Thioredoxin Reductase Plays a Critical Role in IFN Retinoic-mediated Tumor-Growth Control in Vivo

Daniel J. Lindner, Xinrong Ma, Jiadi Hu, Sreenivasu Karra, and Dhananjaya V. Kalvakolanu

Greenebaum Cancer Center, Department of Microbiology and Immunology, Molecular and Cellular Biology Program, University of Maryland School of Medicine, Baltimore, Maryland 21201 [X. M., J. H., S. K., D. V. K.], and Taussig Cancer Center, The Cleveland Clinic Foundation, Cleveland, Ohio 44195 [D. J. L.]

ABSTRACT

The IFN and retinoic acid (RA) combination suppresses cell growth by inducing apoptosis in the cultured tumor cells. Using a genetic technique, we have isolated several “genes associated with retinoid-IFN-induced mortality” (GRIM) that participate in this death pathway. One such gene, GRIM-12, encodes the redox enzyme thioredoxin reductase (TR). Antisense-mediated inhibition of TR abrogates cell death. To test the in vivo relevance of TR for growth suppression, we have conducted the following study. A wild-type TR or a catalytically defective mutant were expressed in MCF-7 breast carcinoma cells and transplanted into athymic nude mice. These mice were treated with IFN-β and all-trans RA combination. Tumors expressing the vector or wild-type TR were readily suppressed by the IFN/RA combination. In contrast, the tumors bearing a mutant TR were resistant to regression. We further show that markers of apoptosis are stimulated in the regressing tumors. These studies show a prominent role for TR in tumor-growth suppression.

INTRODUCTION

The IFN family of cytokines regulates a number of physiological responses including antiviral, antitumor and immune defenses (1–3). IFNs have proven valuable in the therapy of chronic myelogenous leukemia, Kaposi’s sarcoma, lymphomas, hairy cell leukemia, multiple myeloma, gliomas, ovarian cancers, renal cell carcinoma, melanoma and multiple sclerosis (3–6). A number of genes that mediate the pleiotropic actions of IFNs are induced primarily through the Janus kinase-STAT pathway. Indeed, an abrogation of this signaling pathway promotes cell growth. For example, the IFN-γ receptor−/− and STAT1−/− mice (7) are highly susceptible to chemical carcinogenesis. Tumor cells lacking either of these genes are not rejected by the immune system (7), which indicates that IFN-γ governs the surveillance of neoplastic cells in vivo. IFNs also inhibit cell growth by interfering with cell cycle progression. For example, they down-regulate c-myc gene expression (8), inactivate transcription factor E2F (9), and induce/dephosphorylate retinoblastoma protein in lymphoid tumor cells (10, 11). Some IFN-inducible transcription factors of the murine p200 family, such as p202, also suppress cell growth by antagonizing the c-myc and E2F-inducible gene expression (12, 13). Although the expression of some IFN-stimulated genes has been correlated to growth control, the in vivo relevance of these genes to tumor growth control by IFNs has not been clearly elucidated. Although IFNs as single agents impart significant therapeutic benefit to patients with several types of tumors, they have limited effects on many solid tumors (4, 14). Thus, the combination of IFNs with other agents provides an avenue to enhance their growth-suppressive effects.

Retinoids, a group of vitamin A-related compounds, have a profound influence on cell growth and differentiation (15). Prototypic retinoids like all-trans RA bind to nuclear RAR and retinoid X receptor complexes and stimulate the transcription of genes (16) that possess the RA response elements. The retinoid-stimulated gene expression is a more complex process that involves multiple RAR and retinoid X receptor types and subtypes (16) and their differential expression in various tissues. The RARβ gene expression is lost in some cancers (17), which indicates its role in growth suppression. Some studies show that RARexerts antioncogenic effect by inhibiting the myb oncoprotein (18). Others reported that RA inhibits transcription factor E2F by converting it into an inhibitor (19). Because RARs are transcription factors, the antitumor actions are obligatorily dependent on the downstream gene products, whose identity is unknown. Clinically, retinoids are effective in the therapy or prevention of primary cancers of skin, head and neck, and cervix (20, 21). The growth of some neuroblastomas, promyelocytic leukemias, and teratocarcinomas is inhibited by RA in vitro (15). Retinoids are minimally active as single agents in the therapy of advanced lung cancer, squamous cell carcinomas of skin and cervix, and recurrent bladder carcinoma.

