Synergistic Effect of the v-myc Oncogene with H-ras on Radioreistance

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ABSTRACT

Resistance of tumors to irradiation or chemotherapeutic agents is thought to be one of the reasons why patients who present with early malignancies may not be cured. Much is now known about the molecular mechanisms that underlie drug resistance, but until recently little was known about genetic contributions to radiation resistance. Some evidence now links oncogenes, particularly the ras family of oncogenes, to radiation resistance but heterogeneity between tumors and cell lines has complicated this analysis. Primary rat embryo cells have been chosen as a model system in which the effects on radiation resistance of the H-ras oncogene could be studied on a uniform genetic background. These cells offer several useful advantages. The cells prior to transformation are diploid, and because they have been in culture only for a few passages prior to transformation with the oncogene it is unlikely that any preexisting mutation affecting radiation response could be present. Additionally, the use of rat embryo cells permitted the study of the effects of a second oncogene on the appearance of the radioresistant phenotype. The results show that the activated H-ras oncogene is associated with radiation resistance in primary rat cells after transformation but that the effect of the oncogene by itself is small. However, the oncogene v-myc, which has no effect on radiation resistance by itself, has a synergistic effect on radiation resistance with H-ras. There appear to be differences in the phenotype of radiation resistance associated with these two forms of transfectants. Thus, radiation resistance seen with H-ras by itself is characterized by a change in the shoulder of the radiation survival curve at high radiation doses but little or no change within the shoulder region of the radiation survival curve. Radiation resistance seen in H-ras plus v-myc transfectants is also characterized by an increase in the slope of the curve at high doses but there is also a large effect within the shoulder region of the radiation survival curve. These studies led to the following conclusions: (a) the radioresistant phenotype is not due to preexisting genetic heterogeneity in the cells prior to transfection; (b) the radiation resistant phenotype of cells transformed by H-ras is seen to a greater degree in cells which also contain the v-myc oncogene; (c) the v-myc oncogene may play an important role in the phenotype of radiation resistance at low doses that is within the range most critical for clinical practice.

INTRODUCTION

While the role of tissue hypoxia as a mediator of differences among tumors in determining radiation sensitivity has long been considered important (1, 2), it is only recently that investigators have begun to probe the role of intrinsic genetic differences between tumors of similar histology as a determinant of radiation response (3–7). The genetic basis of this intrinsic heritable property of tumor cells is not yet understood but recently several papers have raised the possibility that oncogenes which are thought to play a role in the conversion of a normal cell to a malignant cell may also play a role in the development of radiation resistance at least in a subset of the radioresistant tumors. A rare genetic syndrome has been described, Li-Fraumeni syndrome (8), which is characterized by the dominant inheritance of a propensity to develop neoplasms. Syndromes which predispose to malignancy might be thought to be more likely to be associated with increased radiosensitivity. However, Chang et al. (9) showed that normal fibroblasts from skin biopsies of one of these patients showed radioresistance in tissue culture. They were able to show in these fibroblasts overexpression of the myc oncogene relative to normal levels and also apparent activation of the raf oncogene. Kasid et al. (10) showed that a cell line developed from a clinically radioresistant human squamous cell carcinoma is also characterized by the apparent activation of the raf oncogene. While these studies showed that an activated oncogene could be found in cells which were known to be radioresistant, they did not show that the oncogene was in any way directly connected with the radioresistant phenotype. FitzGerald et al. (11) in a study of NIH3T3 cells containing the human N-ras gene showed that the cells appeared to have increased radioresistance over the untransfected cells at high dose rates but that the effect seemed to disappear at low dose rates. Sklar (12) extended this work when he compared the radiosensitivity of a number of transformants of NIH 3T3 cells containing various forms of the ras oncogene. He showed that all of the lines containing an activated ras oncogene had increased radioresistance in tissue culture but that this was not seen after transfection with the protooncogene or other oncogenes.

The use of NIH 3T3 cells for the study of radioresistance is problematic. They have been in culture for many years, are highly aneuploid, and have a very high rate of spontaneous transformation. There is known to be wide variation among laboratories in the properties of this long established line making comparisons difficult between lines from different sources, controlling for only a single variable (13, 14). Additionally cell lines which have been long established in cell culture and which are used as controls or recipients in transfection experiments cannot be assumed to be normal in all of their properties. Some have speculated that the reason NIH 3T3 cells are so easily transformed by H-ras is because NIH 3T3 cells already have an oncogene-like activity in the cells prior to transformation (15).

