[CANCER RESEARCH 60, 6597–6600, December 1, 2000]

Advances in Brief

Direct Evidence for the Contribution of Activated N-*ras* and K-*ras* Oncogenes to Increased Intrinsic Radiation Resistance in Human Tumor Cell Lines[1]

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Abstract

Transformation with *ras* oncogenes results in increased radiation survival in many but not all cells. In addition, prenyltransferase inhibitors, which inhibit ras proteins by blocking posttranslational modification, radiosensitize cells with oncogenic *ras*. These findings suggest that oncogenic *ras* contributes to intrinsic radiation resistance. However, because introduction of *ras* oncogenes does not increase radiation survival in all cells and because prenyltransferase inhibitors target molecules other than ras, these studies left the conclusion that ras increases the intrinsic radiation resistance of tumor cells in doubt. Here we show that genetic inactivation of K- or N-*ras* oncogenes in human tumor cells (DLD-1 and HT1080, respectively) leads to increased radiosensitivity. Reintroduction of the activated N-*ras* gene into the HT1080 line, having lost its mutant allele, resulted in increased radiation resistance. This study lends further support to the hypothesis that expression of activated ras can contribute to intrinsic radiation resistance in human tumor cells and extends this finding to the K- and N- members of the ras family. These findings support the development of strategies that target ras for inactivation in the treatment of cancer.

Introduction

Past studies have shown that numerous oncogenes, when present in activated form or when overexpressed, can increase radiation survival in experimental cell systems. Radiation resistance was increased in 3T3 cells after transfection with a wide variety of oncogenes including *ras*, *raf*, and *sis* (1–6). Expression of activated oncogenes similarly increased radiation resistance in rodent lymphoid cells (5, 7).

Oncogenic H-ras contributes to increased radiation resistance in rat embryo cells (8–11) and rhabdomyosarcoma cells (12). Further studies in rat embryo cells showed that the increased survival was accompanied by a decrease in apoptosis and a prolonged G_2-M arrest after irradiation (13–15). Treatment with a farnesyltransferase inhibitor that blocks H-ras prenylation and thus inhibits ras signaling reversed ras-mediated radiation resistance and caused increased radiation-induced apoptosis in these cells (16). The reduction of radioresistance and the increase in apoptosis were specific for cells expressing activated H-*ras* oncogenes, and no effect was detected in untransformed rat embryo cells or in cells immortalized with v-*myc*. However, farnesylation of other proteins and thus their function could be affected by farnesyltransferase inhibitor treatment.

In human cells, assessing the contribution of *ras* activation to radiation resistance is more complex, primarily because transformation of human cells with oncogenes is more difficult and requires p53 inactivation and telomerase activation (17). Despite this, several studies have shown elevated resistance to γ-irradiation in human cells transfected with oncogenic H-*ras*. Su and Little (18) showed consistent increases in radiation resistance in cells transfected with SV40T, and further increases were noted in clones isolated from two of three fibroblast lines transfected sequentially with oncogenic H-*ras* and SV40T. The effect was greatest in the cell line that was initially the most radiosensitive (*Do,* 1.17 Gy). The third line, which had a higher initial radiation resistance (*Do,* 1.46 Gy) showed a small increase after transfection with SV40T but no additional effect of oncogenic H-*ras* transfection. The interpretation of these studies is complicated by the report that transformation with SV40 can lead to overexpression of c-*myc*, K-*ras*, and c-*raf* genes, all of which have been implicated in radiation resistance (19). In contrast to this report, Grant *et al.* (20) reported a lack of correlation between *ras* transfection and radioresistance in human retinoblasts. In this study, however, three of nine *ras* + adenovirus *E1a* transfectants showed radioresistance that was elevated (*Do,* 1.55–1.66) compared with six *E1a*-transfected lines (*Do,* 0.88–1.35). It was also noted that of the three radioresistant *ras* + *E1a* transformants, two were transfectants expressing the highest levels of ras protein. Mendonca *et al.* (21) obtained similar results in the human HaCaT keratinocyte line, where modest elevation of resistance was seen in two of three lines expressing high levels of H-ras. More recently, increased survival at doses of radiation above six Gy were observed in human bronchial epithelium cells transfected with H-*ras*, although no difference was observed at lower doses (22). Although these reports were interpreted as negative for a role for activated ras in radiation resistance, in each case some increase in resistance was noted after *ras* transfection. In the one report where ras appeared to be completely without effect in increasing radiation resistance (23), the parental mammary epithelial cell line tested had an initial *Do* of 2.19 Gy, thus demonstrating a high level of radioresistance prior to transfection.