Clinical and experimental models have shown that the

Received 2/5/02; revised 5/9/02; accepted 6/4/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by National Cancer Institute Grants CA 71401 and CA 78282 (to D. V. K.).
2 D. J. L. and X. M. contributed equally to this study.
3 To whom requests for reprints should be addressed, at Greenebaum Cancer Center, Department of Microbiology and Immunology, Molecular and Cellular Biology Program, University of Maryland School of Medicine, Baltimore, MD 21201. Phone: (410) 328-1396; Fax: (410) 328-1397; E-mail: dkalvako@umaryland.edu.
IFN/RA combination is a more potent inhibitor of cell growth (22–31) and tumor-induced angiogenesis (32) than either agent alone. RA induces some ISGs and STAT1 levels directly (33) leading to an enhancement of IFN responses in certain IFN-resistant cells (34, 35). The combination of 13-cis-RA and IFN-α2a showed significantly high overall response rates in advanced squamous cell carcinomas of the skin and cervix and small cell lung carcinomas (27–30). However, the mechanisms responsible for this strong growth-suppressive action of IFN/RA are unclear.

We have initiated a study to identify the mechanisms of IFN/RA-induced tumor growth suppression. To this end, we have used a genetic technique that identifies genes based on their function and have isolated several “genes associated with retinoid-IFN-induced mortality” (GRIM). One such gene, GRIM-12 (TR), modulates IFN/RA induced apoptosis in several tumor cell lines (36). To examine the in vivo relevance of this gene for tumor-growth suppression, we have used the mouse xenograft model described earlier (26). In this study, we show that a functional TR is necessary for mediating the tumor-growth suppression by IFN-β/RA and for up-regulating apoptotic markers in vivo.

MATERIALS AND METHODS

Reagents. Enhanced chemiluminescence reagents and horseradish peroxidase coupled to antirabbit or antimouse antibodies (Amersham Pharmacia Inc.), and human IFN-β (Ares Serono), mouse monoclonal antibodies against actin (Sigma Inc., St. Louis, MO), myc-epitope (Zymed Inc.); and rabbit polyclonal antibodies against caspase-8 (Santa Cruz Biotechnologies, Inc.) were used in these studies. All-trans RA (Sigma-Aldrich Inc.), freshly prepared in ethanol, was used for in vitro studies.

Cell Culture. MCF-7 cells were cultured in phenol red-free EMEM supplemented with 5% charcoal stripped fetal bovine serum and 10−11 m estradiol during treatment with IFN-β and all-trans RA. Cells were grown in phenol red free-media 24 h before treatments were initiated in the tissue culture studies. Cells were examined for Mycoplasma contamination prior to use in animal studies. For implantation, cells were harvested with trypsin-EDTA, washed twice with PBS at pH 7.4. Cell viability was determined by microscopic examination after 0.1% trypan blue staining prior to transplanting in mice. Viable cells (4 × 10⁶) were inoculated into mice in a volume of 0.1 ml of PBS.

Expression of TR Mutants. All of the gene products in this study were expressed using the mammalian expression vector pCXN2-myc, which contains a myc epitope tag in its multiple cloning site (37). The chicken actin promoter and the rabbit β-globin polyadenylation signals permit a consistent and sustained expression of cloned cDNA. A neomycin-resistance gene in this plasmid allows the selection of stably transfected cells. Wild-type and mutant TR inserts were cloned into this vector (37). A mutant TR lacking the active site cysteine residues was generated as published elsewhere (37). Referred to as Cys-mut throughout this study, site-directed mutagenesis resulted in the replacement of the “Cys-Val-Asn-Val-Gly-Cys” sequence with “Gly-Ala” in this construct. The wild-type and Cys-mut constructs were transfected into MCF-7 cells, and stable transfectants were selected using G418.