We therefore set out to devise a model system in which the effects of ras and other oncogenes on radiation resistance could be studied on a uniform genetic background with fewer confounding variables. We chose primary rat embryo cells for our studies because we have previously used these cells for the study of oncogene induced transformation and metastasis (16–18). We have also used them to elucidate the effects of second oncogenes on the transformed phenotype after transformation. Since we have worked extensively with primary rat embryo cells to study metastasis we chose to use this system as the model for our studies on radiation resistance. These cells offer several useful advantages. The cells prior to transfection are diploid and because they have been in culture only for a few passages...
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prior to transfection with the oncogene, it is unlikely that any preexisting mutation affecting radiation response could be present in the cells. Additionally, the use of REC allowed us to view the effects of second oncogenes on the induction of the radioresistant phenotype. Also, we have shown that transformation of primary cells by ras under certain conditions produces specific and unique chromosomal aberrations (17), suggesting that not all of the changes that occur in cells after oncogene transfection need to be attributed directly to the oncogenes themselves but that other nonrandom changes in gene expression may occur at high frequencies secondary to these specific karyotypic alterations which occur in the cells.

MATERIALS AND METHODS

Cell Lines. The derivation of several of the lines used in this study has been described in previous publications (16–18). Primary rat embryo cells from 17-day-old Sprague-Dawley embryos (Flow Laboratories, MacLean, VA) were trypsinized and 2.5 x 10^6 cells were placed in each tissue culture plate in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Calcium phosphate DNA precipitates were formed in a volume of 2 ml with each plasmid DNA as indicated at a final concentration of 25 µg/ml. When more than one plasmid was used the plasmids were mixed in equal amounts by weight in the precipitates. Precipitates were applied to the cells for a period of 4 h, followed by a glycerol shock for 2 min in 3 ml of 15% w/v glycerol in 1 x 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline. After 2 weeks colonies were either picked or stained with crystal violet and scored if they contained more than 50 cells. If selective medium was used, the cells were fed with medium containing 200 µg/ml of neomycin (G418; Gibco). Lines 3R, 4R, and 5R were transformed with H-ras alone, using the plasmids pEJ (human ras derived from the T24 bladder carcinoma cell line) in coselction with pSV2neo, as described previously (16). Cell lines 2.8, 2.10, and 3.7 were transformed by H-ras plus the cooperating oncogene v-myc using the plasmids pEJ and pMC29 (v-myc, the cloned avian myeloblastosis virus) as described previously (17). RC2 was a spontaneously immortalized cell line developed by continuously passing the primary rat embryo cells beyond crisis. The line mycREC, obtained on a Fischer rat background, was immortalized by retroviral transfection with pMV6/c-myc using 10^3 retroviral particles in 2 ml culture medium with 8 µg/ml Polybrene/tissue culture plate. The cell lines RC2 and mycREC were not transformed in tissue culture. They remained contact inhibited and did not form tumors or metastases after injection into nude mice or syngeneic rats. The presence of the transfected sequences was checked by Southern analysis using the method of Dean (19, 20).

RESULTS

Primary rat embryo cells are sensitive to ionizing radiation with a D_0 of 108 cGy. Since the cells will survive for only a few passages in tissue culture it is not possible to assess subclones of the primary cells. However, we studied two immortalized but not transformed lines of REC. The line RC2 is a spontaneously immortalized cell line which we obtained by continuing to passage the REC beyond crisis. This line also retains the radioresensitivity of the parent line with a D_0 of 90 cGy (Table 1). Similarly, the line mycREC, immortalized by myc, also has the same parameters of radiation response as the untransformed cells (Figs. 1 and 2). Thus, these cells do not show heterogeneity in their response to radiation prior to transformation (Table 1, Group 1).

We have examined 3 independently derived clones of rat embryo cells transformed by ras without a second oncogene. In the cell line 3R (see Fig. 3), transformed by H-ras alone (in cotransfection with pSV2neo), there is a small increase in the D_0 to 148 cGy (Fig. 3). When we examine the initial portion of the radiation survival curve we see that this has occurred largely by an alteration in the β component of the radiation survival curve with little alteration in α (Fig. 4). Similar results are seen in the 2 additional independent transformants (see summary in Table 1). One of these lines, 5R, has a D_0 of 127; the other clone, 4R, has a D_0 of 137. The mean D_0 of this group of cell lines independently transformed by ras alone without the use of a second oncogene was 137 (Table 1, Group 2). This is significantly different from the primary cells and the untransformed cells in a Student t test comparing Group 1 versus Group 2 (P < 0.02). The D_0 values of the REC transformed by H-ras without a second oncogene are not remarkably higher than that seen in many normal mammalian cell lines (21) but are slightly higher than that of the untransformed cells or the immortalized clones in this system.

