Farnesyl Transferase Inhibitors Induce Apoptosis of Ras-transformed Cells Denied Substratum Attachment

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ABSTRACT

Farnesyl transferase inhibitors (FTIs) are a novel class of antitumor drugs that block the oncopgenic activity of Ras. Because FTIs lack significant cell toxicity in vitro and in vivo, a significant question is how they cause tumor regression. We now report that FTIs are in fact potent activators of apoptosis in Ras-transformed cells if attachment to substratum is prevented. When cultured at high density or on polyHEMA, a nonadherent substrate, Ras-transformed cells exhibited massive DNA degradation and cell death within 24 h of treatment with the FTI L-739,749. Death was p53-independent and was inhibited by the apoptosis suppressor BCL-XL. Furthermore, apoptosis was significantly attenuated by ectopic expression of a farnesyl-independent form of RhoB, a Rho protein previously implicated as a critical target for inhibition by FTIs. The findings suggest a link between FTIs and Rho-dependent adhesion signaling. Furthermore, our work indicates that FTIs revert cells to a state in which cell-substratum attachment is necessary for viability and suggests that apoptosis forms the basis for drug-induced tumor regression.

INTRODUCTION

FTIs have emerged as promising leads in the search for rationally designed anticancer therapeutics (1). These compounds were originally designed to block the action of oncogenic Ras. A crucial regulator of cell growth, Ras is a guanine nucleotide-binding protein that cycles between GTP- and GDP-bound states, transducing signals between upstream growth factor receptors and downstream kinases (2, 3). Oncogenic activation of Ras, which contributes to a significant percentage of human cancers (4), causes the protein to be constitutively in an active GTP-bound state, which transmits a constant proliferation signal.

Posttranslational farnesylation of Ras has been shown to be essential to its function (5, 6). Although the exact role of farnesylation is unclear, it is thought to facilitate proper membrane association and have a role in mediating protein-protein interactions (7). In the modification reaction, the enzyme FT covalently links a farnesyl group (a 15-carbon isoprenoid) to a cysteine residue located in the carboxy-terminal CAAX motif of Ras (in which C is cysteine, A is an aliphatic amino acid, and X is any amino acid; Ref. 8). FTIs were therefore developed as potential anticancer agents that would block farnesylation and thus inhibit the function of oncogenic Ras. There is emerging evidence that inhibition of Ras farnesylation is not the critical event for the antitumorigenic effects of FTIs (9, 10) and that inhibition or alteration of Ras activity may be crucial instead (9).

Despite questions regarding their exact mechanism of action, FTIs have been shown to exhibit potent antitumorigenic effects in cell culture and antitumor effects in animal models. In cell culture, FTIs induce a rapid morphological reversion and block anchorage-independent growth of Ha-Ras-transformed cells; interestingly, normal cell growth is not significantly affected by treatment (11–13). FTIs have also been shown to block the growth of Ras-transformed cells in nude mice without significant systemic toxicity (14, 15). Additionally, in transgenic mice that develop spontaneous carcinomas due to the presence of an oncogenic v-Ha-ras gene, FTI treatment leads to dramatic, nearly complete tumor regression (16). The basis of this regression, however, is unclear because FTIs have not been reported to induce significant apoptotic or necrotic death in vitro at concentrations significantly beyond the minimal inhibitory concentration required to inhibit Ras transformation (12).

The question of mechanism of tumor regression is made more important by two potentially problematic findings from the transgenic mouse experiments: (a) resistance to FTI-mediated tumor regression was observed in some animals; and (b) even among tumors that seemed to regress completely, cessation of FTI treatment led to a rapid return of the tumor (16); thus some malignant cells seem to persist even though the bulk of the tumor disappears. Tumor persistence, if also seen with the treatment of human cancer, would require continuous long-term FTI treatment that could increase side effects and the development of resistance. Thus, understanding the basis of tumor resistance and persistence may be essential for effective clinical use of FTIs.

In this study, we report that FTIs cause apoptotic death in Ras-transformed cells grown under conditions in which they cannot attach to substratum. Furthermore, we show that this apoptotic response is blocked by BCL-XL, independent of p53 status, and inhibited by a myristylated form of RhoB. Our work provides a potential explanation for FTI-induced tumor regression, as well as tumor resistance and persistence, in the v-Ha-ras mice.

MATERIALS AND METHODS

Plasmid Constructs. A human BCL-XL cDNA (kindly provided by D. Ewert) was subcloned into pcDNA3 (Invitrogen) to make CMV-BCL-XL. The plasmid pLTRcGvall35, which has been described previously, expresses p53V135, a murine temperature-sensitive mutant form of p53 (17). An expression plasmid for Myr-rhoB714 has also been described previously (9).

Cell Culture. All cells were maintained in DMEM containing 10% fetal bovine serum (HyClone) and 50 units/ml penicillin-streptomycin (Life Technologies, Inc.). The Rat1/ras and Rat1/tras/myr-rhoB cell lines have been described previously (9). The cells were transfected by a modified calcium phosphate precipitation method. Stable lines were generated by cotransfection of 15 μg of total DNA that included a 10:l ratio of hygCMV, a hygromycin-resistance gene vector (9), with the construct of interest (either CMV-BCL-XL or pLTRcGvall35). Stably transfected cells were selected in growth media containing 100 μg/ml hygromycin B (Boehringer Mannheim), and cell colonies were ring-cloned and expanded into cell lines.

For culture on the nonadhesive substrate polyHEMA, tissue culture dishes were coated by applying 4 ml of a 10 mg/ml solution of polyHEMA (Sigma) dissolved in ethanol onto the dish, drying, repeating the application, and washing with PBS. In all experiments involving FTI treatment, cells were treated for 36 h with 20 μM L-739,749, a peptidomimetic FTI, unless indicated otherwise.

