Differential Responses to X-Irradiation of Subpopulations of Two Heterogeneous Human Carcinomas in Vitro¹

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ABSTRACT

The responses of two heterogeneous human cancer cell lines and their derivative clones to graded single doses of X-rays were examined in vitro. One system consisted of the human colon carcinoma line DLD-1 and two subpopulations (clones A and D). The second system consisted of the human lung carcinoma line (LX1) and four subpopulations (LX1-1, LX1-2, LX1-3, and LX1-9). These subpopulations have previously been shown to be markedly heterogeneous in terms of such characteristics as karyotype, morphology, drug sensitivity, tumorigenicity, and expression of membrane glycoproteins (such as carcinoembryonic antigen and tumor colon mucoprotein antigen). Exponentially growing cultures were irradiated with graded single doses of 100-kVp X-rays. Survival was assessed using colony formation as the end point, and responses from multiple experiments were fitted to the single-hit, multitarget equation of cell survival. Values for the mean lethal dose (D₀, grays), quasithreshold dose (Dₚ, grays), and extrapolation number (n) were obtained. For the human colon adenocarcinoma system, these values for the three tumor lines were: DLD-1, 0.95, 2.34, and 11.7; clone A, 1.06, 2.23, and 8.20; and clone D, 1.06, 1.89, and 5.80. For the human lung carcinoma system, these values for the five sublines were: LX1, 1.14, 0.19, and 1.20; LX1-1, 0.96, 2.06, and 8.54; LX1-2, 0.96, 0.88, and 2.48; LX1-3, 0.68, 2.05, and 20.3; and LX1-9, 1.12, 0.00, and 1.00. These two human tumor systems therefore exhibit variability in their intrinsic sensitivity to X-irradiation. The data indicate that failure of some human carcinomas to respond to physical treatment modalities can be due to preexisting resistant subpopulations.

INTRODUCTION

Individual animal tumors have been shown to consist of subpopulations of cancer cells with intrinsically different properties. These include in vitro growth characteristics, immunogenicity, tumorigenicity, karyotype, metastasis potential, production of various cellular products, and sensitivity to chemotherapeutic drugs (2, 12, 15, 19, 21, 22, 34, 36). Although much of the work to date has been done with murine tumors, much of the work to date has been done with murine tumors, due to preexisting resistant subpopulations.

Therefore, these human tumor model systems have been extensively characterized with respect to the nature and degree of intrinsic heterogeneity existing within each system. Given this background, the purpose of the experiments described here was to determine whether differences also existed in the responses of each parent line and its subpopulations in each tumor system to another major cytotoxic modality, ionizing radiation.

MATERIALS AND METHODS

Human Colon Adenocarcinoma System. The cultivation and characterization of the DLD-1 cell line have been described previously (5, 10, 14, 41), and the first work on this tumor line appeared in 1977. At the time of establishment, it was apparent that the morphology of the DLD-1 line in tissue culture was heterogeneous and that 2 distinct colony morphologies could be identified. These subpopulations were isolated using soft agar cloning techniques and were found to be morphologically identical to the cells seen in the DLD-1 line. These subpopulations were termed clones A and D (5, 10). These clones have been recloned, and no difference has been seen between the original and the recloned cells (14). Also, the karyotype of the lines has remained unchanged from that determined for early-passage DLD-1 cultures (14). DLD-1 cells have a predominantly diploid karyotype with a few hyperdiploid cells present, and they produce moderately to poorly differentiated colon carcinomas in nude mice. Clone D cells are diploid and produce moderately differentiated carcinomas in nude mice. Clone A cells are hyperdiploid (75% of clone A cells have 46 to 60 chromosomes) and produce poorly differentiated carcinomas in nude mice. All colon carcinoma cell lines are maintained in Rosewell Park 2656

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The abbreviations used are: DMF, N,N-dimethylformamide; PI, propidium iodide.

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Memorial Institute Tissue Culture Medium 1640 supplemented with 10% fetal calf serum and antibiotics (10). The cultured lines have remained very stable in tissue culture over the past 5 years. Similarly, tumor production in nude mice occurs with the same growth rate and histology, and the histology remains representative of the morphologies seen in vitro. This DLD-1 system therefore appears to be a good model for investigation of such parameters as intrinsic radiation sensitivity.

