The Farnesyltransferase Inhibitor L744,832 Reduces Hypoxia in Tumors Expressing Activated H-ras

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ABSTRACT

Many tumors contain extensive regions of hypoxia. Because hypoxic cells are markedly more resistant to killing by radiation, repeated attempts have been made to improve the oxygenation of tumors to enhance radiotherapy. We have studied the oxygenation of tumor xenografts in nude mice after treatment with the farnesyltransferase inhibitor L744,832. Hypoxia was assessed by measuring the binding of the hypoxic cell marker pentafluorinated 2-nitroimidazole. We show that xenografts from two tumor cell lines with mutations in H-ras had markedly improved oxygenation after farnesyltransferase treatment. In contrast, xenografts from two tumors without ras mutations had equivalent hypoxia regardless of treatment. The effect on tumor oxygenation could be detected at 3 days and remained after 7 days of treatment. These results indicate that treatment with farnesyltransferase inhibitors can alter the oxygenation of certain tumors and suggest that such treatment might be useful in the radiosensitization of these tumors.

INTRODUCTION

Radiation oncologists and radiobiologists have long been interested in tumor oxygenation because of evidence that hypoxic cells may limit radiation treatment outcome. Hypoxic cells survive irradiation to a significantly greater extent than cells in an oxygenated environment. The magnitude of this difference in survival can be on the order of a factor of 2–3-fold in vitro (1). Human tumors containing hypoxic cells are more prone to recurrence following radiation therapy. Gatenby et al. (2) demonstrated that the short-term clinical response to radiation of well-oxygenated cervical node metastases from head and neck tumors was superior to that of poorly oxygenated lymph nodes. Brizel et al. (3) found that disease-free survival in patients with head and neck cancer was better if the tumor was less hypoxic. The average tumor median pO2 for relapsing patients was 4.1 mm Hg and 17.1 mm Hg in nonrelapsing patients. Additionally patients with hypoxic cervical carcinoma or high-grade soft tissue sarcomas had significantly worse disease-free and overall survival probabilities compared to patients with nonhypoxic tumors after radiotherapy (4–6). Thus, enhanced oxygenation of tumors prior to irradiation could improve tumor cell killing by radiation. The sensitivity of normal tissues would be expected to be unaltered, since normal tissues are usually not hypoxic.

H-ras is one of a number of oncogenes which, when activated can contribute to an increased radiation survival in transformed cells (7). Increased clonogenic survival of rodent cells and human cells has been reported after transfection with activated ras (8–11) and inhibition of ras activity in human cells expressing activated ras can radiosensitize these cells (11–14). Since activating mutations of the ras family occur in approximately 30% of human tumors, ras is an attractive target for therapeutic intervention. The ras pathway can also be activated by overexpression of ras or through cell surface receptor mutations that lead to deregulated signaling through ras in the absence of ras mutation. Thus, inhibiting ras activity may be a therapeutic strategy for a larger number of tumors than those expressing oncogenic ras.

H-ras is prenylated by farnesyltransferase. This is the first and obligate step in ras processing that results in a functional protein (reviewed in Refs. 15–17). Because the addition of a prenyl side chain is a requirement for oncogenic ras transformation, several groups have developed inhibitors specific for one or the other of the two prenyltransferases involved in ras processing. These inhibitors have been isolated as plant metabolites from drug screens and also developed to mimic either the prenyl-group substrate or the tetrapeptide CAAX recognition sequence on ras that is targeted by prenyltransferases. (reviewed in Refs. 18 and 19). By inhibiting ras posttranslational processing, these compounds inhibit ras activity. Farnesyltransferase inhibitors (FTIs) were initially shown to block the growth of ras-transformed mouse cells in soft agar and reverse the transformed morphology of v-H-ras-transformed fibroblasts, but not to inhibit the growth of src- or raf-transformed or nontransformed fibroblasts. FTIs have also been shown to inhibit spontaneous tumors in ras-transgenic mice (20–22), as well as certain human tumor xenografts in nude mice (23–25).

