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Evidence Supporting a Signal Transduction Pathway Leading to the Radiation-Resistant Phenotype in Human Tumor Cells

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A signal transduction pathway, involving oncogenes and their normal counterparts the proto-oncogenes, analogous to that for cell growth and differentiation has been proposed to lead to the phenotype of cellular radioresistance (RR). In this report we provide evidence demonstrating the existence of such a pathway by using antisense oligonucleotides (ASO) to reverse the RR phenotype. Utilizing ASO directed against the *raf*-1 gene, a central component of this proposed pathway, we were able to reverse the RR phenotype of human tumor cell lines having elevated HER-2 expression or a mutant form of Ha-*ras,* two genes upstream of *raf*-1 in signal transduction. Additionally, anti-*ras* ASO were able to radiosensitize HER-2 overexpressing cells. These results, which verify the presence of a signaling pathway leading to cellular RR, also have possible clinical implications for the use of ASO as a means to sensitize radioresistant tumors to radiation therapy.

The failure of a significant number of tumors to respond to radiation therapy is a serious problem in the treatment of cancer. A better understanding of the mechanisms leading to this cellular radiation resistance will result in the development of more effective treatment modalities. We have previously reported that the radiation resistant (RR) phenotype appears to be linked to the activation of specific protooncogenes in a signal transduction pathway analogous to that described for cell growth and differentiation (1). Disruption of the pathway therefore should lead to reversal of this phenotype and increased sensitivity to radiation therapy. A specific strategy to interfere with the signaling is to modulate the expression of specific genes in the pathway at the mRNA level using antisense oligonucleotides (ASO). Short antisense DNA oligonucleotides selectively bind to cellular mRNA targets through complementary sequence-specific Watson-Crick base pairing. The hydrogen-bonded antisense molecule can modulate the expression of the targeted gene product (2). We and others have demonstrated the ability of antisense oligonucleotides and their modified analogues to specifically inhibit *ras* p21 protein synthesis in *in vitro* translation, in cell culture, and in tumorigenesis in nude mice (3-9). Additionally, ASO against genes such as c-*myb,* c-*myc,* c-*fos,* BCR-ABL and the IGF receptor, have also been shown to suppress human tumor cell growth *in vitro* and in some cases are currently in clinical trials as anti-cancer therapeutics (10-12).

The serine/threonine kinase Raf-1 protein appears to be a central component of multiple signal transduction pathways in the cell (Reviewed in 13-14) including that for RR. Consequently, the use of ASO against *raf*-1 itself, or against upstream effectors of *raf*-1, to impeded signaling through this gene should result in increased radiosensitivity. Here, therefore, we employ ASO targeted against specific protooncogenes in the proposed signal transduction pathway leading to the RR phenotype in an effort to further define the early steps in this pathway.

The results presented here demonstrate that treatment with specific antisense oligonucleotides is able to revert the RR phenotype of head and neck, ovarian, and bladder carcinoma cell lines confirming our hypothesis of a signal transduction pathway leading to radioresistance, and suggest that the use of ASO in the clinical setting may be an effective means to not only to inhibit tumor cell growth, but also to increase the effectiveness of current radiotherapeutic modalities.

MATERIALS AND METHODS

Oligonucleotides. Phosphorothioated ASO directed at or near the initiation codon were synthesized by Midland Certified Reagent Co.,

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Midland, TX for the *raf*-1 (5′-TCCCTGTATGTGCTCCAT-3′), Ha-*ras* (5′-TATTCCGTCAT-3′), and HER-2 (5′-TCCATGGTGCTCACT-3′) genes.

Two controls for each gene, either a sense and a scrambled (for *raf*-1) or two different scrambled (for Ha-*ras* and HER-2) oligonucleotides were also synthesized. The scrambled oligomers have the same base composition as antisense but in a different, random order. The sequences for the *raf*-1 controls are 5′-ATGGAGCACATACAGGGA-3′ (sense) and 5′-CTAGCCTATCTGTCTTCG-3′ (scrambled); for Ha-*ras* 5′-TTATACGTCCT-3′ (scrambled 1) and 5′-TTATACGTCCT-3′ (scrambled 2); and for HER-2 5′-CACTGGTTGCACCTT-3′ (scrambled 1) and 5′-CTAGCCATGCTTGTC-3′ (scrambled 2).