Expression of the wild-type or Cys-mut did not alter the expression of endogenous TR (38). The Cys-mut acted as a dominant-negative inhibitor and strongly suppressed the TR enzymatic activity and cell death, compared with the cells transfected with the expression vector alone (37, 38). Wild-type TR promoted the enzymatic activity and hyperstimulated cell death in response to IFN/RA, compared with the vector. To avoid a clonal bias, all of these experiments used pools of ~200 individual colonies transfected with the recombinant plasmids. Endogenous TR was detected by using TR-specific antibodies as described earlier (36).

Death Assays. Cell death was monitored using annexin-V-binding assays. After treatment with the indicated agents, cells were stained using a commercially available kit (Trevigen Inc.) per manufacturer’s recommendation. FITC-positive cells (apoptotic) were quantified using flow cytometry (FACScan, Becton-Dickinson).

Enzymatic Assay. TR activity was determined as described previously (39). Twenty μg of tumor extract were incubated with insulin, NADPH and thioredoxin in 0.2 m HEPES (pH7.6) for 20 min at 37°C. Reactions were terminated after the addition of 6 m guanidinium hydrochloride/0.4 mg/ml dithio-bis(2-nitrobenzoic acid) prepared in 0.2 m Tris pH 8.0. Absorbance at 412 nm was measured. In each case a corresponding control without thioredoxin was used to determine the basal level of TR activity (caused by endogenous Trx and NADPH). Absorbance values obtained from these controls were subtracted from those obtained with the reactions that contained Trx and NADPH. A negative control reaction without cell extracts but with all of the reaction components was also used. Triplicate samples were measured for enzymatic activity.

Caspase-8 Assay. Caspase-8 activity was determined using a commercially available kit as recommended by the manufacturer (Biosource International Inc.). Briefly, tumor cell lysates were prepared by homogenization, and a comparable quantity of lysate (~25 μg) from each sample was incubated with a synthetic substrate, IETD-pNA at 37°C for 3 h. The release of chromophore, pNA, from these substrates was quantified by monitoring the absorbance at 405 nm in a microplate reader.

Western Blot Analyses. Equal quantities of cell extracts were separated on 10–12% SDS-PAGE and were Western blotted onto nylon membranes. Specific primary antibodies were incubated with the blots after blocking as described previously (Ref. 36). These blots were washed and incubated with an appropriate secondary antibody tagged with horseradish peroxidase. Protein bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Inc.).

Mice. Three-to-4-week-old female athymic nude (nu/nu) BALB/c mice (Taconic) were housed at a density of four mice in each cage with microisolator tops and autoclaved bedding. They were fed autoclaved Purina Lab Rodent Chow 5010 (Memonone Falls, WI) and HCl-acidified (pH 3) tap water ad libitum and were placed in rooms with controlled temperature (22°C–24.5°C) and humidity (40%) and 12-h light-dark cycles. Mice were housed in the animal facility for at least a week prior to use in the study.
Animal Procedures. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies.5

Tumors. Mice were inoculated with the vector (pCXN2myc), TR, or Cys-mut expressing MCF-7 cell lines. Each experimental group contained 10 mice, each bearing one tumor. Tumor cells (4 × 10⁶) were inoculated into flanks in the mid-axillary line. Tumors were allowed to grow to a volume of ~300 ± 50 mm³ in some experiments when required. Combination treatment for 12 weeks was begun with human IFN-β (Ares Serono) 10³ units/day s.c. and RA (Sigma) 0.3 mg/day oral gavage. Tumor volume was calculated using caliper measurements and the formula for a prolate spheroid: volume = (4/3) × πa²b, where 2a = minor axis, 2b = major axis of prolate spheroid. Each experimental group contained 10 mice, and each experiment was performed twice. Student’s two-tailed t test was used to assess the statistical significance of difference between pairs of means of tumor volume.