Human Lung Carcinoma System. The human lung carcinoma parent line LX1 was originally established in tissue culture from a solid tumor growing in a nude mouse by Dr. B. Giovanella (17). We received our cultures from Dr. A. Bogden of the Mason Research Institute, Worcester, Mass., in 1978. This original culture was immediately cloned using soft agar techniques, and the clones with the most distinctive morphologies were then established as individual tumor lines. These subpopulations were termed LX1-1, LX1-2, LX1-3, and LX1-9. Chromosomal analyses and karyotypic determinations were made on all tumor lines. Morphologically, the LX1 parent culture was seen to contain 2 different morphologies, one being round and epithelial and the other being fibroblastic. The LX1-1 line is round and epithelial; LX1-2 is epithelial with a tendency to pile up; LX1-3 is fibroblastic with a tendency to pile up; and LX1-9 is fibroblastic. Exponentially growing cells from all lines exhibit cell culture-doubling times of approximately 20 hr, and all cell lines contain between 80 and 90 chromosomes. Upon establishment of these tumor subpopulations, all lines were preserved in liquid nitrogen. These lines all produce solid tumors in immunosuppressed mice, and in our laboratory these tumors have been histopathologically described as undifferentiated epidermoid carcinomas (4). The X-ray experiments and the determination of DNA contents of the lung tumor lines were all done on cultures that had been established by removal from liquid nitrogen storage immediately prior to investigation. As stated in the "Introduction," these lines exhibit differential responses to selected chemotherapeutic agents (3, 4, 6, 7).

X-Irradiations. All cell irradiations were performed using a Picker X-ray machine operated at 100 kvp and 10 ma. The target-to-sample distance was approximately 33 cm with 1 mm aluminum added filtration. Exposure doses in roentgens were converted to absorbed doses using a roentgen-to-rad conversion factor of 0.96. Exponentially growing monolayer cultures were exposed in Falcon T-30 plastic flasks (Falcon Plastics, Oxnard, Calif.) containing 5 ml of complete medium, using a roentgen-to-rad conversion factor of 0.96. Exponentially growing cells from all lines exhibit cell culture-doubling times of approximately 20 hr, and all cell lines contain between 80 and 90 chromosomes. Upon establishment of these tumor subpopulations, all lines were preserved in liquid nitrogen. These lines all produce solid tumors in immunosuppressed mice, and in our laboratory these tumors have been histopathologically described as undifferentiated epidermoid carcinomas (4). The X-ray experiments and the determination of DNA contents of the lung tumor lines were all done on cultures that had been established by removal from liquid nitrogen storage immediately prior to investigation. As stated in the "Introduction," these lines exhibit differential responses to selected chemotherapeutic agents (3, 4, 6, 7).

RESULTS

Intrinsic Radiosensitivities of Tumor Subpopulations. In Table 1, we have summarized all of the survival data obtained from experiments in the 2 human carcinoma systems. These data are displayed in Charts 1 and 2. With regard to survival curve parameters in the colon system, the parent DLD-1 line has the highest extrapolation number, although the 95% confidence limits on the value of n overlap for all of the colon lines. The D0 values also are not significantly different among the lines. However, the parent line (DLD-1) and clone A have D0 values which are significantly greater than is that for clone D. The DLD-1 line is not statistically different in its D0 value from clone A. Therefore, the major difference among the colon cancer sublines lies in the fact that clone D has a statistically significant lower value for D0. This finding suggests that clone D has a decreased ability to accumulate sublethal radiation injury in comparison to clone A or to the parent DLD-1 line. The plating efficiencies for all 3 lines are high (ranging from 68.6 to 80.8%) and are not statistically different.

For the human lung carcinoma system, with regard to survival curve parameters, there is a much greater absolute variation. In terms of extrapolation numbers (n), values range from 1.00 (LX1-9) to 20.3 (LX1-3). The parent LX1 line also shows a small extrapolation number (1.20), and n values for the 2 lines (LX1 and LX1-9) are statistically smaller than are those for the other 3 cell lines. LX1-2 has an intermediate and statistically distinct value of n (2.48), as do LX1-1 and LX1-3. With the exception of LX1-3, the other lines have similar values for the D0 values ranging from 0.96 to 1.14 grays. Line LX1-3, which

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Data Analysis. In the analysis of the responses to ionizing radiation, data from individual experiments were combined, and survival curves were constructed by plotting the average surviving fraction as a function of dose. The slopes of the linear portions of the survival curves were fitted using a least-squares linear regression analysis, and the extrapolation number (n), quasithreshold dose (Dq, grays), and the mean lethal dose (D0, grays) for each subline was calculated. The 95% confidence limits on these values were obtained using standard parametric methods (18), and the data points were used with equal weights in the analyses. This approach (the so-called single-hit, multitarget model of cell inactivation) is a conventional means of presenting survival data.