Cell culture and treatment. Squamous cell carcinoma of the head and neck (SCCHN) cell lines JSQ-3 (15), SQ-20B (16) and SCC-61 (16), which were kind gifts from Dr. Ralph Weichselbaum, University of Chicago, were maintained in Minimum Essential Medium with Earle's salts (EMEM), supplemented with 10% heat inactivated fetal bovine serum; 50 μg/ml each of penicillin, streptomycin and neomycin; 2 mM L-glutamine; 0.1 mM non-essential amino acids, 1 mM pyruvate and 0.4 μg/ml hydrocortisone. Human ovarian (SK-OV-3) and bladder (T24) carcinoma cell lines (obtained from ATCC, Rockville, MD) were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum, 50 μg/ml each of penicillin, streptomycin and neomycin and 2 mM L-glutamine. Normal human non-tumor breast cell line MCF 10A (ATCC, Rockville, MD) was maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 5% horse serum, 20 ng/ml epithelial growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, 50 μg/ml each of penicillin, streptomycin and neomycin and 2 mM L-glutamine.

For oligonucleotide treatment, the cells were plated at 1×10^5 cells/well in 6-well tissue culture plates. Twenty-four hours later, at approximately 40-60% confluency, the cells were transfected with the oligonucleotides, facilitated by Lipofectin Reagent, using essentially the protocol supplied by the manufacturer, Life Technologies, Inc. After 6 hours, the lipofection solution was removed and the monolayer washed with fresh medium containing 8 mM L-glutamine and 20% serum. The cells were then incubated for an additional 16-18 hours in 1 ml of this medium.

Radiobiology. Cellular response to radiation was evaluated by the colony survival assay. Exponentially growing monolayer cultures of each cell line were treated with the oligonucleotides as described above. The cells were harvested 24-48 hours later, suspended in fresh medium and irradiated at room temperature with graded doses of ^{137}Cs γ rays at a dose of approximately 36 Gy/minute in a J.L. Shepard and Associates Mark I irradiator. Afterward, the cells were diluted and plated at a concentration of 300 to 5000 cells per well in a 6-well tissue culture plate. Two to three days after plating, the cells were supplemented with 0.5 ml of serum plus 5 μg/ml hydrocortisone. Approximately 7-14 days later, the cells were stained with 1% crystal violet and colonies (comprising 50 or more cells of normal appearance) were scored. Survival curves were plotted as the log of the survival fraction versus the radiation dose using Sigma-Plot Graphics program. D_{10} (the dose required to reduce survival to 10%) values were calculated from the initial survival data.

Protein analysis. After oligonucleotide treatment, cells for protein analysis were trypsinized, pelleted, rinsed with PBS and lysed in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 30 μg/ml aprotinin and 1 mM sodium orthovanidate in PBS) (Santa Cruz Biotechnology, Inc). After shearing with a 26 gauge needle, 100 μg/ml Phenylmethylsulfonyl fluoride (PMSF) was added, the lysate incubated on ice for 30-60 minutes and centrifuged at 13,000 $\times$ g for 20 minutes at 4°C to pellet insoluble material. Protein concentration was determined using the micro-BCA Protein Assay Kit (Pierce Biochemicals).