Treatment. Human IFN-β (Ares Serono), specific activity 3.2 × 10⁸ IU/mg protein, was diluted in normal saline to a concentration of 10⁶ IU/ml. Mice received 10⁵ IU IFN-β in a single dose once a day (0.1 ml/day s.c.). All-trans RA (Sigma) was suspended in 2% (w/v) hydroxy propyl cellulose vehicle at 3 mg/ml. Mice received 0.1 ml (0.3 mg) by gavage daily. Because MCF-7 tumors do not grow without estradiol, all of the athymic oophorectomized nude (nu/nu) mice inoculated with MCF-7 tumors received s.c. estradiol capsule implants 7 days prior to tumor inoculation. Estradiol (17β; Sigma) was mixed in 2-cm long, 0.125-inch outer diameter silastic tubing and the ends sealed with silicone adhesive. Estradiol-containing capsules were sterilized by γ-irradiation. After anesthesia with 0.3 ml of avertin i.p., the capsules were inserted into the dorsal s.c. region through a 4-mm incision, which was closed with a stainless steel clip. Depleted estradiol capsules were removed and replaced with fresh ones after 6 weeks. Xenograft experiments used two different dose schedules, early and delayed. Mice in the early schedule received IFN/RA beginning 2 days after tumor cell inoculation. In the delayed schedule, tumors were allowed to grow without IFN or RA treatment for 6 weeks, at which time, treatment was started.

RESULTS

Expression of Transfected Mutants and Their Relative Sensitivity to IFN/RA-induced Cell Death in Vitro. Before transplanting the MCF tumor cell lines expressing the TR and its mutant products into mice, cell lines were examined for two characteristics: (a) expression of the transfected gene product and (b) death sensitivity as measured by annexin-V staining. For the former, equal amounts of protein extract from the transfec-

ies. Because the TR and its catalytically inactive Cys-mut are expressed as myc-epitope-tagged proteins, they can be detected readily with this antibody. Both the mutant and the wild-type TR can be detected in the respective transfectants and not in the vector-transfected cells (Fig. 1A). Wild-type TR was expressed at levels that were 3- to 4-fold lower than the Cys-mut. This indicates that high-level TR expression is incompatible with cell growth. Indeed, TR-expressing cells grow slower than the control vector transfectants (37). In an earlier study, we have shown that expression of the transfected genes had no effect on levels of endogenous TR protein (38). Furthermore, wild-type TR-expressing cells had a significantly higher enzymatic activity compared with transfection with vector alone. In contrast Cys-mut expression caused a strong suppression of TR activity in the cells (37).

Our previous studies (26) have shown that, at equivalent doses, IFN-β was a stronger suppressor of tumor growth than IFN-α was, both in vivo and in vitro. Therefore, throughout this study, IFN-β was used. For analyzing their relative sensitivities, cells were exposed to IFN-β (500 units/ml) and RA (1 µM) combination for 72 h, stained with FITC-annexin-V and subject to flow cytometry to detect apoptotic cells. Although a significantly higher percentage of wild-type TR-expressing cells became annexin-V positive after IFN/RA treatment, fewer of the Cys-mut-expressing cells were found to be annexin-V positive compared with the vector transfectants. In the basal state, TR-expressing cells were slightly more annexin-V positive (2-fold) compared with control (Fig. 1B).
Effect of IFN/RA Combination on Tumor Xenografts.

