Geranylgeranylated RhoB Mediates Suppression of Human Tumor Cell Growth by Farnesyltransferase Inhibitors

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Abstract

Farnesyltransferase inhibitors (FTIs) are in clinical trials, but their mechanism of action is not fully understood. We have shown that FTI treatment rapidly elevates the level of geranylgeranylated RhoB in cells and that this event is sufficient to inhibit cell cycle transit and reverse malignant transformation without affecting normal cells. However, because these observations were made in rodent fibroblast models in which transformation was driven by defined genetic alterations, it remained to be established whether RhoB-GG was relevant to the antineoplastic effects of FTIs in human epithelial tumor cells with diverse genetic backgrounds. In this study, we show that elevated levels of RhoB-GG are sufficient to block the proliferation of FTI-sensitive but not FTI-resistant human carcinoma cells. RhoB-GG induced the cell cycle kinase inhibitor p21WAF1 in a p53-dependent manner, similar to FTI treatment, but this event was dispensable because RhoB-GG could still inhibit the growth of p53-null cells that lacked p21WAF1 activation. Consistent with actions beyond G1-phase arrest, certain cell lines exhibited accumulation in G2-M phase or an increased apoptotic index in response to RhoB-GG. We concluded that RhoB-GG suppressed human tumor cell proliferation by more than one mechanism and that it promoted apoptosis as well as inhibited cell cycle transit in malignant epithelial cells. These findings suggest how FTIs suppress the growth of human tumor cells that lack Ras mutations.

Introduction

The mechanism underlying the antineoplastic properties of FTIs4 is not fully understood. Although FTIs were developed with the aim of inhibiting the posttranslational prenylation and therefore the oncogenic activity of Ras (1–4), it has become apparent that proteins other than Ras are crucial pathophysiologic targets for alteration in malignant cells (5–7). Prenylated proteins are modified by COOH-terminal C15 (farnesyl) or C20 (geranylgeranyl) isoprenoids that are transferred by the housekeeping enzymes FT, GGT-I, or GGT-II (Rab GGT). Whereas FT and GGT-I are closely related heterodimeric enzymes, GGT-II is an unrelated enzyme that acts in a mechanistically distinct fashion (8, 9). Prenylation promotes membrane and protein-protein associations (9), and geranylgeranylation is the predominant type that occurs in cells (10, 11). Compounds that specifically inhibit FT were sought because oncogenic Ras must be farnesylated to promote transformation without affecting normal cells. However, because these observations were made in rodent fibroblast models in which transformation was driven by defined genetic alterations, it remained to be established whether RhoB-GG was relevant to the antineoplastic effects of FTIs in human epithelial tumor cells with diverse genetic backgrounds. In this study, we show that FTIs not only block Ras-mediated cell transformation and tumorigenesis but also pinpoint a specific feature of neoplastic pathophysiology (13–23).

Whereas preclinical proof of principle has been established, questions about the physiological mechanism have been raised because there is extensive evidence that FTIs do not have to inhibit Ras function to suppress malignant growth (6, 7). We previously provided evidence that the antineoplastic properties of FTIs are mediated by effects on the prenylation and function of certain farnesylated members of the Rho family of small GTPases (the FTI-Rho hypothesis). Investigations of the Rho protein RhoB as a paradigm corroborate this hypothesis and suggest a gain of function mechanism, based on the accumulation of geranylgeranylated RhoB species in FTI-treated cells as a growth inhibitory principle (24). Rho proteins regulate cytoskeletal actin dynamics and many cellular properties, including adhesion, receptor-mediated internalization, motility, transformation, invasion, survival, and transcription. Rho functions are accentuated or overexpressed in cancer cells, and these events correlate with malignant progression and invasive capacity (25–29). RhoB is unique among prenylated proteins in that it exists normally in vivo in two populations that are either farnesylated or geranylgeranylated (RhoB-F or RhoB-GG; Ref. 7 and 30). FT is responsible for the generation of RhoB-F, whereas GGT-I is responsible for the generation of RhoB-GG (11). RhoB has a short half-life (31), therefore the steady-state levels of RhoB-F decrease rapidly in FTI-treated cells. However, because of its rapid synthesis rate, there is a simultaneously rapid elevation in the steady-state levels of RhoB-GG because newly synthesized protein continues to serve as a substrate for GGT-I. This alteration correlates with a change in cell localization of RhoB and a loss of its growth-promoting capacity (11, 31), representing a gain of function consequence of FTI treatment. Indeed elevation of RhoB-GG is sufficient to mediate phenotypic reversion and growth inhibition in Ras-transformed fibroblasts (24). However, it was unclear whether RhoB-GG would be relevant to the response of epithelial-derived tumors with diverse genetic alterations. In this report, we show that RhoB-GG is sufficient to mediate growth inhibition of such cells. Susceptibility to inhibition by RhoB-GG or FTIs paralleled each other, corroborating the role of RhoB-GG in mediating the antineoplastic effects of FTIs. The findings of this study corroborate and extend the FTI-Rho hypothesis for drug mechanism and explain how FTIs inhibit the growth of tumor cells that lack Ras mutations or Ras involvement.