Studies inhibiting oncogenic ras expression or function have shown that radiation resistance in human cells can be lowered as a consequence. Studies in which inhibition of ras processing was accomplished using pharmacological inhibitors such as lovastatin or prenyltransferase inhibitors demonstrated reduced radiation survival in cells expressing activated ras (24, 25). Similar findings were obtained using antisense-mediated inhibition of ras expression in cells with activation of the Her-2/Neu receptor, which signals through the ras pathway (26). All of these manipulations could, however, induce nonspecific changes in the treated cells that might alter radioresistance.

To better define the role of activated ras in the intrinsic radiation resistance of human tumors, we have now examined a panel of human cells derived from the HT1080 and DLD-1 human tumor lines for clonogenic survival after irradiation. The parental lines contain one activated allele of N- or K-ras, respectively (27). The radiation survival of the parental lines was compared with the survival of cell lines

Received 5/1/00; accepted 10/17/00.

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[1] These studies were supported by NIH Grant CA75138 (to W. G. M., R. J. M., and E. J. B.) Grant CA73820 (to E. J. B.), and Grant CA69515 (to E. J. S.). Farnesyltransferase inhibitors were provided by Merck and Co., Inc.

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in which the activated allele of ras was lost, but wild-type ras expression was maintained. In this way, the contribution of an activated *ras* oncogene to radiation resistance in human cells was addressed directly.

Materials and Methods

Cell Lines. Cells were cultured at 37°C in a water-saturated 5% CO_2 incubator. HT1080 cells having lost the activated N-*ras* allele were obtained as described previously (SG-2 published as MCH603c8; Ref. 27). In the DLD-1 cell-derived lines, the activated allele K-*ras* allele was disrupted by targeted knockout, yielding a line expressing only the normal allele as described by Shirasawa *et al.* (28). Cells were maintained in DMEM high glucose medium (Life Technologies, Inc.). Media were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were negative for *Mycoplasma*.

Radiation Survival Determination. Clonogenic survival was determined at radiation doses from 1 to 8 Gy. Cells from logarithmically growing cultures were plated, allowed to attach, and then irradiated with a Mark I cesium irradiator (J. L. Shepherd, San Fernando, CA) at a dose rate of 1.6 Gy/min. Colonies were stained and counted 14–21 days after irradiation. The surviving fraction at a given dose is defined as: number of colonies formed/(number of cells plated) $\times$(plating efficiency). Each point on the survival curves represents the mean surviving fraction from at least three dishes.

Apoptosis Detection. Twenty-four and 48 h after irradiation with 10 Gy, both adherent and nonadherent cells were harvested from irradiated and sham-irradiated cultures. Cells were stained with propidium iodide in an NP-40 buffer as described previously (16). Cell counts were performed within 5 min of staining. A minimum of three independent fields of 100 cells was counted for each sample.

Growth Determination and Flow Cytometry. Log phase growth cultures were plated at 2×10^5 cells/dish in 60-mm dishes. Replicate dishes were harvested for cell counts and flow cytometric analysis of DNA content (29). Flow cytometry analysis was carried out using the ModfitLT version 2.0 program. Where indicated, cells were treated with 2.5 μM farnesyltransferase inhibitor L779–575 (Merck & Co., Inc.).