Flow Cytometric Determination of DNA Content of Human Tumor Cell Lines. Exponentially growing cells of all tumor subpopulations were seeded into T-75 plastic flasks (Falcon Plastics) at concentrations of 1 x 10^6 cells/flask and allowed to multiply for 48 hr (about 2.5 cell doublings). Cells were then removed from flasks using the same procedure as in the X-ray experiments. The cellular pellets were resuspended in phosphate-buffered saline (0.9% NaCl) (Grand Island Biological Co.) and recentrifuged twice. After the washings, the cells were resuspended in a PI solution (Cambioehem-Behring Corp., San Diego, Calif.) containing PI, 0.05 mg/ml, in 0.1% sodium citrate, according to the method of Krishan (27). The cells were incubated in PI staining solution for 10 min at 4° and were then fixed by very quickly adding an equal volume of 90% ethanol and mixing quickly. The stained and fixed cells were then analyzed for DNA content using an Ortho Instruments (Westwood, Mass.) ICP 22A Cytofluorograph in conjunction with a mercury arc lamp for stain excitation and a Model 2150 computer system with image-forming module for data display and analysis. All determinations of tumor cell DNA content, biological standardizations were done using human peripheral blood lymphocytes stained at the same time and in the same manner as were the tumor cells.

It is important to note that, in all of the DNA and X-ray experiments, growth conditions and the times between preparation of experimental cultures and analysis of treatment were rigorously standardized, because parameters such as cell volume and distribution of cells through the cell cycle are strongly dependent upon stage of growth.
Table 1

Radiation survival parameters of human colon adenocarcinoma and lung carcinoma sublines

<table>
<thead>
<tr>
<th>Tumor Subline</th>
<th>Plating Efficiency</th>
<th>Extrapolation Number (n)</th>
<th>Dq (grays)</th>
<th>D0 (grays)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent (DLD-1)</td>
<td>68.6 ± 5.2*</td>
<td>11.7</td>
<td>2.34</td>
<td>0.95</td>
</tr>
<tr>
<td>Clone A</td>
<td>80.8 ± 6.2</td>
<td>8.20</td>
<td>2.23</td>
<td>1.06</td>
</tr>
<tr>
<td>Clone D</td>
<td>75.7 ± 7.1</td>
<td>5.80</td>
<td>1.89</td>
<td>1.08</td>
</tr>
<tr>
<td>Lung carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent (LX1)</td>
<td>15.6 ± 2.4</td>
<td>1.20</td>
<td>0.19</td>
<td>1.14</td>
</tr>
<tr>
<td>LX1-1</td>
<td>12.8 ± 1.8</td>
<td>8.54</td>
<td>0.06</td>
<td>0.96</td>
</tr>
<tr>
<td>LX1-2</td>
<td>10.7 ± 1.9</td>
<td>2.48</td>
<td>0.88</td>
<td>0.96</td>
</tr>
<tr>
<td>LX1-3</td>
<td>17.4 ± 2.1</td>
<td>20.3</td>
<td>2.05</td>
<td>0.68</td>
</tr>
<tr>
<td>LX1-9</td>
<td>16.2 ± 1.8</td>
<td>1.00</td>
<td>0.00</td>
<td>1.12</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
Numbers in parentheses, 95% confidence limits.

Chart 1. Survival response of human colon adenocarcinoma sublines (A, DLD-1; O, clone A; •, clone D) to 100-kVp X-irradiation. Points, mean survival values from replicate experiments; bars, S.E.

Chart 2. Survival response of human lung carcinoma sublines (O, LX1; •, LX1-1; A, LX1-2; •, LX1-3; A, LX1-9) to 100-kVp X-irradiation. Points, mean survival values from replicate experiments; bars, S.E.

has the statistically smallest value for D0 (0.68 gray), also shows the largest extrapolation number. Significant differences may also be seen with regard to the Dq values. LX1-9 appears to have no capacity to accumulate sublethal radiation injury (D0 = 0.19 gray), and the parent LX1 line has a very small value (Dq = 0.00 gray). LX1-2 is intermediate in the value of this parameter (Dq = 0.88 gray), whereas lines LX1-2 and LX1-3 have the highest values (Dq = 2.06 and 2.05 grays, respectively) and are not statistically different from each other. In this system, the plating efficiencies are lower (ranging from 10.7 to 17.4%), and there are significant differences between the lowest (LX1-2) value and the values for LX1, LX1-9, and LX1-3. No other comparisons are statistically different. Thus, there are marked differences between the DLD-1 and the LX1 system in terms of the colony-forming ability of unirradiated cells. However, as survival responses after X-irradiation were all normalized to the appropriate colony-forming efficiency from control cultures, this has no effect on the shape of the dose-survival curves.