To determine the in vivo relevance of TR to IFN/RA therapy, MCF-7 tumor cells expressing TR and the Cys-mut were transplanted into oophorectomized athymic female nude mice (26). We have chosen MCF-7 for this study because many of our earlier observations were made with this cell line and it is quite sensitive to IFN/RA-induced growth suppression. Because our previous observations (26) have also shown that IFN-\(\beta\)/H9252 is a more potent inhibitor of tumor growth than IFN-\(\beta\)/H9251 in vitro and in vivo, we have used the IFN-\(\beta\)/RA combination for this study. However, IFN-\(\beta\) also exhibited similar effects on tumor growth. Tumor cells characterized in Fig. 1 were transplanted into mice and their growth was monitored in the absence (Fig. 2A) and presence (Fig. 2B) of IFN/RA. Human IFN-\(\beta\) was administered to the mice s.c. at a site distal to the tumor, and RA was given via oral gavage. As shown in Fig. 2A, the vector- and Cys-mut-expressing cell grew at comparable rates in the absence of any treatment. In contrast, the TR-expressing cells grew slower than vector-expressing cells. At the end of 12 weeks, the TR-expressing tumors grew to a \(\approx 2.5\)-fold smaller volume than the vector-expressing cells. These data are consistent with our earlier in vitro studies that TR expression slows cell growth significantly.

IFN/RA treatment strongly suppressed the growth of wild-type and vector-expressing tumors. At the end of 12 weeks, there was no detectable tumor growth in either group (Fig. 2B). In contrast, a significantly different profile of tumor growth was observed in the case of Cys-mut-expressing cells. These tumors continued to grow even in the presence of IFN/RA combination. At the end of 12 weeks, the Cys-mut tumors grew to 45% of the volume of untreated tumors. These data suggest that apoptotic pathways are only partially suppressed in the Cys-mut tumors. The significant inhibition of Cys-mut tumor growth by IFN/RA indicates the operation of other TR-independent tumor suppressor pathways. We have also monitored the effect of withdrawal of IFN/RA on the tumor growth in mice transplanted with TR- and vector-expressing tumor cells. After initial therapy with IFN/RA for 12 weeks, mice were left untreated for an additional 3 months (data not shown). Tumors did not reappear after the cessation of therapy. Thus, the transplanted tumors were eliminated, probably because of the induction of apoptosis.

IFN/RA Enhances Tumor Regression in the Presence of Wild-Type TR. In the experiment shown in Fig. 2, IFN/RA therapy was initiated 1 day after tumor implantation. In this case, it is probable that tumors were small enough for IFN/RA to suppress their growth readily. To demonstrate tumor regression, we used mice with established tumors (Fig. 3A). Because TR-expressing tumors grew slowly, they had to be grown for 6 weeks to obtain a comparable size tumor prior to initiating the therapy with IFN/RA. The vector and Cys-mut-expressing tu-

---

**Fig. 2** Effect of IFN-\(\beta\) and RA on TR-expressing MCF-7 human breast tumors. Each mouse was transplanted with \(4 \times 10^6\) MCF-7 cells (one site per mouse) expressing various TR mutants on day 0. All of the mice had estradiol (14 mg) capsule implants. In A, mice were left untreated. In B, mice were treated with RA (0.3 mg/day p.o.) by gavage + 10^5 IU/day IFN-\(\beta\) s.c. Mean tumor volume \(\pm\ SE\) for each group was plotted (n = 10).

