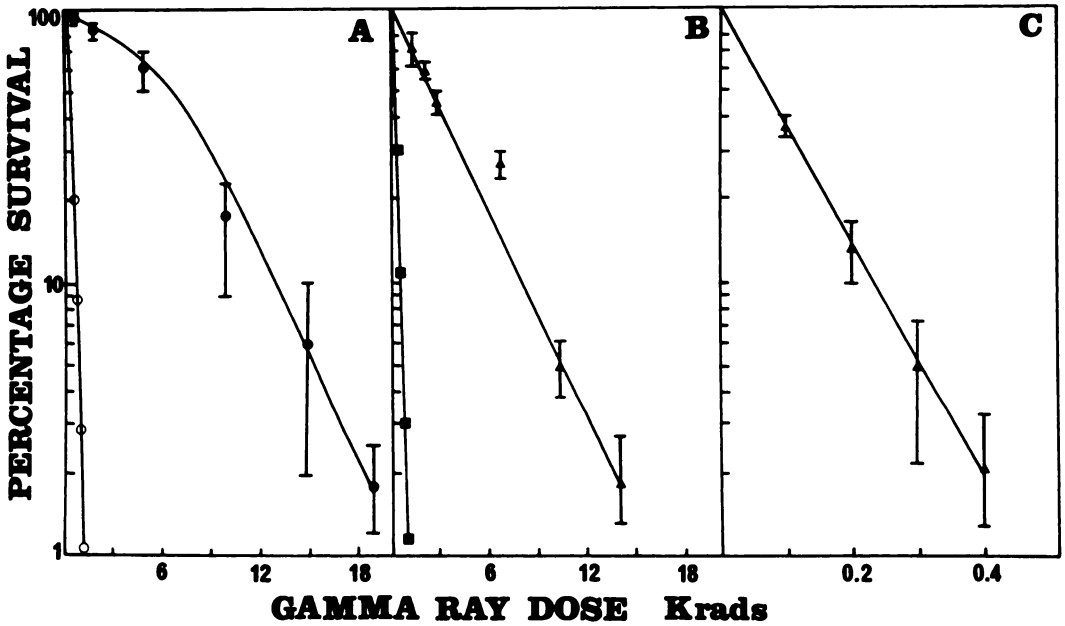

Chart 3. Survival of melanoma, HeLa, and lymphoblastoid (JHP) cells after exposure to γ-rays. *A*, MM96 cloned in agar (●); MM96 cloned on plates (○); *B*, HeLa-QB1 cloned in agar (▲); HeLa-QB1 cloned on plates (■); *C*, JHP cloned in agar (△).

tion. Exposure of melanocytes *in situ* to X-rays or to UV leads to increased tyrosinase activity, indicating a labilization of melanosomal membranes (39). Wills and Wilkinson (45) concluded that inhibition of hydroxylation reactions after X-irradiation of microsomes *in vitro* is a result of partial disintegration of the endoplasmic reticulum. Phase-contrast microscopy of erythrocytes after low doses of irradiation (50 to 100 rads) demonstrated an increase in the number of irregularly shaped cells with folded and ruffled membranes as well as some hemolysis (24). Other data suggest that the increased release of hydrolytic enzymes following ionizing radiation is due to a membrane effect (11, 41). Increased permeability of membranes to ions has been observed in tissues from irradiated animals (15), in human erythrocytes (37), and in mitochondria (43).

Damage to membranes could contribute to the difference in survival of irradiated melanoma cells when cloned in agar and on plates. Changes in permeability and surface conformation after irradiation could lead to detachment from plates and cell death. However, when cells are cloned in agar, this medium would provide a supportive matrix so that the surface properties of the cells might be less immediately critical to survival. Since repair of damage to DNA is critical to cell survival, the increased resistance observed in agar must also depend on an inherent ability to cope with damage to DNA. That higher viability may be obtained when cells are cultured in a solid support is evident from a recent report (29) in which primary cultures of parenchymal liver cells were successfully maintained on collagen gels for up to 3 weeks. While this viability was established for unirradiated cells, it nevertheless stresses the importance of cell attachment in survival.

The levels of survival observed in our experiments with agar are extraordinarily different from those reported from a variety of studies of both normal and tumor cells in which survival was determined after ionizing radiation both *in vitro* and *in vivo* (5, 9, 13, 18, 26, 46). In these studies *in vitro* survival was assayed by cloning on plates. There is some evidence that cells *in vivo* can accumulate more sublethal damage than can cells grown on plates *in vitro*, which makes difficult any extrapolation from the one situation to the other (17).

As noted previously melanomas *in vivo* respond poorly to ionizing radiation. More recent studies (38) on the response of a human malignant melanoma cell line to high LET radiation suggest that the radioresistance observed *in vivo* may be mediated in part by the *in vivo* environment. The increased survival observed in agar in these experiments may reflect similar protection.

Because the incidence of human melanoma correlates with exposure to sunlight (10, 19, 21, 28), it has been proposed that UV may be involved in the genesis of these tumors. If the UV resistance of melanoma cells in culture, as judged by agar cloning (7), reflects an *in vivo* property, it is conceivable that UV has a role in the progression of the tumor as well as in the extent of metastasis.

In summary we suggest that the difference in survival of melanoma cells after both UV and ionizing irradiation, when cloned by different methods, is related to membrane damage as well as chromosomal damage. Growth of irradiated cells in agar appears to minimize the effects of membrane damage on survival, permitting recognition of the inherent radioresistance of melanoma cells. The finding that HeLa-QB1 cells are similarly resistant to ionizing radiation when cloned in agar indicates that this phenomenon is not restricted to melanoma. At the same time, the sensitivity of the lymphoblastoid line JHP when cloned in agar indicates that relative survival in this medium reflects an intrinsic property of each cell line.

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