We have shown that inhibitors of ras prenylation radiosensitize H-ras-transformed rat embryo fibroblasts (26) and human tumor cell lines expressing either activated H- or K-ras in vitro (13). In our studies, radiosensitization appears to be specific for cells expressing mutated ras and independent of growth inhibition. The radiosensitivity of immortalized cells and primary cells is not diminished by prenyltransferase inhibitor treatment. We have further demonstrated radiosensitization of tumors with mutated H-ras by FTIs in vivo (27). While part of this effect may be due to alteration in intrinsic radiosensitivity, here we show that treatment of mice with FTIs also leads to reduction of hypoxia in tumors with H-ras mutations.

MATERIALS AND METHODS

Tumor Xenograft Generation and FTI Treatment. Pathogen-free Ncr-nu/nu mice were obtained from Taconic Farms (Germantown, NY) and housed aseptically. At 5–7 weeks of age, mice were implanted by trocar with 1-mm3 tumor fragments. Animals were randomly assigned to treatment groups 1–2 weeks after tumor implant when tumors had attained a volume of 100–200 mm3. FTI treatment consisted of continuous infusion with L744,832 (20, 28) (40 mg/kg/day) or carrier (DMSO:water, 1:1) delivered by Alzet micro-osmotic pump (Alza Corporation, Palo Alto, CA) for 3 to 7 days. Mice were then injected with 10 mm pentafluorinated 2-nitroimidazole (EF5) in 0.9% saline i.v. (0.01 ml/g body weight), followed by an equal volume i.p. injection 30 min later. Three hours after the first EF5 injection, mice were euthanized

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3 The abbreviations used are: FTI, farnesyltransferase inhibitor; EF5, pentafluorinated 2-nitroimidazole.
and tumors were excised. Samples for Western blot analysis, immunohistochemistry, and flow cytometry were harvested and processed immediately upon sacrifice of the animal. Ras farnesylation was assessed by direct Western blotting of tumor lysates using monoclonal LA069 (Viromed Biosafety, Camden, NJ). Antibody binding was detected using enhanced chemiluminescence (Amersham, Piscataway, NJ).

**EF5 Detection of Hypoxia.** Frozen tissue sections (10 mm) were cut from the tumor onto poly-L-lysine-coated slides, fixed in 4% paraformaldehyde for 1 h, and then rinsed and blocked for 2 h at room temperature. After removing the block, sections were incubated for 90 min with rat antimouse CD31 (platelet/endothelial cell adhesion molecule 1) monoclonal antibody (PharMingen, San Diego, CA) followed by Cy5-conjugated Affinipure mouse antirat IgG (Jackson ImmunoResearch, West Grove, PA) overnight at 4°C. Slides were then refixed in 4% paraformaldehyde before performing anti-EF5 staining with Cy3-conjugated ELK3–51, a mouse monoclonal antibody to EF5.
TUMOR OXYGENATION AND FARNESYLTRANSFERASE INHIBITORS

Activity of the FTI L744,832 in Vivo. Pumps to deliver L744,832 were placed in animals bearing xenografts of tumors derived from T24, HT29, and 141-1. After 7 days of L744,832 (40 mg/kg/day continuous infusion), the animals were sacrificed and the tumors were evaluated for inhibition of H-ras farnesylation. Farnesylated H-ras migrates more rapidly in PAGE than unprocessed H-ras. Fig. 1 shows that treatment of the tumor-bearing animals resulted in accumulation of unprocessed ras in the tumors. This was true regardless of whether the H-ras gene contained a mutation and showed that this methodology effectively delivers L744,832 to the tumor.