**Fig. 3** Effect of IFN-\(\beta\) and RA on established tumors. Each mouse was inoculated with \(4 \times 10^6\) MCF-7 cells (one site per mouse) for weeks prior to the initiation of treatment. For TR-transfected tumors, inoculation was performed 6 weeks before treatment. Established tumors were visible and palpable at the beginning of treatment. In A, mice received IFN-\(\beta\) + RA as in Fig. 2. In B, no treatment was given. Mean tumor volume \(\pm\ SE\) for each group was plotted (n = 10).
mors were allowed to grow 4 weeks prior to initiation of therapy. Once palpable tumors were established, mice received either saline injections or IFN/RA. Tumors comprised of MCF-7 cells expressing pCXN2myc vector alone were arrested by IFN/RA treatment. There was a slight decrease in tumor growth with no further increase in tumor volume during the 12 weeks of treatment. Tumor volume for both pCXN2myc and TR groups at the end of the experiment was unchanged compared with the initial tumor volume ($P > 0.20$ for both groups). The TR-expressing tumors started to regress 3 weeks after therapy and disappeared by 9 weeks. These tumors underwent regression, with tumor volume decreasing from $0.311 \pm 0.006 \text{ cm}^3$ to $0.029 \pm 0.020 \text{ cm}^3$ over 12 weeks ($P < 0.001$). This represents a 91% reduction in tumor volume. In contrast, Cys-mut-expressing tumors were highly resistant to combination drug treatment. Despite daily treatment, tumor volume increased 2.59-fold over 12 weeks. The untreated tumors continued to grow, and the TR-expressing tumors grew somewhat more slowly (Fig. 3B).

**Tumor Regression Is Caused by the Continued Expression of Transfected Gene.** To clearly demonstrate that the tumors retained the expression of transfected genes at the time of therapy, in a parallel experiment (similar to the one shown in Fig. 3), transplanted tumors were harvested 7 weeks after therapy, and the expression of transgenes was monitored by Western blot analysis using myc (for TR and its mutant) and actin (internal control) antibodies. Tumors were homogenized, and equal amounts of cell lysates from them were used in the experiment. A representative blot is shown in Fig. 4. Both the treated and the untreated tumors expressed the wild-type and Cys-mut. The low intensity of the bands in the treated tumor is attributable to the fact that a significant reduction in tumor volume occurred at this stage in the TR-expressing tumors, and very small amounts of lysate could be obtained from these tumors.

Similarly, we have also measured the TR enzymatic activity in the tumor lysates. As shown in Fig. 4C, TR enzymatic activity was high in the vector- and TR-expressing tumors. The latter tumors have higher enzyme activity, as expected. Enzyme activity was significantly diminished in the Cys-mut-expressing tumors. Thus, a dominant inhibition of TR enzymatic activity by Cys-mut directly correlates with tumor resistance to growth suppression. Lastly, we have measured the levels of endogenous TR protein by Western blot analysis of the tumors expressing vector, TR, and the Cys-mut. The endogenous TR protein level was not altered because of the expression of myc-tagged TR and its mutant (Fig. 4D). In the TR- and Cys-mut-expressing tumors, a slow migrating band can be seen, in addition to a fast migrating one. This band is absent in the vector-transfected tumors. This slow migrating species represents the plasmid-derived myc-tagged protein. The fast migrating band is the endogenous TR.

**Tumor Regression Is Caused by Induction of Apoptotic Pathways.** We next examined whether tumor regression was caused by a triggering of apoptotic pathways. Tumor lysates were examined for the activation of caspase-8 by Western blot analysis using caspase-8-specific antibodies (Fig. 5A). There was no detectable activation of caspase-8 in untreated tumors. However, in the case of IFN/RA-treated vector and TR-expressing tumors, activation of caspase-8 could be seen. There was a significantly higher activation of caspase-8 cleavage in the TR-expressing tumors compared with the vector control. No caspase-8 cleavage was detected in Cys-mut tumors.

The caspase-8 activation was further confirmed by measuring its activity. Tumor cell lysates were incubated with a caspase-8-specific synthetic substrate, IETD-pNA, and the release of chromophore-pNA was quantified (Fig. 5B). These assays demonstrated high caspase-8 activity in TR-expressing tumors treated with IFN/RA compared with the vector transfectants. There was no significant activation of caspase-8 in the Cys-mut-expressing tumors after IFN/RA therapy. A marginally higher basal caspase-8 activity was noted in the untreated TR-expressing tumors compared with those expressing vector or Cys-mut.