A contrasting result is seen in 3 independently derived cloned cell lines transformed by H-ras plus the oncogene v-myc, as seen in Fig. 5. In cell line 2.10, the D_0 of the transformed line is increased to 176 cGy. Similar results are also seen in two additional H-ras plus v-myc transformed lines (see Table 1, Group 3). This alteration in slope also seems to be accompanied by an alteration in the shoulder region of the radiation survival curve.
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Table 1 Comparison of the radiobiological parameters of all the cell lines used in this study

$D_0$ values are given in cGy. To reduce observer bias $D_0$ was calculated by a least squares regression of all the data points for each cell line for doses above 300 cGy regardless of the apparent location of the shoulder. Each experiment consisted of at least six 10-cm tissue culture plates of at least two cell dilutions for each dose point. Eight dose points (0, 100, 200, 300, 400, 600, 800, 1000 cGy) were examined for each survival curve and all plates were counted. Each curve has been repeated at least twice as noted. The cell lines have been arranged in three groups: Group 1, untransformed cell lines, i.e., the primary REC and the two immortalized lines (RC2 and mycREC); Group 2, three independently derived transformed lines containing H-ras without a second oncogene (3R, 4R, 5R). Group 3, 3 independent cell lines transformed with H-ras in cotransfection with the v-myc oncogene (2.8, 2.10, 3.7). The standard error for each set of data is shown.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell line</th>
<th>% of plating efficiency</th>
<th>$D_0$ (mean ± SE)</th>
<th>No. of experiments</th>
<th>Oncogene</th>
<th>Mean $D_0$ of group ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>REC</td>
<td>24</td>
<td>108 ± 9</td>
<td>2.97</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>RC2</td>
<td>56-92</td>
<td>90 ± 5</td>
<td>5.53</td>
<td>None</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>mycREC</td>
<td>43-86</td>
<td>108 ± 13</td>
<td>4.47</td>
<td>myc</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>3R</td>
<td>47-72</td>
<td>148 ± 12</td>
<td>3.26</td>
<td>H-ras</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4R</td>
<td>16-42</td>
<td>137 ± 8</td>
<td>3.63</td>
<td>H-ras</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5R</td>
<td>47-82</td>
<td>127 ± 7</td>
<td>3.19</td>
<td>H-ras</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>2.8</td>
<td>57-88</td>
<td>168 ± 10</td>
<td>2.57</td>
<td>H-ras + myc</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2.10</td>
<td>54-57</td>
<td>176 ± 14</td>
<td>2.91</td>
<td>H-ras + myc</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td>42-48</td>
<td>217 ± 13</td>
<td>1.97</td>
<td>H-ras + myc</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 1. Clonogenic radiation survival curves for primary rat embryo cells and rat embryo cells immortalized with the cloned myc oncogene (mycREC). The exponential portion of the curve is fitted by a least squares regression of all data points above and including 300 cGy. This procedure was used for all the survival curves shown regardless of the apparent location of the shoulder. The shoulder on the survival curve is drawn by interpolation.

Fig. 2. Radiation survival curves for primary rat embryo cells and the cell line mycREC using the transformed survival expression

$-(\ln SF)/D = a + \beta D$

Lines are a least squares fit to the transformed survival expression. Bars, SE.

The smallest effect is seen in line 2.8 which has a smaller change in $D_0$ and a lesser alteration in the shoulder region of the curve. The greatest effect is seen in clone 3.7 which had a $D_0$ of 217. Examining the initial portion of the radiation survival curve for this cell line shows that there has been a decrease in the $a$ component of the radiation survival curve in this line (Fig. 6). The mean $D_0$ of this group of cells is 187 cGy and this is significantly greater than that of the cells which contain ras alone in a Student $t$ test comparing Group 2 versus Group 3 ($P < 0.01$). All three of these lines show increases in $D_0$ which are larger than any seen in the primary parent line or the two independent immortalized lines or any of the 3 clones transformed by H-ras alone. These results are summarized in Table 1 and seem to indicate a synergy between
correlation could be seen between any changes in the radiation effects could account for our results by doing a cell cycle analysis with the phases of the cell cycle and increases markedly in early ras and v-myc in the association with the radiation resistant line 3.7 as described in the legend to Fig. 3.

genes. 3.7 is from a separate transfection experiment. Rat embryo cells are shown been performed for these cell lines; therefore it remains possible radioresistance. However, radiosensitivity may vary even within the resistant part of the cycle, did not correlate with increased survival curves and changes in cell cycle distribution (Table 2).