Protein lysate (40 μg for Ha-*ras*, 5 μg for *raf*-1 and HER-2) was mixed with an equal volume of 2$\times$ protein sample buffer (0.05 M Tris (pH 6.8), 3% SDS, 20% Glycerol, 6% 2-Mercaptoethanol and 0.001% Bromophenol blue) boiled for 5 minutes, loaded on a 12% (5% stacking gel) SDS/Polyacrylamide gel and electrophoresed at 200V for 8 hours. The protein was transferred to nitrocellulose membrane as previously described (17). Preparation of membrane and incubation with the primary and secondary antibodies was performed essentially as described in a protocol supplied by Santa Cruz Biotechnology, Inc., with the exception that incubation with the primary antibody was extended to 1 hour for *raf*-1, 2 hours for HER-2 and 4 hours for Ha-*ras* and wash times of 15 minutes per wash were used. The primary antibodies for HER-2 (*neu* C-18) and Ha-*ras* (*ras* C-20) were obtained from Santa Cruz Biotechnology, Inc. The anti-*raf*-1 antibody was a kind gift from Dr. Andrew Laudano, University of Vermont (18). The washings after addition of the secondary antibody (Anti-mouse IgG-HRP, Santa Cruz Biotechnology, Inc.) were also lengthened to 15 minutes per wash.

Visualization of the protein was accomplished using the ECL Chemo-luminescent Western Blotting Kit (Amersham) according to the manufacturer's protocol.

RESULTS

Human tumor cell lines JSQ-3 and SQ-20B, which display a high level of radiation resistance, were established from SCCHN tumors which failed radiotherapy (15, 16). An activated form of the *raf*-1 oncogene was isolated from these cell lines via the NIH 3T3 transfection assay (19). These and other studies with radioresistant non-cancerous skin fibroblast cell lines from a cancer-prone family (20, 21) have clearly linked activation of *raf*-1 to increased RR. We, therefore, wished to determine if treatment of these cell lines with anti-*raf*-1 ASO would inhibit *raf*-1 expression and revert this phenotype. Consequently, JSQ-3 and SQ-20B were treated with increasing concentrations of anti-*raf*-1 oligonucleotides and the level of the *raf*-1 protein expression determined. Since we have previously shown that lipofectin enhanced uptake of these compounds resulting in lower effective doses (manuscript in preparation), a commercially available liposome preparation (Lipofectin) was used in these and all subsequent experiments to facilitate delivery of the oligonucleotides. As shown in Figure 1A, Raf-1 protein expression in JSQ-3 cells is completely inhibited by treatment with 1 μM of antisense *raf*-1, with significant inhibition evident at a concentration as low as 0.1 μM. The specificity of the inhibition was demonstrated by treating the cells with Lipofectin alone (0) or with a *raf*-1 sense oligonucleotide (S). No decrease in protein expression as compared to the untreated control cells (C) was observed in either case. A similar pattern of results was observed with cell line SQ-20B. However, with this cell line, a 3 μM concentration was necessary to effect complete inhibition of *raf*-1 protein, with only approximately 50% inhibition observed at 1 μM. Also shown in Figure 1A is the effect of *raf*-1 ASO on a radiosensitive SCCHN cell line, SCC61. Here also treatment with *raf*-1 ASO was able to specifically inhibit *raf*-1 protein expression.

We next examined the effect of anti-*raf*-1 on the RR level of these cells. Figure 1B demonstrates a dramatic