Materials and Methods

Plasmid Constructions. We have previously described an expression vector, pCMV-3-RA-hoB-GG, that encodes an influenza virus HA epitope-tagged RhoB polypeptide that is solely geranylgeranylated in cells (11, 24). The HA-robB-GG insert from this vector was subcloned into the zinc-inducible vector pCB6+ (32) to generate pCB6+/HA-robB-GG or subcloned into the murine retroviral vector pMSCVpac (33) to generate pacMSCV-HA-robB-GG. pCB6+ contains the sheep metallothionein promoter that permits tighter suppression in untreated cells than the analogous mouse promoter and includes a neomycin resistance gene cassette. pacMSCV includes a puromycin resistance gene cassette. pacMSCV-HA-robB-GG was further altered by swapping an expression cassette for a membrane-bound GFP (34) in place of the puromycin resistance (pac) cassette, generating gfpMSCV-HA-robB-GG.

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4 The abbreviations used are: FTI, farnesyltransferase inhibitor; FT, farnesyl transferase; GGT, geranylgeranyl transferase; HA, hemagglutinin; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

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**Tissue Culture and Cell Assays.** The cell lines used in this study were obtained from the American Type Culture Collection and cultured in DMEM (Life Technologies, Inc.) containing 10% fetal bovine serum (Cellgro) and 10 units/ml penicillin-streptomycin (Cellgro). For colony formation assays, cells were transfected with pCMV3-HA-rhoB-GG or pcDNA3 vector using Lipofectamine (Life Technologies, Inc.) and conditions recommended by the vendor. Cells were passaged the day after transfection at ratios of 1:10 to 1:30 into 6-cm dishes and cultured in media containing 0.5 mg/ml G418 the following day for a period of 2–3 weeks. G418-resistant colonies were scored by crystal violet staining. To generate zinc-inducible cell lines, 10^6 MDA-MB-231 cells were transfected with 10 μg of pCB6+ vector or pCB6+/HA-RhoB-GG using Lipofectamine, selected in 0.5 mg/ml G418 as described above, and rinsed and expanded into mass culture. Western blot analysis of cell extracts to document the steady-state levels of protein expression was performed as described previously (24). Cell proliferation and viability were measured by the MTT assay (35). Briefly, cells were seeded at 1000 cells/well in 96-well culture plates in quadruplicate in the presence or absence of 100 μM ZnSO₄. At various points, the medium was removed, and cells were incubated with 180 μl of RPMI 1640 containing 5% FCS and 0.25 mg/ml MTT (Sigma) at 37°C for 4 h, followed by solubilization in 20% SDS and 50% dimethyl formamide in water for 4–16 h at 37°C. The absorbance of each well was measured using a microplate reader at 595 nm and 655 nm dual wavelength. For soft agar culture, 1 ml of DMEM containing 10% fetal bovine serum and 0.5% NuSieve-agarose (FMC Biochemicals) was used to coat the bottom of each well in 6-well culture dishes. After hardening, 5 × 10⁴ cells were suspended per ml of DMEM containing 10% fetal bovine serum, 0.5% NuSieve agarose, and 100 μM ZnSO₄ or no addition. One ml of the cell suspension was added to each well and allowed to set for 30 min at room temperature, and then the dishes were moved to 37°C. Cells and cell colonies were photographed using an Olympus microscope with a 35-mm camera attachment. For GFP experiments, cells were seeded and transfected as indicated with 20 μg of gfpMSCV-HA-RhoB-GG or myristoylated GFP empty vector using Lipofectamine. Twenty-four h later, cells were harvested, fixed in ethanol, stained with propidium iodide, and processed for flow cytometry as described (24). Green fluorescent cells were gated for propidium iodide fluorescence, and >2000 events were counted.