DNA Content of Tumor Subpopulations. The DNA content of the tumor lines, expressed as the ratios of the G1 DNA peaks for the tumor cells to human peripheral blood lymphocytes for the DLD-1 system were: DLD-1 cells, 3.3 (10.1%); clone A, 4.9 (6.1%); and clone D, 2.7 (11.1%). For the LX1 system, values were: LX1, 3.3 (9.3%); LX1-1, 2.8 (5.9%); LX1-2, 3.1 (10.3%); LX1-3, 3.1 (5.6%); and LX1-9, 3.0 (6.2%). The values in parentheses represent the coefficient of variation of these values expressed as a percentage of the G1 peak. For peripheral blood lymphocytes, the percentage coefficient of variation was about 9.

It is apparent that the DLD-1 human colon tumor system subpopulations vary significantly in their DNA content. In contrast, the variability in DNA content is not as great in the LX1 lung tumor system. Indeed, on the basis of the coefficient of variation seen in the DNA histograms, these LX1 lines are not significantly different in their respective DNA contents.
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The data summarized in Table 1 and illustrated in Charts 1 and 2 show that the subpopulations of tumor cells from the human colon and human lung carcinoma systems differ significantly with regard to their sensitivities to X-irradiation. These data have several important implications, which are discussed below.

It is interesting to attempt to relate the differences seen in intrinsic radiation survival parameters to the content of DNA, the prime target for the action of ionizing radiation. For the DLD-1 system, the greatest amount of DNA is found in clone A, with the parent DLD-1 line containing only about two-thirds of the content shown by clone A and clone D containing roughly about one-half of the DNA content shown by the clone A line. From Table 1, it is obvious that none of the survival parameters used in the single-hit, multitarget formalism exhibit total correlation with DNA content. In contrast, the LX1 subpopulations, which all exhibit approximately the same extents of aneuploidy, vary significantly in their radiation survival parameters. Such differences in correlation of ploidy or DNA content to inherent radiation sensitivity have been pointed out by other investigators (9, 40) and illustrate the inherent weakness of certain survival models (the single-hit, multitarget formalism) which do not explicitly include terms which relate to the fact that both the number and nature of radiation lesions produced and the fidelity and activity of intracellular repair systems acting on such lesions must be considered. While some "repair"-type models have been advanced, they are complex and are still in their infancy (42).

It should be noted that, in both these human tumor systems, the position of the parent survival curve in relationship to those of its clones (Charts 1 and 2) cannot be predicted; i.e. the shapes and slopes of the DLD-1 and LX1 curves (and the values of their parameters) are not the averages of the survival curves of each set of subpopulations. In principle, one might speculate that, if the radiosensitivity of each clone were known as well as its proportion in the entire tumor, then the survival curve of the parent line might be predicted from a knowledge of the survival curves of the clones. Our data show that this is not the case with either system; with the colon tumor the parent line appears too sensitive, and with the lung carcinoma it appears too sensitive. One explanation is that we have not isolated all of the subpopulations in each of the 2 heterogeneous tumors. This is certainly possible, although there is good evidence that the DLD-1 line is comprised primarily of clone A and clone D cell types (14). Another interpretation of the positioning of the survival curve of each parent with respect to those of its subpopulations is that subpopulations in a heterogeneous neoplasm interact in such a way as to make the response of the entire tumor unpredictable even if the responses of the isolated clones are known (5, 13).

One approach for successful treatment of a heterogeneous tumor would be to modify the more resistant subpopulations in such a way as to make them more sensitive to a treatment modality. The use of biological modifiers to induce a more benign phenotype in cancer cells has been extensively documented (5, 8, 11, 24, 26, 28, 37) and, in this regard, we have used the polar solvent DMF to induce a better differentiated, less malignant phenotype in DLD-1 clone A and clone D cells (10, 20). Perhaps, as we have suggested previously, treatment with DMF or other modifiers would force a convergence of subpopulations from a heterogeneous neoplasm to a more common phenotype, which is more sensitive to X-irradiation than were the more radioresistant clones prior to induction (5, 13). We have recently reported that a radioresistant subpopulation isolated from a spontaneously occurring mouse mammary tumor (12) can be sensitized to X-ray treatment by prior exposure to DMF (13, 30), and recent data from our laboratory indicate that DMF can also sensitize clone A and clone D colon cancer cells to ionizing radiation (32). At present, we do not know if DMF is affecting the number of radiation-produced lesions in the cell, the repair of lesions in the cell, or possibly the accessibility of repair enzymes to damaged target material (e.g., DNA).