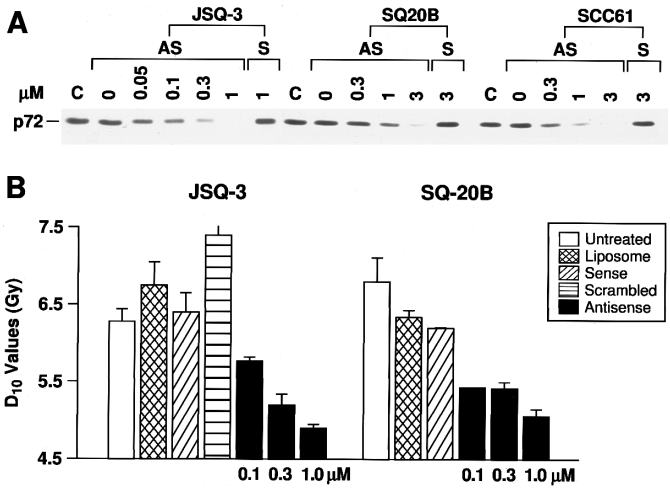

FIG. 1. The effect of anti-*raf*-1 oligonucleotides on Raf-1 p 72 protein synthesis and the radiation resistance levels of SCCHN cell lines JSQ-3 and SQ-20B. A - Western blot analysis of Raf-1 protein synthesis inhibition by increasing concentrations of *raf*-1 oligonucleotides. C = untreated cells; 0 = cells treated with liposomes but no oligonucleotides; AS = antisense; S = sense. B - Histogram demonstrating radiosensitization with increasing concentrations (0.1, 0.3, 1 μM) of anti-*raf*-1 ASO. As controls, the cells were treated with 1 μM of either a sense or a scrambled *raf*-1 oligonucleotide. Radioresistance levels are given as D_{10} values. Error bars represent the standard error of the mean (S.E.M.) of 2 to 13 values.

increase in radiosensitivity for both cell lines after ASO treatment. This response, particularly in the JSQ-3 cells, is dose dependent. The D_{10} value for JSQ-3 drops from the highly resistant level of 6.3 $\pm$ 0.16 Gy to 4.9 $\pm$ 0.05 Gy, a value much closer to the level considered to be radiosensitive, after treatment with 1 μM *raf*-1 ASO. Even a dose as low as 0.3 μM is capable of significantly sensitizing these cells to killing by γ-radiation. Similarly, the resistance level of SQ-20B is reduced from 6.8 $\pm$ 0.31 Gy to 5.1 $\pm$ 0.09 Gy. This change of approximately 1.5 Gy was found to be highly statistically significant ($p < 0.001$). Here also, the specificity of the oligonucleotide is evident since treatment with either Lipofectin (Liposome) alone, a sense, or a scrambled oligomer had minimal or no effect on the RR level of the cells. Moreover, the differences between JSQ-3 and SQ-20B with respect to their level of sensitization after ASO treatment correlates with that observed in the protein analysis, indicating that this decrease in radioresistance is directly related to Raf-1 expression. An example of the survival curves produced in these experiments is given as Figure 2A.

By contrast, treatment of SCC61 cells, which are highly radiosensitive, with 1 μM of *raf*-1 ASO had no significant effect on their radiation response level. The D_{10} value of the control and sense treated cells was found to be 3.3 $\pm$ 0.4 Gy and 3.4 $\pm$ 0.06 Gy, respectively, while that of the ASO treated was 3.0 $\pm$ 0.4 Gy indicating a slight, but not significant, sensitization of the cells by the *raf*-1 ASO.

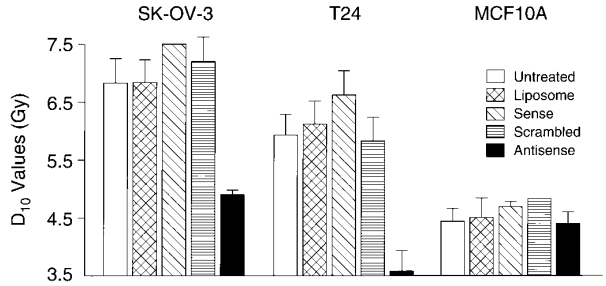

FIG. 2. Histogram demonstrating the effect of 1 μM anti-*raf*-1 ASO on SK-OV-3, T24 and MCF10A cells. As controls, the cells were treated with 1 μM of either a sense or a scrambled *raf*-1 oligonucleotide. Radioresistance levels are given as D_{10} values. Error bars represent the S.E.M. of 2-6 values.

Our previous studies have placed *raf*-1 in a central role in a proposed signal transduction pathway leading to cellular RR. If this hypothesis is correct then treatment with *raf*-1 ASO of cells which have activated or abnormally expressed genes upstream of *raf*-1 in this pathway should block signaling leading to decreased radioresistance. Therefore, we transfected human tumor cell lines SK-OV-3 and T24 with *raf*-1 ASO. These two cell lines possess either elevated levels of HER-2 (22) or a mutated Ha-*ras* genes (23), respectively. Both of these genes have been placed upstream of *raf*-1 in the signal transduction pathway (14, 24). The presence of 1 μM antisense *raf*-1 was able to significantly reduce the RR level of both cell lines from 6.83 $\pm$ 0.42 Gy to 4.90 $\pm$ 0.08 Gy for SK-OV-3 and from 5.93 $\pm$ 0.36 Gy to 3.58 $\pm$ 0.36 Gy for T24 (Figure 3). As before, treatment with either the sense or scrambled oligo did not decrease on the RR level of the cells. This sensitization is also evidenced by the differences in the survival curves between control SK-OV-3 cells, and those treated with 1 μM *raf*-1 antisense oligonucleotides (Fig-

