Fibroblast Growth Factor-4 Enhanced G₂ Arrest and Cell Survival Following Ionizing Radiation

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ABSTRACT

Fibroblast growth factors (FGFs) bind to cell membrane receptors and activate signal transduction pathways related to cell growth, angiogenesis, and tumorigenesis. FGFs have been shown to be abundantly expressed in some of the human tumors, which are known to be poorly responsive to radiation therapy. Using adrenal cortical carcinoma cells genetically engineered to express FGF-4, we have tested cellular survival following exposure to ionizing radiation.

We report here that FGF-4 enhances cellular capacity to survive ionizing radiation. Furthermore, cell cycle analysis shows a pronounced increase in the duration of G₂ arrest, suggesting perturbation of a cell cycle checkpoint. These findings implicate fibroblast growth factor-mediated signal transduction in cellular resistance of human tumors to radiation therapy.

INTRODUCTION

FGFs are a family of cytokines that bind to cell membrane receptors and activate signal transduction pathways. Cellular responses to FGFs may include the activation of pathways related to growth, differentiation, metastasis, and angiogenesis (1). FGF-4 is a fibroblast growth factor cloned from NIH/3T3 cells transformed with DNA from a Kaposi’s sarcoma (2). Independently, it was also cloned from a human gastric tumor, hence its original designation as K-fgf/hsf (3). Expression of FGF-4 in permissive cells results in oncogenic transformation, characterized by anchorage independent growth in soft agar and tumorigenicity in nude mice (4).

FGF-2 (basic FGF) has been implicated in protection of bovine endothelial cells from the lethal effects of ionizing radiation (5). Furthermore, both FGF-1 (acidic FGF) and FGF-2 expression have been reported in abundant amounts in primary human brain tumors, known for their poor responsiveness to radiation therapy (6, 7). These findings suggest that FGFs may be important determinants of the radiocurability of such tumors.

We decided to determine whether FGFs play a role in the relative resistance of some tumor cells to killing by ionizing radiation. Previous work had shown that a cell line (SW13) derived from a human cortical carcinoma is sensitive to FGFs at picomolar concentrations (4). In the absence of growth factors, SW13 cells do not form colonies in soft agar and are nontumorigenic in nude mice. In the presence of FGFs, both soft agar colonies and tumorigenicity in mice are observed. Using FGF-4 transfected cells, we studied the radiation survival, cell cycle distribution, and DNA repair kinetics. We observed a radiation resistant response by cells expressing FGF-4 with a change in the shape of the survival curve. Cell cycle analysis in the postirradiation interval revealed a pronounced G₂ arrest, suggesting perturbation of a cell cycle checkpoint.

MATERIALS AND METHODS

Cell Lines and Culture. SW13 adrenal carcinoma cells were purchased from the American Type Culture Collection. KS-6 and KS-1 are clonally derived cell lines from SW13 transformed with pCNCB8 mammalian expression vector containing the full length FGF-4 cDNA (4). SWED-1 is transformed SW13 cells with the vector alone. Cells were maintained in Iscove’s minimal essential medium supplemented with 5% fetal bovine serum, 1% l-glutamine, and 500 units/ml penicillin-500 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Rnase Protection Assay. Thirty µg total RNA from each cell line were hybridized with 32P-labeled riboprobes for glyceraldehyde 3-phosphate dehydrogenase (104-base pair protected fragment) and FGF-4 (312-base pair protected fragment) as described previously (8). Single stranded RNA was digested and the samples were subjected to denaturing polyacrylamide gel electrophoresis (6% polyacrylamide, 7 µm urea) followed by autoradiography.

Radiation Survival Curves. Logarithmically growing cells were harvested and seeded into tissue culture flasks at various cell numbers depending upon the radiation dose which the flask was to receive (i.e., more cells for higher doses), so that the final number of survivors in each flask would be similar. After 6 h of incubation, the flasks were irradiated to various doses using a clinical cobalt-60 machine (Theratron 80) and returned to the incubator for 2 weeks. The flasks were stained to reveal colonies produced from clones of surviving cells and then counted. The fraction of survivors relative to the original number of cells seeded was calculated and then normalized to the zero dose plating efficiency to determine the surviving fraction at each dose. The data were fitted to the single-hit multitarget curve model (9).

Strand Break Repair Assays. Repair of radiation-induced DNA single strand breaks was measured using the alkali-unwinding technique as described previously (10). DNA single strand breaks result in a reduction of the amount of double stranded DNA recovered after a mild alkali treatment of the cells. Repair is measured as an increased recovery of double stranded DNA. Cells were irradiated with 15 Gy on ice. Following irradiation, the cells were transferred to a 37°C water bath and held for various intervals before assaying for strand breakage.