The MCF-7 tumor cells have an endogenous wild-type p53. We next examined whether the presence of TR activated p53-dependent gene expression. For this purpose, we examined the levels of Bax, a known p53-inducible protein in the tumor cell...
lysates (Fig. 5C). A significantly higher induction of Bax was noted in the treated TR-expressing tumor, compared with the vector transfectants. Bax levels remained unchanged in the Cys-mut-transfected cells.

**DISCUSSION**

Like many cancer therapeutics, IFNs and retinoids both exhibit antitumor effects, which are likely to be a result of apoptosis. Vitamin A deficiency is associated with a greater incidence of a number of carcinomas (15, 40) and abnormal myelopoiesis, which can be suppressed by RA (41). Retinoids also prevent the development of some carcinomas in rats (42). RA strongly inhibits acute promyelocytic leukemias carrying the promyelocytic leukemia-RARα gene translocation. A number of IFN-inducible transcription factors are necessary for the expression of gene products involved in apoptotic responses. For example, deletion of the IRF-1 gene causes myelodysplasia in humans (43); and IRF1<sup>−/−</sup> genotype causes a loss of apoptotic responses and increases the cellular propensity to oncogenic transformation (44). Deletion of ICSBP, a member of the IFN-gene regulatory factor family, causes chronic myelogenous leukemia-like disease in mice (45) and ICSBP suppresses tumor BCR-abl-induced cellular transformation (46). Lastly, STAT1 controls the expression of several caspases and a loss of STAT1 expression results in a loss of apoptosis (47, 48). Some human melanomas (49) and cutaneous lymphomas (50) have an extremely low level of STAT1, resulting in resistance to IFN-induced growth suppression.

We have shown earlier that the IFN/RA combination, but not the individual agents, induce death in a number of tumor cell lines (26). The IFN- and RA-induced responses cross-talk even though they clearly use different signal transduction pathways. For example, the PML-RARα gene is induced by IFNs, and it participates in the anticellular actions of IFN-α (51). The PML gene is directly induced by IFNs through STAT1 (33). PML forms a nuclear body that consists of several IFN-inducible gene products (52, 53). Indeed, IFN can suppress the growth of RA-resistant acute promyelocytic leukemias (53). An RA-inducible gene, RIG-G, is a homologue of ISG-561 (54), which inhibits protein synthesis (55). Similarly, RA can promote IFN response by up-regulating STAT1 in some IFN-resistant cell types (34, 35, 56). Because STAT1 is a transcription factor, its effects are dependent on the downstream products. The gene products that mediate the IFN/RA-induced tumor growth suppression and their mechanisms of action are unclear.

To understand the molecular basis for the activation of cell death, we have used a genetic technique and identified several GRIM that potentially mediate the death regulatory functions of IFN/RA. One such gene, TR1, mediates cell death (36) via an up-regulation of caspase-8-dependent cell death (37). This enzyme modulates the redox status of several intracellular proteins (57). Consistent with our in vitro studies (37), caspase-8 activity and its cleave occurred in the regressing wild-type and TR-expressing tumors but not in the Cys-mut-expressing ones. These tumors also retained the transfectected TR gene expression at the time of tumor regression. This observation directly establishes the dependence of tumor regression on a functional TR. We also observed that TR-expressing tumors underwent complete regression (Fig. 2B), and they did not reappear after cessation of IFN/RA therapy. If there were residual viable tumor cells, the growth stimulus provided by the implanted estradiol capsule would have caused tumor recurrence. Thus, the transplanted tumors were eliminated, probably because of the induction of apoptosis. In mammals, two other isoforms of TR, which are expressed in an organelle- and tissue-specific manner, have been reported (57–59). The physiological roles of these new TRs are unclear. It is possible that they may also participate in growth regulation. A future study is required to examine their role in apoptosis. However, with respect to IFN/RA treatment, the blockade of TR1 by antisense or by dominant-negative Cys-mut is sufficient to ablate apoptosis (36, 37).