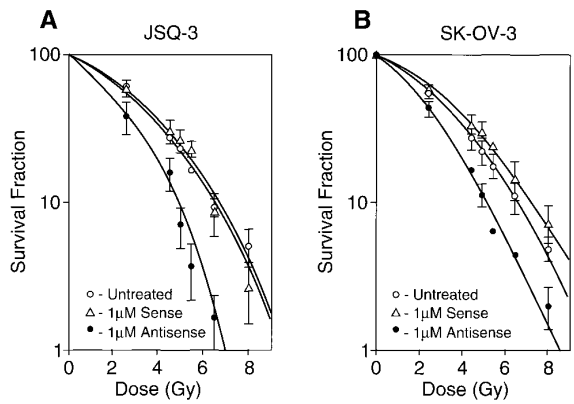

FIG. 3. Survival curves, after graded doses of γ-radiation, for A - JSQ-3 and B - SK-OV-3 cells untreated or treated with either 1 μM *raf*-1 antisense or sense oligonucleotides. Curves are plotted as the log of the surviving fraction vs. radiation dose in Gy. Points are plotted as the S.E.M. of 2-13 values.

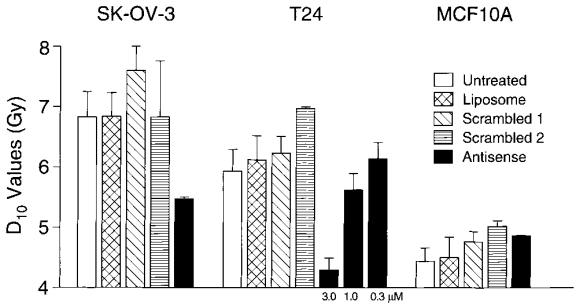

FIG. 4. Histogram demonstrating radiosensitization by Ha-*ras* ASO. The concentration of antisense *ras* oligonucleotide used to treat SK-OV-3 and MCF10A was 3 μM. As controls, the cells were treated with 3 μM of two different scrambled Ha-*ras* oligonucleotides. Radioresistance levels are given as D_{10} values. Error bars represent the S.E.M. of 2-7 values.

ure 2B). As a control, a normal radiosensitive breast epithelial cell line, MCF10A, was also used. As with SCC61, no effect on the D_{10} values was observed with this cell line, again indicating no further sensitization of the cells at this concentration of ASO.

To further confirm the role of these activated oncogenes in signal transduction and the RR phenotype, SK-OV-3, T24 and MCF10A cells were also treated with ASO against the Ha-*ras* gene. Since, as a growth factor receptor, HER-2 is upstream of *ras* in the proposed signalling pathway, it would be expected that the Ha-*ras* ASO would affect both the HER-2 expressing cells (SK-OV-3), and the cells containing mutant Ha-*ras* (T24). Figure 4 shows this to be the case. D_{10} for the 3μM ASO treated T24 cells is decreased from the control value of 5.93 $\pm$ 0.36 Gy to the significantly more radiosensitive value of 4.29 $\pm$ 0.20 Gy, while that for SK-OV-3 is lowered from 6.83 $\pm$ 0.42 Gy (Control) to 5.47 $\pm$ 0.03 Gy after introduction of the anti-*ras* molecule. As before, there was no significant decrease in radiation survival in the control MCF10A cells after ASO treatment.