In Charts 1 and 2 and in Table 1, we have summarized and illustrated the survival data of these heterogeneous tumor lines after X-irradiation. While it is apparent that there is a large absolute biological variability in response among clones within each system, it is possible to calculate the average radiation dose needed to produce any given level of survival for all of the clones within a system. From the different dose values obtained, we may then calculate the 95% confidence limits on the dose needed to produce this level of survival, using the appropriate 2-tailed t value (2.78 and 4.30, respectively, for the lung and colon systems), with $n - 1$ d.f. (18). This statistical approach yields a model of the intrinsic heterogeneity of the 2 systems and allows them to be intercompared. While we cannot be assured that all radiation sensitivities are represented since we may not have isolated all subpopulations, this statistical model allows us to predict what the responses of a larger population of sublines might be. In Chart 3, we have generated a heterogeneity ratio plot: (a) to compare the total variability between these heterogeneous tumor systems; and (b) to describe specifically the variability in the direction relevant to the clinical use of ionizing radiation (i.e., in the direction of radiosensitivity). An index of the total variability is made by taking the ratio of doses represented by the maximum and minimum extremes of the calculated 95% confidence limits on dose at any given level of tumor cell survival. This ratio is then plotted versus the average dose also at this given level of survival (Chart 3, Curve A). The therapy-related variability is defined as...
the ratio of the maximum value of the 95% confidence value to the average dose value, and this ratio is also plotted versus the average dose (Chart 3, Curve B). The generation of these ratios is similar to the generation of the well-known relative biological effectiveness and oxygen enhancement ratio plots in radiation biophysics (16).

Several points of interest are evident from Chart 3. (a) It is striking that the shapes of these heterogeneity ratio plots for either the maximum total or the therapy-related variabilities are extremely similar. This similarity suggests that, at least for these 2 heterogeneous human tumor systems, the intrinsic variability in their responses to ionizing radiation is extremely comparable, even though the absolute levels of survival after a given dose of radiation are clearly different (Charts 1 and 2). (b) It is possible to obtain a value for this heterogeneity ratio for any administered dose. For example, at a dose level of 2 grays, which represents the most often used clinical radiation dose per treatment, values of about 2.1 and 1.4 are obtained for the total and therapy-related ratios of variability present in these 2 tumor systems. Interpretation of the value of 1.4 means that 40% of the total variability in response seen is in a direction greater than that of the average response, i.e., in the direction of radioreistance. A corollary to this interpretation is, unfortunately, that there is likely to exist in a heterogeneous system sublines with radiobiological survival characteristics similar to, or even more resistant than, those of the limiting normal tissue(s) in the treatment volume.

Our ability to kill these resistant subpopulations will determine the efficacy of radiotherapy of a heterogeneous human tumor, and, of course, if the dose needed to eradicate the radioreistant tumor cells exceeds the normal tissue tolerance, therapy will fail due to unacceptable toxicity. Also, a tumor containing such resistant subpopulations might be expected to recur after exposure of the neoplasm to a given tolerable total dose, even though most of the tumor burden would have been eliminated. We realize that there are other environmental factors that will impinge on the outcome of therapy (e.g., the extent of tumor hypoxia). However, we feel that our description of intrinsic variability provides at least one reason for the failure of some neoplasms to respond to radiation therapy. In a pertinent study, Hill et al. (23) investigated the responses of clonal lines of 10 B16 mouse melanoma lines to X-irradiation. The authors noted a considerable variability in radiation response among the melanoma clones and commented that the radiation-resistant fraction of subclones within a human melanoma may be in part responsible for the known radiation resistance of human melanomas.

In summary, we have described the intrinsic radiation sensitivities of subpopulations isolated from 2 original different human tumors, a lung and a colon carcinoma. Significant differences were found in absolute sensitivities, although the internal variability of the 2 systems appears very comparable. In this regard, it must be noted that it has also been established that the ranking of responses of tumor sublines in such heterogeneous systems is different depending on the cytotoxic agents used (5, 13, 29, 31). Thus, we have as yet found no correlation between sensitivity of a given subpopulation to chemotherapeutic drugs and sensitivity to ionizing radiation. Therefore, a logical approach to the phenomenon of intrinsic tumor heterogeneity is the use of multimodality or multianti therapy. Our ability to detect, characterize, and manipulate subpopulations of tumor cells with such differential properties (e.g., X-ray sensitivity, drug sensitivity, metastatic potential) is clearly an important area of research with direct clinical significance.

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