Because the IFN-β used in this study is species specific and nude mice lack CTL responses, we believe that observed tumor regression is caused by the direct effects on the tumor cells. This
is corroborated by the in vitro culture studies (37). Although these mice have some NK cell activity, murine NK cells do not respond to human IFN-β. Athymic mice with tumors had NK activity levels that did not significantly change with increasing retinol doses (60). NK cell activity was increased only at a very high dosage of retinyl palmitate (61). The dosage of the all-trans RA used in our study is considered a low-to-moderate dose. However, one cannot completely rule out that RA-augmented NK response may contribute a fraction to the overall tumor growth inhibition by IFN/RA in vivo.

Although the data from one study that showed Trx1 (62) overexpression promotes tumor growth in vivo are in contrast to our data, there are several differences between those and the present studies: (a) we have used the enzyme and its mutant in our study, whereas the other study used the substrate Trx1; (b) the previous study used clonal isolates that expressed a marginally higher Trx1 (62); (c) although Northern blot analysis was shown, the expression of Trx protein was not confirmed in that study; and (d) other investigators could not obtain high Trx expression because a high-level Trx expression promotes cell death (62). This observation is consistent with our demonstration that Trx1 is a growth inhibitor (63). In the previous study (62), no attempt was made to demonstrate that the tumors, indeed, expressed Trx at the time of growth.

On the basis of the correlative expression pattern of thioredoxin-1, a substrate for TR, and of chemical inhibition, Trx1 has been suggested to act as a growth promoter (57). Although some studies showed the inhibition of apoptosis by Trx (64), it should be noted that the cells used in those studies are different from ours, and most data are derived from cells in tissue culture. Furthermore, those studies did not use growth-inhibitory cytokines such as IFNs. It should be noted that Trx overexpression did not prevent dexamethasone-, etoposide-, and doxorubicin-induced death (65). It is possible that TR/Trx can regulate both pro-growth and pro-death pathways depending on the physiological status of the cells. In an analogous manner, epidermal growth factor, c-myc, and transcription factor E2F, which are thought to promote cell growth, can also induce apoptosis (47, 66–71), depending on the metabolic state of the cells.

In contrast to the above reports, genetic studies by us and other groups have shown that the TR-Trx system functions as growth suppressor. These observations include: deletion of the Trx gene in yeast accelerates the cell cycle (72); deletion of the TR gene leads to a loss of p53-dependent gene expression in yeast (73, 74); antisense-mediated inhibition of TR1 (36) and Trx (75) leads to growth promotion in response to IFN/RA and IFN-γ in several cell types; microinjection of Trx into Xenopus oocytes causes an arrest of DNA synthesis (76); and chemical inhibitors of TRs block IFN/RA-induced cell death (36). Direct evidence that the TR-Trx system modulates caspase activities have been shown by us and others (37, 63, 77). We and others have also shown that TR and Trx promote p53-regulated gene expression to enhance apoptosis (38, 78), although p53 is not absolutely required for IFN/RA-induced cell death to occur (38). However, under these conditions, no increase in p53 levels occurred. p53 induces the expression of several proapoptotic proteins (Bax, Noxa, and PUMA) and enzymes (79–83) that alter the mitochondrial permeability transition leading to the release of apoptogenic molecules that execute the cell (84). In recent years, several novel synthetic retinoids with potent anti-tumor activities were identified. Some of them are cytotoxic without activating the receptors (85, 86). It remains to be seen whether TR also participates in these novel retinoid-induced death activities. In light of our observations and clinical studies that show marginal IFN/RA-induced antitumor responses (87–89), it would be appropriate to examine the utility of novel retinoids (20, 90) and pegylated IFNs (91) as potential combinations for tumor therapy.

REFERENCES

Redox Enzyme and Tumor-Growth Suppression


Thioredoxin Reductase Plays a Critical Role in IFN Retinoid-mediated Tumor-Growth Control *in Vivo*

Daniel J. Lindner, Xinrong Ma, Jiadi Hu, et al.

*Clin Cancer Res* 2002;8:3210-3218.