These same three cell lines were also treated with antisense oligonucleotides directed against HER-2. Over 80% inhibition of HER-2 protein was observed in the SK-OV-3 cells with HER-2 ASO at a concentration as low as 0.3 μM (Figure 5A). However, significant HER-2 protein inhibition in the T24 cells is found only at 3 μM and none is evident in MCF10A, even at this relatively high concentration of ASO. The effect of HER-2 ASO on the RR level of these cells was also examined (Figure 5B). While treatment with 0.1 and 0.3 μM HER-2 ASO had some effect on the radiosensitivity of the SK-OV-3 cells, treatment with 1 μM HER-2 ASO significantly sensitized the SK-OV-3 cells, reducing the D_{10} value from 6.83 $\pm$ 0.42 Gy to 4.88 $\pm$ 0.43 Gy, a result virtually identical to that observed after treatment of SK-OV-3 cells with 1 μM anti-*raf*-1 ASO. This change of approximately 2 Gy is highly statistically significant ($p < 0.001$) and represents a 5 fold increase in sensitivity to radiation killing these cells. Surprisingly, the radiosensitivity of the T24 cells was also altered by treatment with 1 μM HER-2 ASO.

DISCUSSION

In our previous studies we examined the relationship between activation of oncogenes and the phenomenon of cellular radiation resistance (1, 21). We proposed, based upon our findings and those of other researchers, the presence of a signal transduction pathway, analogous to that for cell growth and differentiation, leading to radiation resistance to killing by ionizing radiation (1). In the studies described above, we present evidence confirming such a pathway. Activation of the *raf*-1 gene has been shown to be related to radiation resistance in SCCHN and in the non-cancerous skin fibroblasts from a cancer-prone family with Li-Fraumeni syndrome (19-21, 25). Raf-1 is also known to play a central part in signal transduction via the MAP Kinase pathway (13, 14). In this Ras/Raf/MEK/ERK pathway, a small guanine nucleotide-binding protein links receptor tyrosine kinase activation to a cytosolic protein kinase cascade

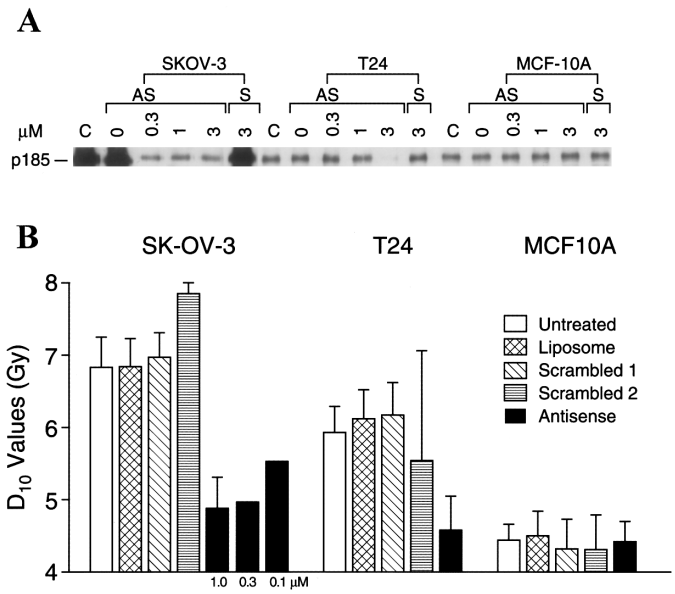

FIG. 5. The effect of anti-HER-2 oligonucleotides on p185 HER-2 protein synthesis and the radiation resistance levels of SK-OV-3, T24 and MCF10A cells. A - Western blot analysis of HER-2 protein synthesis inhibition by increasing concentrations (0.3, 1.0 & 3.0 μM) of HER-2 oligonucleotides. C = untreated cells; 0 = cells treated with liposomes but no oligonucleotides; AS = antisense; S = scrambled. B - Histogram demonstrating radiosensitization by HER-2 ASO. The concentration of antisense HER-2 oligonucleotide used to treat T24 and MCF10A was 1 μM. As controls, the cells were treated with 1 μM of two different scrambled HER-2 oligonucleotides. Radioresistance levels are given as D_{10} values. Error bars represent the S.E.M. of 2-7 values.