Results

We examined three cell lines derived from the human HT1080 fibrosarcoma that differ in activated N-ras expression for clonogenic survival after irradiation. The parent line (SG-1) expresses one activated N-*ras* allele ($Gln^{61} \rightarrow Lys^{61}$). This allele was lost in the SG-2 line and reintroduced by transfection in SG-6 cells. As shown in Fig. 1, the SG-1 cell line showed significantly greater survival than SG-2 cells. Survival of SG-6 cells was also significantly greater than that of SG-2 cells, although somewhat lower than the survival of the parent line. These results demonstrate increased radiation clonogenic survival in the lines expressing activated N-ras, either as a result of a naturally occurring mutation in one endogenous N-*ras* allele or as a result of reintroducing the activated N-*ras* allele by transfection after it had been lost.

Radiation-induced apoptosis is reduced in ras plus myc-transformed rat embryo fibroblasts relative to rat embryo cells immortalized with myc alone. To determine whether apoptosis was influenced by ras expression in the human HT1080 cells, it was measured was scored 24 and 48 h after irradiation with 10 Gy (Fig. 2). Apoptosis in unirradiated cultures from all three lines was $<4\%$. The mean number of apoptotic cells rose to 7% in both the SG-2 and SG-6 cells at 24 h. SG-2 cells showed a further increase to 15% at 48 h, whereas SG-6 cells retained a 6% apoptotic cell fraction. SG-1 cells, in contrast, showed no increase in apoptotic fraction, remaining $<5\%$ throughout the course of the experiment. Thus, the extent of apoptosis after irradiation in cultures of these cells was reduced in a manner that was in accordance with the clonogenic survival of the cultures. Cells expressing activated N-ras demonstrated slightly reduced radiation-induced apoptosis, but this reduction was small relative to the difference in clonogenic survival between HT1080 cells expressing mutant ras and those cells having lost expression of this oncogene.

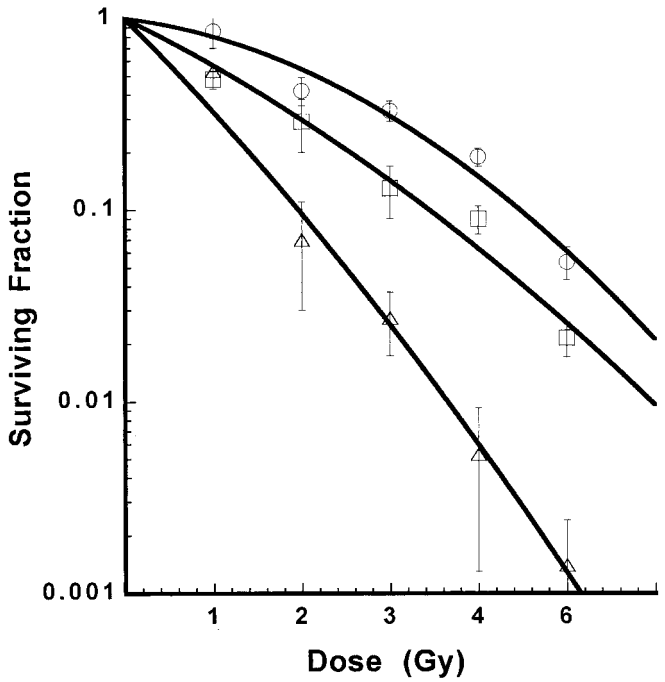

Fig. 1. Clonogenic survival of HT1080 cells is reduced after loss of activated N-ras. Cells in log phase growth were plated and irradiated for clonogenic survival at the doses indicated. After 14–21 days, plates were stained and scored for colony formation. SG-1 (○), HT1080 cells expressing both activated and wild-type endogenous N-*ras* alleles. SG-2 (Δ), HT1080 expressing only the wild-type endogenous N-*ras* allele. SG-6 (□), HT1080 expressing the wild-type endogenous N-*ras* allele and an activated N-ras introduced by transfection. *Bars*, SD.

Radiation survival was also assessed in cells derived from the human DLD-1 colorectal adenocarcinoma tumor line that expresses one activated K-*ras* allele and one wild-type K-*ras* allele. The parental SG-3 line expresses an activated K-*ras* allele, whereas SG-4 and SG-5 cell lines express only wild-type *ras* alleles because of homologous recombination knockout of the mutant allele. As seen in Fig. 3, The SG-3 line showed greater clonogenic survival than either the SG-4 or SG-5 cells after irradiation. Radiation-induced apoptosis in the DLD-1-derived lines never exceeded 5% in either SG-3, SG-4, or SG-5 cells after irradiation (not shown). Thus, apoptosis does not appear to determine the differences in clonogenic survival obtained in either the DLD-1 or HT1080 cell lines because the levels of apoptosis are too low to account for these differences.