Repair of radiation-induced DNA double strand breaks was measured using pulse field gel electrophoresis as described by Ahn et al. (11). DNA double strand breaks result in a reduction in the amount of large molecular weight DNA that is retained in the wells during electrophoresis. Repair is measured as an increase in the amount of DNA retained in the well at intervals following irradiation. Cells were exposed to 200 cGy, or sham irradiated. The cells were then held at 37°C for intervals between assay.

Potentially lethal radiation damage repair assay was performed as follows. Logarithmically growing cells were harvested and seeded into 25-cm² tissue culture flasks at 5 x 10⁶ cells/flask. The flasks were incubated without any medium changes, until they were in 3 to 4 days of plateau phase growth, as determined by daily cell counts of a parallel cohort of flasks. Cells were either irradiated with 6 Gy or sham-irradiated and returned to the incubator. At various intervals, 3 irradiated and 3 unirradiated flasks were removed from the incubator, trypsinized, and seeded onto 60-cm² tissue culture flasks at 3 x 10⁶ cells/flask. The survival fraction at zero incubation time.

Cell Cycle Analysis. Cells were harvested in logarithmic monolayer growth and resuspended into 25-cm² tissue culture flasks, at 3 x 10⁶ cells/flask. The flasks were incubated at 37°C for 24 h and then either irradiated to 10 Gy or sham-irradiated. Following irradiation, the cells were returned to the incubator. At various intervals postirradiation, pairs of irradiated and unirradiated
flasks were harvested. Cell nuclei were prepared for flow cytometric analysis using the procedure of Vindelov et al. (12). The nuclei samples were analyzed on a Becton-Dickinson FACStar™ instrument and the percentage of nuclei with G2/M DNA content was determined by computer analysis of DNA histograms with Modfit software (Verity Software House, Inc.).

RESULTS AND DISCUSSION

SW13 cells were previously transformed with the pCNCEB8 mammalian expression vector containing the full-length FGF-4 cDNA (4). Two clonally derived cell lines (KS-1 and KS-6) constitutively expressing abundant amounts of FGF-4 were established and confirmed to be tumorigenic in nude mice. As controls, SW13 cells were transfected with vector alone, using the pCNCEB8 and the pCNOT constructs (13). Fig. 1 shows FGF-4 expression as determined by the RNase protection assay. Both, KS-1 and KS-6 cells express high levels of FGF-4 while the control showed no measurable expression.

To examine the radiosensitivity, cells were exposed to graded doses of ionizing radiation and clonogenic survivals were determined (14). The data were plotted on semilogarithmic coordinates (Fig. 2). Such curves demonstrate an initial gradually curving (shoulder) region and a subsequent exponential killing (terminal slope) region. KS-6 cells, expressing FGF-4, show an enhanced survival due to a pronounced broadening of the shoulder region of the radiation survival curve, as compared to controls. Since these results were also seen with KS-1 and another control line (data not shown), it is unlikely that these observations are merely the result of clonal heterogeneity (15).

We next considered possible mechanisms for this resistance to radiation killing. Cells exposed to ionizing radiation may experience “mitotic” or “apoptotic” death. Analyses of levels of DNA damage and its repair provide radiobiological evidence for prevailing mechanisms of cell killing and possible insight into enhanced survival. The shoulder of the radiation survival curve has been attributed to cellular capacity to accumulate DNA damage. Single strand breaks are a common index for overall DNA damage and repair quantification. Double strand breaks in DNA have been correlated to the lethal effects of ionizing radiation (16). A general test used in our laboratory for assessment of DNA strand breaks and repair is the alkaline unwinding assay (10). We investigated the relative levels of DNA single and double strand breaks in KS-6 and control cells. Both FGF-4 expressing cells and controls showed the same initial levels of DNA single strand breaks and similar rates of rejoicing (Fig. 3A). Subsequent analysis of double strand breaks in these cells also showed no differences in accumulation or rejoicing of these lesions (Fig. 3B).

An empirical test for repair capacity, previously invoked to explain the enhanced recovery from radiation killing of bovine endothelial cells following exposure to FGF-2, is the assay for potentially lethal damage repair (5). This test is performed by exposing cells to radiation and then comparing survival fractions of cells plated immediately for colony formation to those held under growth inhibitory conditions prior to plating (17). Fig. 3C shows that there is a suggestion of an increase in potentially lethal damage repair of FGF-4 expressing cells, but the difference was not statistically significant from controls.