In particular, the length of the S phase, thought to be the most resistant part of the cycle, did not correlate with increased radioresistance. However, radiosensitivity may vary even within a phase of the cycle, and age response experiments have not been performed for these cell lines; therefore it remains possible that a more subtle cell cycle effect may be present. The cell cycle times for all the cell lines in this study were in the range of 16–18 h. No obvious correlation can be seen between any alteration in radiation resistance and the number or configuration of the chromosomes in the cell. All of the cell lines were diploid or pseudodiploid even after transformation (17, 18).

### DISCUSSION

Our results show that primary rat fibroblasts after transfection by the activated H-ras oncogene are radiation resistant but that the effect seen in the cells transformed by the ras oncogene by itself is small. The $D_0$ of the cells is increased from 108 cGy to 130–150 cGy. However, the oncogene v-myc, which has no effect on the slope of the curve of radiation resistance by itself, has a synergistic effect with ras on radiation resistance. This effect is seen in all 3 of the independently derived clones we have tested. The myc oncogene belongs to a class of oncogenes which have become known as “cooperating oncogenes” because they greatly increase the efficiency of transformation by the ras oncogene. We have elected to call the effect of myc on radiation resistance synergy rather than cooperation to avoid confusion with the term as it applies in transformation assays.

In a study of NIH 3T3 cells transformed with ras, Sklar (12) found that all of the ras genes with the exception of the ras protooncogene were associated with an increase in radiation resistance in the transfected NIH 3T3 cells. In examining this effect, however, others have found variability among subclones of NIH 3T3 cells prior to transfection.6 Todaro and Green (22) in their initial description of the isolation of the 3T3 cell line in 1963 pointed out that the cells became highly karyotypically unstable with continued passage in culture and that “the cells of the long established line are different genetically and metabolically from both the original euploid cells and the early established cells (22).” Other evidence would suggest that this tendency to genetic instability has not diminished with the passage of time (13, 14) and remains one of the caveats that governs the use of this cell line in biological experimentation. This consideration led us to devise a model system in which the effects of H-ras on radiation resistance could be studied on a uniform genetic background. Primary REC have been used for the study of oncogene induced transformation. They have also been used to elucidate the effects of second oncogenes on transformation and on the transformed phenotype after transformation. These cells offer several useful advantages. The cells prior to transformation are diploid and because they have been in culture for less than 4 passages prior to transformation with the oncogene it is unlikely that any preexisting mutation affecting radiation response would be present, nor could genetic heterogeneity preexisting transfection explain any results we might see in transfectants. In this system the activated H-ras oncogene is associated with radiation resistance but the effect of the oncogene by itself is small. However, the oncogene v-myc which has a minimal or no effect on radiation resistance by itself has a synergistic effect on radiation resistance with H-ras. Furthermore, there appear to be differences in the phenotypes of radiation resistance in these two types of transfectants. Thus, radiation resistance in cells transformed by H-ras by itself is characterized by a change in $D_0$, but little or no change within the shoulder region of the radiation survival curve. Radiation resistance in cells transformed by H-ras plus v-myc is characterized by a uniformly greater increase in $D_0$, and there is also

6 A. Dritschilo. personal communication.


43Oncogene None

200 400 600 800 1000 1200

Dose (cGy)