HT1080 cells expressing mutant N-*ras* alleles proliferate faster than those lacking mutant *ras* (27). Although SG-1 cell cultures grew more rapidly than SG-2 cultures (Fig. 4), the cell cycle distribution of SG-1 and SG-2 cells in log phase was equivalent (Table 1). SG-3 and SG-4 cultures showed comparable growth rates (Fig. 3). The cell cycle distribution of these cells was also similar (Table 1). Thus, the overall cell cycle distribution and the proportion of cells in S-phase in particular did not significantly differ between either DLD-1 or HT1080 cells expressing mutant ras and those expressing only wt ras. Furthermore, SG-1 cells treated with farnesyltransferase inhibitor showed no reduction in growth rate after treatment for up to 72 h, whereas treatment of these cells with farnesyltransferase inhibitor for as little as 24 h reduces survival at two Gy of radiation (SF_2) from 0.71 to 0.31 (not shown). Taken together, these data argue against a role for differences in cell cycle distribution contributing to the observed differences in radiosensitivity in this experimental system.

Discussion

We have demonstrated that loss of an activated *ras* allele is sufficient to significantly reduce radiation survival in two human cell lines. The approach used in the current report differs from previous studies in that the survival of the cell lines was established from cells lacking an endogenous, activated oncogene rather than from cells into which an activated oncogene was introduced. This allowed the determination of the contribution of activated ras to radiation survival without the significant perturbations induced in cells secondary to transfection with activated oncogenes. Furthermore, inhibitors and introduction of exogenous DNA in the form of antisense constructs were not used, thus avoiding the nonspecific effects of these manipulations. The cells were thus minimally and equally perturbed prior to, during, and after the time of irradiation.

In this report, we have shown that both N- and K-*ras* activation can contribute to radiation resistance in human tumor cells. H-*ras* has been the focus of most prior studies in *ras* oncogene-mediated radiation resistance. N-*ras* was the first *ras* oncogene demonstrated to influence radiation survival in rodent cells by FitzGerald *et al.* (1) in 1985; however, its contribution to radiation resistance in human cells has never been reported previously. Similarly, few studies have examined the contribution of K-ras to radiation resistance. We showed previously that prenyltransferase inhibition can reduce the clonogenic survival of human tumor lines expressing activated K-ras (24). Inhibiting K-ras prenylation required inhibiting both farnesyl- and geranylgeranyltransferase enzymes. Because over 200 proteins are prenylated by these enzymes, the combined effects of these inhibitors could potentially affect many other essential proteins in addition to ras, thus complicating the interpretation of the observed radiosensitization. The demonstration that loss of an activated K-*ras* allele results in reduced radiation survival thus confirms and strengthens the earlier observation obtained with inhibitors of ras function.

Finally, we have shown that increased radiation-induced apoptosis does not fully account for the results obtained by clonogenic survival in these cells. An association between the extent of apoptosis and clonogenic survival results was seen in cells derived from HT1080 but not DLD-1. As has been pointed out, apoptosis can neither predict nor substitute for the results of long-term assays, such as clonogenic survival assays, for measuring radiosensitivity (30).

Demonstrating that intrinsic radiosensitivity is reduced after loss of N- and K-*ras* in human cells adds to the findings obtained with H-*ras* in the radioresistance of human cells. Because the most prevalent *ras* mutations in human solid tumors are in K-*ras*, the current results strengthen the argument that strategies targeting ras activity or expression may have clinical relevance for the treatment of radiation resistant tumors expressing ras mutations.