A FGF preparation extracted from brain tissue has been reported to inhibit apoptosis in serum-depleted endothelial cells (18). Furthermore, a possible mechanism for the enhanced radiation survival of bovine endothelial cells in the presence of FGF-2 may be the result of protection from apoptosis (19). Neither the SW13 cells nor the KS-6 cells showed evidence of apoptosis as determined by the lack of DNA laddering on agarose gel electrophoresis at intervals up to 24 h following radiation exposure (data not shown).

Following exposure to ionizing radiation, most eukaryotic cells exhibit delays in the G1 to S and in the G2 to M cell cycle transitions. These checkpoints are postulated to be important for maintaining the fidelity of the genomes of cells that are progressing through the cell cycle (20). Delay in the G1 to S transition has been proposed to require
participation of the wild-type p53 protein (21). However, the parental SW-13 cells used in the transfections are known to contain a mutant p53 (22). Immunostaining of parental cells confirmed elevated levels of mutant p53 in SW13 cells (data not shown). Failure to fully utilize this checkpoint, however, has been postulated to relate more to genomic instability than to radiation lethality (23).

There are several lines of evidence supporting the hypothesis that the extent of G2 arrest may be mechanistically related to the radiation sensitivity of cells. In yeast, mutants deficient in G2 arrest show a more pronounced sensitivity to killing by ionizing radiation (20). In the human genetic mutant syndrome of ataxia telangiectasia, the exquisite radiation sensitivity of cultured cells has been attributed to failure to undergo G2 arrest (24). Finally, the enhanced radiation resistance of rodent cells transfected with the myc and the ras onco-

genes has been related to an enhanced G2 arrest (25).

We addressed cell cycle arrest in FGF-4 expressing cells using flow cytometry. Table 1 shows the cell cycle distributions of asynchronous unirradiated cells. There are no evident differences in G1, S, or G2/M to explain the observed radiation responses. We then followed the distributions of cells following exposure to ionizing radiation. Preliminary experiments indicated that all 4 cell lines underwent a similar rate of G2 accumulation at intervals to 24 h after irradiation; however, the extent and duration of G2 arrest appeared greater in FGF-4 expressing cells (data not shown). We selected 10-Gy exposure as the optimal dose for demonstrating G2 arrest; however, qualitatively similar results were observed at lower doses (data not shown). Additional experiments defined the exact shape of the G2 arrest curve at intervals from 12 to 72 h. Cells were exposed to 10 Gy of ionizing radiation, and fractions in G2 and M phases of the cell cycle were determined at the indicated time points (Fig. 4). FGF-4 expressing cells demonstrate a pronounced enhancement of G2 arrest that differs from control cells in magnitude and duration. We interpret the differences observed in the G2/M fractions between FGF-4 expressing cells and controls as allowing an increased time for the FGF-4 expressing cells to recover from DNA damage prior to entry into mitosis, thereby resulting in enhanced clonogenic survival. These data extend the concept of radioprotective G2 arrest to the shoulder region of the radiation survival curve. Whereas increased radioprotection has been attributed to enhanced G2 arrest and reduced terminal slope of the radiation survival curve (26), our data show that the shoulder is primarily affected, suggesting that mechanisms not fully understood are at play.

The clinical implications of a change in cellular response to radiation, such as presented here, must be underscored. Since clinical radiation therapy is delivered in small daily fractions (200 cGy or

![Table 1 Cell cycle distributions of asynchronous unirradiated cells](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS-1</td>
<td>45.0 ± 3.0</td>
<td>34.7 ± 3.7</td>
<td>20.0 ± 2.1</td>
</tr>
<tr>
<td>SWEB</td>
<td>52.0 ± 11.1</td>
<td>32.5 ± 9.5</td>
<td>15.7 ± 5.6</td>
</tr>
<tr>
<td>KS-6</td>
<td>44.0 ± 2.9</td>
<td>39.5 ± 2.8</td>
<td>17.3 ± 1.2</td>
</tr>
<tr>
<td>SWNT</td>
<td>55.0 ± 2.9</td>
<td>33.8 ± 4.19</td>
<td>11.8 ± 2.0</td>
</tr>
</tbody>
</table>

- Mean ± SD.
less), the enhancement of the shoulder region of the survival curve may result in an incurable tumor by conventional radiation therapy fractionation schedules. The family of FGFs exhibits considerable cross-reactivity in binding to FGF receptors. Therefore, these experiments using FGF-4 in the model SW13 cell system offer insight into mechanisms that may be relevant to other FGFs, particularly FGF-1 and FGF-2. Expression of both of these factors has been reported in human brain tumors, particularly glioblastomas. These tumors are essentially incurable by conventional, fractionated radiation therapy.

Further knowledge of mechanisms of FGF modulation of cellular radiation response may offer novel targets for improvements in therapeutic intervention.

REFERENCES

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