Oxygenation of Tumors after Treatment with FTI. The extent of hypoxia was evaluated in tumors derived from T24 after 3 or 7 days of L744,832 infusion. Control tumors received the delivery vehicle for the same time. Three hours before sacrifice, the hypoxic cell marker EF5 was administered. Hypoxia was evaluated by fluorescent antibody staining for EF5 bound to tumor cells. Fig. 2, A and C, show that control tumors after either 3 or 7 days have substantial regions of hypoxia. In contrast, the treated tumors (Fig. 2, B and D) showed little evidence of hypoxia even with photographic exposure times three to five times longer than controls. The peak fluorescent intensity adjusted for exposure times was calculated by scanning a representative area of 1.05 × 0.7 mm from each of the four tumors in each group. These numbers are plotted in Fig. 3 and indicate that the average peak intensity was reduced 6-fold after treatment with FTI L744,832. FTI L744,832 treatment also reduced hypoxia in tumors derived from 141-1, a murine prostate carcinoma cell line transformed by mutant H-ras and v-myec. Representative images are shown in Fig. 2, E and F (the exposure time for the treated tumor is 8.5 × the control exposure). Quantitative image analysis from the tumors is shown in Fig. 3. Reduced hypoxia was also observed in tumors derived from human prostate cells transfected with H-rasY512C,3 HT29 (a human colon carcinoma cell line wild type in H-ras) resulted in tumors with hypoxia comparable to that seen in T24 and 141-1, yet treatment with L744,832 did not alter this hypoxia (Fig. 2, G and H, Fig. 3). Tumors from RT4 (a human bladder carcinoma cell line with wild type H-ras) were less hypoxic (Fig. 3). The exposures shown in Fig. 2, I and J, are approximately ×8 the exposure used for HT-29 (Fig. 2, G and H). This prolonged exposure shows that treatment of mice bearing RT4 tumors with FTI did not alter their hypoxia, which was confirmed with quantitative analysis (Fig. 3).

We confirmed the reduction in tumor hypoxia observed in sections of T24 tumors after FTI treatment by performing flow cytometry on cells isolated from T24 tumors after 7 days of FTI treatment. Cells from L744,832-treated tumors contained only 42% positive cells (Fig. 4B) compared to 71% in the untreated tumors (Fig. 4A), and the most hypoxic cells (seen at intensities of 10^3-10^4) were drastically reduced.

DISCUSSION

Enhanced of oxygenation of tumors has been a therapeutic goal for many years. In this study, we have shown that FTI treatment of mice bearing xenografts achieved this result. Augmented oxygenation occurred after 3 or 7 days of FTI treatment in mice with tumors derived from cells with activating mutations in H-ras. We have previously shown that treatment of tumor cells in cell culture with FTI led to radiosensitization that was dependent upon the presence of a ras mutation (13, 26). This sensitization, while significant, was modest and might not have led to detectable radiosensitization in vivo after a single dose of radiation, but might have been expected to be revealed only after fractioned treatment. Nonetheless, Cohen-Jonathan et al. (27) demonstrated radiation sensitization of H-ras-bearing tumors in vivo after 3 days of FTI followed by a single dose of radiation. Whereas alteration of the sensitivity of the tumor cells themselves certainly may contribute to the effectiveness of the combined therapy in vivo, the results seen here suggest that the anticipated effect on tumor oxygenation may also contribute to radiosensitization in vivo. In support of this, Taxol has also been shown to radiosensitize murine mammary carcinomas coincident with reoxygenation of these tumors (30).

An estimated 30–40 proteins, many unidentified, are farnesylated

Footnotes:

Fig. 4. The severity of hypoxia and the percentage of hypoxic tumor cells both decreased after FTI treatment of T24 tumor-bearing mice. Mice were treated for 7 days, as described in Fig. 2 legend, with L744,832 or carrier. Tumors were harvested, enzymatically dissociated, and stained for flow cytometry analysis of EFS binding. Results for T24 control (A) and FTI-treated (B) tumors show reduced EFS binding after FTI treatment.

(Reviewed in Ref. 31), raising the possibility that proteins other than ras are the target of FTI. Rho B for example has been implicated in the reversal of transformed morphology and the induction of apoptosis by FTI (32–34). The inhibition of tumor cell proliferation also appears to be independent of ras (35). The experiments presented here demonstrate that FTIs can cause increased tumor oxygenation. Although the results are consistent with the idea that H-ras inhibition mediates enhanced tumor oxygenation, they do not prove this point. The results do however suggest that treatment with FTIs may enhance the radiosensitivity of certain tumors by increasing their oxygenation and may thus be useful adjuvants for radiotherapy.

The alterations seen in the tumor microenvironment after FTI treatment might be due to decreased oxygen consumption by the treated tumor cells or altered oxygen delivery. Oncogenic ras has been shown to influence tumor cell metabolism and oxygen consumption (36) and inhibiting ras could decrease oxygen consumption. However, FTI treatment of either T24 or 141-1 cells in vitro did not reduce oxygen consumption (data not shown). Inhibiting ras could also influence tumor vasculature (37) or tumor growth leading to altered tumor oxygenation. We are currently investigating the possible mechanisms of FTI-mediated oxygenation of tumors with ras mutations.

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