(26). The protein-protein interaction between Ras and Raf, through the CRI region on Raf-1 and the effector site of Ras, leads to a partial activation of Raf-1. Full activation of Raf-1 is achieved by another tyrosine kinase generated signal (26, 27) and leads to the phosphorylation and activation of MEK, its only known physiological substrate. This in turn results in the activation of ERK1 and/or ERK2. The substrates for the ERKs in the nucleus are transcription factors, activation of which can set in motion a wide range of events. Raf-1 has also been shown to be a key component in the mammalian response to damage by ultraviolet light (28, 29). This "UV response" has been proposed to have a protective function, in a manner analogous to that of the bacterial "SOS" system. It was shown by Devary et al. that this pathway originates at the cell membrane and includes activation of Src, and Ha-Ras as well as Raf-1 in a signaling cascade leading to activation of transcription factor AP-1 and nuclear factor kappa B (28).

Protooncogenes and their oncogenic counterparts such as HER-2 (a homologue to an epidermal growth factor receptor) and *ras* are known to be upstream of *raf*-1 in the Map Kinase pathway (14, 24). The ability, as demonstrated here, of antisense oligonucleotides directed against *raf*-1 to revert the RR phenotype of cells containing activated *ras* or overexpressing HER-2 is clear evidence of signaling through *raf*-1 leading to RR. This is further supported by the ability of antisense *ras* oligomers to sensitize HER-2 overexpressing SK-OV-3 cells to γ-radiation killing. Although HER-2 is upstream of *ras* in the signal transduction pathway, ASO directed against HER-2 was also able to affect the RR level of *ras* transformed T24 cells. These findings may be explained in part by the established interaction between the EGF receptor and adapter protein/guanine nucleotide exchange factor (Grb2/Sos). Buday and Downward have shown that EGF-induced activation of nucleotide exchange on $p21^{ras}$ proceeds through recruitment of Sos to a complex with the EGF receptor and Grb2 at the plasma membrane and that inhibition of this Grb2-EGFR interaction can inhibit activation of *ras* (30). Therefore, it is conceivable that inhibition of the HER-2 protein by ASO can disrupt this interaction, and thus $p21^{ras}$ nucleotide exchange, and interfere with signaling through *ras* resulting in decreased RR.

Further support for the existence of the pathway leading to RR is found in the work of Morrison, et al. (31) and Haimovitz-Friedman, et al. (32). These investigators found that bFGF, synthesis of which is stimulated in epithelial cells by γ-irradiation (32) and which in turn activates Raf-1 protein kinase (31), can protect against radiation-induced cell killing (32). Our hypothesis of a signal transduction pathway is further confirmed in a recent report by Kasid *et al.* which showed that Raf-1 is phosphorylated/activated after exposure to ionizing radiation by upstream protein tyrosine kinases (33).

These studies, supporting a pathway, with *raf*-1 as a central element, leading to cellular radioresistance are also clinically significant in a number of ways. Radiation is one of the major forms of adjuvant therapy for various types of cancer. Understanding the molecular mechanisms leading to the failure of a significant fraction of tumors to respond to radiotherapy opens the door to the development of new methods of intervention to radiosensitize tumors, resulting in more effective cancer treatments. In this vein, our use of antisense oligonucleotides to radiosensitize human tumor cells not only establishes the signal transduction pathway, but also demonstrates the potential of these molecules as cancer therapeutic agents showing that ASO directed against a focal point in the pathway can be effective in a number of different tumor types. In a similar way, using mouse m5S cells, Taki *et al.* (34) also recently found that ASO against RAD51, a gene involved in recombination and DNA repair, could increase radiosensitivity.

The use of liposome facilitated delivery of the ASO permits significantly lower effective concentrations of oligomers to be used, a step towards eliminating one of the major drawbacks to the clinical use of antisense therapy. The efficacy of ASO is also advantageous for clinical use. At the concentrations employed in these studies, none of the ASOs increased the sensitivity of control radiosensitive cell lines SCC61 and MCF10A, thereby demonstrating that the use of ASO to ameliorate radioresistance is not deleterious to normal tissues thereby strengthening the potential usefulness of ASO in cancer treatment.

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