**Results and Discussion.**

To assess the ability of RhoB-GG to influence the proliferation of malignant epithelial cells, a colony formation assay was performed in a set of human carcinoma cell lines (MCF7, MDA-MB-231, LoVo, DU145, and HeLa), the FTI susceptibilities and genetic profiles of which have been documented previously (22). The Ras and p53 status and FTI susceptibility of each cell line are shown in Table 1. Two cell lines (MDA-MB-231 and LoVo) harbor mutated Ki-Ras, the prenylation of which is not inhibited by FTI treatment because Ki-Ras becomes alternately geranylgeranylated by GGT-I in FTI-treated cells (36–39). Cells were transfected with expression vectors that included a neomycin resistance gene cassette and a HA epitope-tagged RhoB-GG gene or no insert. Colony formation efficiency was scored as the number of G418-resistant colonies produced by the HA-RhoB-GG vector relative to the empty vector. The experiment was performed at least three times. As shown in Fig. 1, the RhoB-GG vector produced 4- to 10-fold fewer colonies relative to the empty vector in MCF7 breast, LoVo colon, and DU145 prostate carcinoma cells, all of which are sensitive to growth inhibition by FTI treatment (22). A 2-fold reduction in colony formation was seen in the FTI-sensitive breast cell line MDA-MB-231 (data not shown). In contrast, the colony formation efficiency of the RhoB-GG or empty vector was similar in HeLa cells, which are resistant to growth inhibition by FTIs (22). Western blot analysis confirmed the accumulation of exogenous RhoB-GG in cell colonies emerging from FTI-resistant HeLa cells, but not in any of the FTI-sensitive cells tested (data not shown). Thus, RhoB-GG was not generally growth inhibitory to human epithelial cells, which is consistent with previous observations in fibroblasts (24); however, elevation of RhoB-GG was sufficient to mimic the response to FTI treatment. To further address the specificity of this effect, a similar experiment was performed using vectors that expressed a HA-tagged prenylation-deficient mutant of RhoB, HA-RhoB-S, or a similarly tagged V14-activated RhoA mutant. HA-RhoB-S did not suppress colony formation in either FTI-sensitive or resistant cell lines, but activated RhoA inhibited all of the cell lines tested, including HeLa cells, which did not respond to RhoB-GG (data not shown). These results argued that prenylation was crucial for the action of RhoB and that RhoB-GG acted by a specific mechanism that correlated with FTI susceptibility and not Ras, p53, or gross malignant status.

To investigate how RhoB-GG inhibited tumor cell growth, we generated cell lines stably transfected with a zinc-inducible vector containing the sheep metallothionein promoter. In its uninduced state, this vector was sufficiently leaky that stable cell lines were obtained

<table>
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<th>Cell line</th>
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<th>FTI susceptibility</th>
<th>RhoB-GG susceptibility</th>
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<tr>
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*DU145 cells used in this study differ in sensitivity to those reported in Ref. 22. Null due to expression of human papilloma virus E6 protein.

Fig. 1. RhoB-GG inhibits the growth of FTI-sensitive human tumor cells without regard to p53 or Ras status. The cell lines indicated were transfected with 10 μg of pCMV3-HA-RhoB-GG or pcDNA3 (empty vector), and G418-resistant colonies were scored 2–3 weeks later. Results from at least three trials in each cell line were obtained. A, relative colony formation efficiency. Colony number is depicted on the X axis as the proportion of colonies formed by pCMV3-HA-RhoB-GG relative to those formed by pcDNA3. B, photograph of a representative set of dishes fixed with methanol and stained with crystal violet.