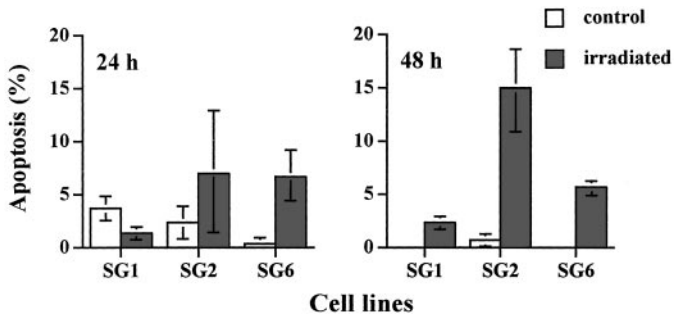

Fig. 2. Radiation-induced apoptosis after loss of activated N-ras expression. Cells in log phase were irradiated with 10 Gy and scored for apoptotic changes in nuclear morphology at 24 and 48 h after irradiation.

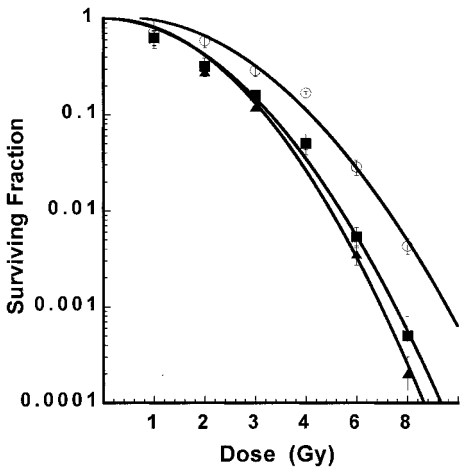

Fig. 3. Clonogenic survival of DLD-1 cells is reduced after loss of activated K-ras. Cells in log phase growth were plated and irradiated for clonogenic survival at the doses indicated. Three weeks after irradiation, plates were stained and scored for colony formation. SG-3 (○), DLD-1 cells expressing both activated and wild-type endogenous K-*ras* alleles. SG-4 (▲) and SG-5 (■), DLD-1 cells expressing only the wild-type endogenous K-*ras* allele. *Bars*, SD.

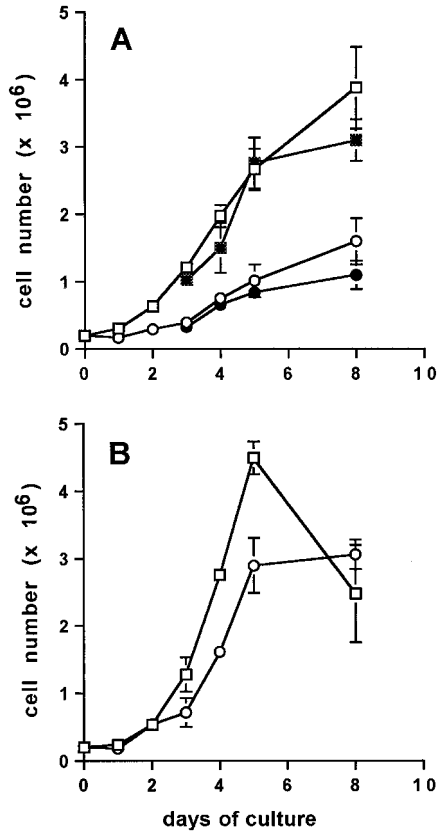

Fig. 4. Cell proliferation in cultures of HT1080 and DLD1. Cells were plated at 2×10^5 cells/dish on day 0 and counted on the days indicated. *A*, SG-1 (□) and SG-2 (○) were treated with farnesyltransferase inhibitor beginning on day 2 of culture (■, ●). *B*, SG-3 (□) and SG-4 (○). Data represent the means of three replicate counts. *Bars*, SD.

Table 1 *Cell cycle distribution of log phase cultures*

Cell cycle distribution was determined from flow profiles obtained on day 5 of culture (Fig. 3) by analysis of DNA content from replicate dishes of the cells used for growth curves.

Cell line	G_0-G_1	S	G_2-M
SG-1	46.1	28.3	25.6
SG-2	50.0	31.4	18.6
SG-3	47.2	28.7	24.1
SG-4	51.7	27.7	20.6

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Cancer Res 2000;60:6597-6600.