•¿ 3.7

•¿ 2.10

» 2.8

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Table 2 Comparison of cell kinetic measurements for selected cell lines from each class of resistant cells used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>G2+G1</th>
<th>S</th>
<th>G2-M</th>
<th>S + G2-M</th>
<th>Oncogene</th>
</tr>
</thead>
<tbody>
<tr>
<td>REC</td>
<td>45.4</td>
<td>38.1</td>
<td>16.5</td>
<td>54.6</td>
<td>None</td>
</tr>
<tr>
<td>mycREC</td>
<td>53</td>
<td>34</td>
<td>13</td>
<td>47</td>
<td>myc</td>
</tr>
<tr>
<td>3R</td>
<td>65</td>
<td>19.7</td>
<td>15.4</td>
<td>35.1</td>
<td>H-ras</td>
</tr>
<tr>
<td>4R</td>
<td>60.3</td>
<td>23.9</td>
<td>15.8</td>
<td>39.7</td>
<td>H-ras</td>
</tr>
<tr>
<td>2.8</td>
<td>38.6</td>
<td>45.5</td>
<td>15.9</td>
<td>61.4</td>
<td>H-ras + myc</td>
</tr>
<tr>
<td>3.7</td>
<td>57</td>
<td>23</td>
<td>20</td>
<td>43</td>
<td>H-ras + myc</td>
</tr>
</tbody>
</table>

ras and v-myc in the association with the radiation resistant phenotype.

Radiation resistance in mammalian cells is known to vary with the phases of the cell cycle and increases markedly in early S phase. We considered the possibility that these cell cycle effects could account for our results by doing a cell cycle analysis under the conditions used for killing curve analysis. No obvious correlation could be seen between any changes in the radiation survival curves and changes in cell cycle distribution (Table 2). In particular, the length of the S phase, thought to be the most resistant part of the cycle, did not correlate with increased radioresistance. However, radiosensitivity may vary even within a phase of the cycle, and age response experiments have not been performed for these cell lines; therefore it remains possible

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a large effect within the shoulder region of the radiation survival curve. These studies lead to the conclusion that radiation resistance is a phenotype of cells which is associated with H-ras but which appears to be more fully seen in the presence of the v-myc oncogene and that the v-myc oncogene may play an important role in the phenotype of radiation resistance at low doses.

The mechanism that underlies these effects remains to be elucidated. It does not appear to be mediated through any consistent change in cell cycle parameters; however, this has been examined only in exponentially growing cultures. It is possible that a more thorough analysis would reveal differences. For example irradiation is known to induce a prolongation in the transit time through the G2 phase of the cycle. It is possible that a study of this G2 delay would reveal differences among the cell lines. Most radiation killing in mammalian cells is secondary to the creation of peroxides and free radicals in the cell but preliminary studies in our laboratory do not reveal any significant alteration in the levels of radical scavenger species such as glutathione or in levels of glutathione S-transferase.

A central question which remains to be answered is whether the oncogenes induce the phenotypic alteration in the transfected cells which results in the radioresistant phenotype or whether we are, by selecting for transformed cells, selecting for a preexisting phenotype which exists prior to transfection. In the experiments with NIH 3T3 cells it was possible that this was due to genetic heterogeneity among the cells prior to transfection. This cannot be the explanation for our results since we start with primary diploid cells from an inbred strain of rats. It remains possible that the effects which we see are a result of preexisting epigenetic effects or to epigenetic changes induced by oncogene transfection. By immunohistochemical techniques all of the cell lines show characteristics of fibroblasts and all form fibrosarcomas on injection into syngeneic animals. However, it is possible that heterogeneity in the original fibroblast population may have contributed to our results. It seems unlikely that the effects of the oncogenes on radiation resistance are a direct effect of the oncogene gene products themselves. ras p21, the product of the ras gene, is a cell surface glycoprotein of unknown function although since it has GTPase activity it is thought perhaps to be a G protein involved in signal transduction (23); therefore, it is an unlikely candidate to be a direct mediator of radiation resistance. myc codes for a nuclear protein which may be involved in the control of DNA replication (24), and it is possible that its effect on radiation resistance could be mediated through its effects on the control of DNA replication, but it seems to us more likely that its effect is through alteration in the expression of other cellular genes. This effect of myc is of great interest to us because Mitchell (25) has noted a similar effect in the shoulder region of the radiation survival curve in some human small cell lung cancer cell lines which carry amplified copies of the myc oncogene although in these data the effect does not appear to be uniform. The mechanism of action of the myc gene in altering the expression of other cellular genes is currently unknown. Recently we have made an observation which suggests a possible mechanism for this alteration. In 7 independently derived clones of primary rat embryo fibroblasts transformed by H-ras plus the cooperating oncogene v-myc, we find frequent, but nonrandom, karyotypic alterations, and we find what seems to be a highly preferred integration site for the H-ras oncogene in the proximal region of the long (q)

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