**Table 1 Cell lines**

Human carcinoma cell lines derived from various epithelial tissues were chosen for analysis based on Ras and p53 genotypes as well as FTI susceptibility (derived from Ref. 22). Susceptibility to RhoB-GG is summarized from results shown in Fig. 1. WT, wild-type; Ki, Kirsten Ras; null, nullizygous; S, sensitive; R, resistant.
only from MDA-MB-231, the cell line that was less inhibited than the other FTI-sensitive cell lines. MCF7 and DU145 cell lines lost inducible expression within 1–2 weeks of mass culture and were used for molecular but not biological experiments (see below), whereas no LoVo cell lines were obtained. Western blot analysis confirmed the induction of RhoB-GG in MDA-MB-231, MCF7, and DU145 cell lines after the addition of 50 μM ZnSO₄ (see Fig. 2). Using the MDA-MB-231 cell lines, we examined the effects of induced RhoB-GG on anchorage-dependent and anchorage-independent growth using MTT or soft agar colony formation assays, respectively. Similar results were obtained using several sublines, and representative results are presented. Induction of RhoB-GG was correlated with a reduction in the rate of anchorage-dependent cell proliferation relative to vector control lines (see Fig. 3A). The reduction was modest but similar in degree to that produced in MDA-MB-231 and other tumor cell lines by FTIs, which retard cell proliferation but did not cause cytostasis in anchorage-dependent cultures (22). Similar levels of growth inhibition were also observed in experiments in which RhoB-GG was transferred by amphotropic retroviruses (data not shown). No changes were observed in the cell cycle profile as measured by flow cytometry, consistent with evidence that whereas FTIs inhibit the growth of MDA-MB-231 cells, this effect is not associated with the accumulation of cells in either the G₁ or G₂-M phases (40). RhoB-GG had more pronounced effects on anchorage-independent proliferation measured by soft agar cultures, similar to FTIs. Uninduced cells exhibited a marked reduction in colony size relative to vector control lines, due to leaky expression from the vector. The addition of 50 μM ZnSO₄ augmented this effect in the absence of any effect on control cells, such that elevated levels of RhoB-GG inhibited colony formation significantly (see Fig. 3B). We concluded that the elevation of RhoB-GG levels elicited by FTI treatment was sufficient to suppress anchorage-dependent and anchorage-independent proliferation of FTI-susceptible tumor cells.

Both FTI treatment and RhoB-GG elevation can activate p21WAF1 in certain settings (24, 41). In particular, FTIs have been reported to induce p21WAF1 in breast tumor cell lines with wild-type p53 (41). We investigated whether there was a correlation between p21WAF1 activation and RhoB-GG-mediated growth inhibition in the Zn²⁺-inducible cell lines generated. Western blot analysis of cell extracts prepared from cells that were untreated or treated for 24 h with 50 μM ZnSO₄ was performed with a p21WAF1 antibody (see Fig. 4A). RhoB-GG did not have any detectable effect on p21WAF1 in DU145 cells, which are nullizygous for p53, but activated p21WAF1 in MDA-MB-231 cells to low but detectable levels. Uninduced MCF7 cells exhibited higher basal levels of p21WAF1 than did vector cells, but upon RhoB-GG induction, levels of p21WAF1 decreased slightly. Because all these cell lines were susceptible to growth inhibition by RhoB-GG, these data argued that although p21WAF1 might be sufficient, it was not necessary for biological response. This interpretation was consistent with data from fibroblast models, which showed that growth inhibition by FTIs and RhoB-GG could be separated from p21WAF1 activation (24). The likelihood that RhoB-GG acted at other levels also was supported by additional experiments in which cells were subjected to transient LipofectAMINE-mediated transfection with vectors that included an expression cassette for a myristoylated GFP that is targeted to the plasma membrane. Cells collected 24 h after transfection were subjected to propidium iodide staining and flow cytometry, gating on green fluorescent cells to assess cell cycle distribution and apoptotic profile in the transfected population. In MCF7 cells, which harbor wild-type ras and p53 genes, introduction of RhoB-GG vector caused a 3-fold increase in the relative number of cells in G₂-M phase of the cell cycle relative to empty vector control (see Fig. 4C). This result was consistent with evidence from several studies showing that FTIs can inhibit G₂-M transition in certain settings (40–43). Increased apoptosis was not evident in LoVo cells in response to RhoB-GG (data not shown). The possibility that p53 status might influence the apoptotic response to RhoB-GG or FTI treatment is supported by recent analysis of cytokine-dependent FTI-induced apoptosis in fibroblast models (44). We concluded that RhoB-GG was sufficient to mediate growth inhibition by FTIs in a manner that may be independent of the effects of each agent on p21WAF1 activation.

This report corroborates and extends the hypothesis that the antineoplastic action of FTIs can be traced to gain of function effects on RhoB or related proteins that are elicited by FTI treatment (7, 24). We found that elevation of RhoB-GG, a rapid effect of drug treatment, is sufficient to inhibit FTI-sensitive but not FTI-resistant human carcinoma cells without reference to Ras or p53 status. These findings establish a mechanism through which FTIs can block the growth of human tumor cells that lack Ras mutations or are dependent on K-Ras or N-Ras, each of which remains active in the absence of FT activity due to crossprenylation by GGT-I (17, 18, 20, 36–39, 45). FTIs are largely cytostatic in xenograft models (20, 23, 38, 46, 47), possibly reflecting evolution in the antiproliferative capacity of human tumor cells and the largely cell cycle-inhibitory effects of the FTI mediator RhoB-GG (24). Analogous to the effects of FTIs and RhoB-GG in
transformed fibroblasts, growth inhibition in MDA-MB-431 breast carcinoma cells correlated with elevation of cell division kinase inhibitor p21WAF1 (24). This observation was consistent with a report that p21WAF1 is activated by FTIs in malignant cells that have wild-type p53 (41). However, although p21WAF1 might be part of the mechanism through which RhoB-GG acts, not all of the tumor types elevated p21WAF1 in response to RhoB-GG and this event was dispensable for growth inhibition. Thus, p21WAF1 appears to respond to rather than regulate growth inhibition mediated by FTIs and RhoB-GG in certain settings. In support of another mechanism of action, G2-M phase arrest and increased apoptosis in LoVo or MCF7 cells were observed in response to RhoB-GG. The basis for these effects remains to be determined. However, given that p53 status can partly influence the cytokine-dependent apoptotic response to FTIs in transformed fibroblasts (44), it is tempting to speculate that p53 status may also influence apoptosis elicited by RhoB-GG in malignant epithelial cells. One caveat is that these effects may reflect higher levels of expression than those achieved by FTI treatment. Nevertheless, this study establishes in principle a potential role for RhoB-GG in apoptosis. Identification of RhoB-GG as a mediator of FTI action does not immediately shed light on why malignant cells should respond so selectively to FTI treatment. Because Rho proteins have an important role in adhesion signaling, we hypothesize that

Fig. 3. Induction of RhoB-GG expression inhibits anchorage-dependent and anchorage-independent growth of estrogen-independent MDA-MB-231 breast carcinoma cells. A, growth curve. Cells (10^5) were seeded overnight and left untreated or treated at 48-h intervals with 100 μM ZnSO₄. Cells were counted at the intervals indicated, and the mean ± SE derived from four individual measurements was plotted. B, anchorage-independent growth. Cells (10^5) were seeded in soft agar media in the absence or presence of 100 μM ZnSO₄ and photographed 2 weeks later.

Fig. 4. Effects of RhoB-GG on cell cycle transit and apoptotic index. A, effects on p21WAF1 expression. Cells (10^5) were seeded overnight and then left untreated or treated for 24 h with 100 μM ZnCl₂. Cell lysates were prepared, and 40 μg/sample were processed for SDS-PAGE and Western blot analysis with an anti-p21 antibody. Vect, cell lines generated by transfection of empty vector only. B, increased apoptotic index in MCF7 cells. A total of 10^6 MCF7 or HeLa cells were transfected with LipofectAMINE-mediated transfection with the myristoylated-GFP vectors indicated. Twenty-four h after transfection, cells were processed for propidium iodide staining and flow cytometry, and the green fluorescing cells exhibiting sub-G₁-phase DNA were scored as apoptotic. Data are presented as the total proportion of apoptotic cells in the cell population assayed. C, increased accumulation of LoVo cells in G₂-M phase. The same experiment as described above was performed, except that LoVo cells were recipients for transfection.
RhoB-GG may interfere in a dominant inhibitory manner with certain integrin-dependent controls of cell cycle and survival that involve Rho and that are specific or more important to malignant cells (48–52). In future work, it will be important to test this hypothesis and to identify the Rho effector pathways that RhoB-GG influences in cancer cells.

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