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3 The abbreviations used are: FTI, farnesyl transferase inhibitor; FT, farnesyl transferase; PI, propidium iodide.
Acridine Orange Staining. Cells were harvested, washed in PBS, and fixed in 1% paraformaldehyde. The cells were then stained for 10 min with acridine orange (10 μg/ml) and analyzed via fluorescence microscopy. Quantitative analysis was done by counting at least 100 cells and scoring for apoptosis based on chromatin condensation.

Flow Cytometric Analysis. Cells (3 × 10⁶) were seeded onto polyHEMA-coated dishes in the presence or absence of FTI, unless otherwise indicated. After 36 h, cells were harvested, washed twice with PBS, and fixed in 70% ethanol overnight. The cells were then stained in PBS containing 50 mg/ml PI, 100 units/ml RNase A, and 0.1% (v/v) glucose for 30 min at room temperature. Fluorescence-activated cell sorting analysis was done essentially as described (18), using an EPIC/XL cell analyzer (Coulter).

Western Analysis. Cells were washed in PBS and harvested in NP40 lysis buffer containing phenylmethylsulfonyl fluoride, pepstatin, and leupeptin (19). Cell lysates were clarified by centrifugation and quantitated by Bradford assay. Cellular protein (50 μg) was fractionated by SDS-PAGE, blotted onto nitrocellulose membranes, and analyzed as described (19). The anti-p53 antibody pAb-421 (Calbiochem) was used as recommended by the vendor, with an anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Boehringer Mannheim) and chemiluminescence kit (Pierce Chemical Co.) used for detection.

RESULTS

FTIs Induce Apoptosis in Ras-transformed Cells Cultured at High Density. We have previously demonstrated that Rat1/ras cells, a Rat1 fibroblast line transformed with the v-Ha-ras oncogene, revert to a flattened, enlarged phenotype resembling normal Rat1 cells when treated with the peptidomimetic FTI L-739,749 (12). In previous experiments in which subconfluent cells were treated with FTI, no cell death or toxicity was observed at doses up to 25 times the minimal inhibitory concentration required for morphological reversion (12).

However, we observed that at higher cell densities (greater than 100% confluence), treating Rat1/ras cells with 20 μM L-739,749 resulted in significant cell death. To examine this response, cells plated at different densities were treated with L-739,749 for 36 h and then analyzed via flow cytometry for DNA content; the amount of death was taken as the percentage of cells with a DNA content <2N. As shown in Fig. 1A, the percentage of dead cells increased markedly with increasing density from under 3% in subconfluent cells to almost 50% in cells at the highest density.

To determine if the cell death that occurred at high density was apoptotic, we extracted genomic DNA from high- and low-density cells that had been treated with L-739,749 for 36 h. DNA extracted from the high-density cells showed the distinctive oligonucleosomal pattern characteristic of apoptosis (data not shown). To confirm apoptotic death, cellular DNA in intact cells was stained with acridine orange and examined by immunofluorescence microscopy. As expected, high-density L-739,749-treated cells showed chromatin condensation and segregation characteristic of apoptosis (Fig. 1B).

L-739,749 Induces Apoptosis in Rat1/ras Cells Cultured on polyHEMA, a Nonadherent Substratum. L-739,749 treatment at high density seemed to affect cells in two different ways, depending on their relative location in the culture. Cells that were in contact with the substratum reverted and survived the treatment. However, cells that did not contact the substratum due to the high density were killed by FTI treatment.

To explicitly test whether the lack of substratum attachment rendered cells sensitive to FTI-induced apoptosis, Rat1/ras cells were cultured on polyHEMA-coated plates. Due to its uniformly nonionic nature, polyHEMA blocks cell attachment by preventing matrix deposition (20). Flow cytometry was used to analyze cells cultured on polyHEMA or plastic in the presence or absence of L-739,749. As expected, untreated Rat1/ras cells grown on polyHEMA proliferated as a suspension culture showing little cell death after 36 h (Fig. 2A).

In contrast, L-739,749-treated cells underwent dramatic apoptosis. Unlike high-density cells on plastic, where many cells adhere and survive, the complete denial of substratum attachment caused nearly complete apoptosis in the cell population.

Although the loss of anchorage was sufficient to cause sensitivity to FTI-mediated apoptosis, the increased cell-cell contact associated with high cell density did not seem to be important in promoting the apoptotic response. The density of the cells during polyHEMA culture had no effect on apoptosis; in addition, cells that had been growing at different densities before culture on polyHEMA showed the same apoptotic response to FTI (data not shown). Therefore, we concluded that lack of substratum attachment, rather than increased cell-cell contact, was critical for the apoptotic response.
Farnesyl transferase (FTI) is a family of enzymes that play a crucial role in cell signaling and cancer. These enzymes add farnesyl groups to proteins, which is important for their proper function. FTIs can induce apoptosis and reversion in certain cell lines, but the mechanisms underlying these effects are not fully understood.

**Apoptosis and Reversion**

Apoptosis and reversion are similar processes that involve cell death. The ability of cells to attach to substratum determines the response to drug treatment. The BCL-XL gene family is involved in many types of apoptosis. One member of this family, BCL-XL, is often overexpressed in certain carcinomas. To study the effects of BCL-XL on apoptosis, researchers generated stable Rat1/ras cell lines that overexpress BCL-XL. Expression was confirmed by Northern analysis. The apoptotic response of these cells (Rat1/ras/bclxl) was tested by seeding cells on polyHEMA in the presence or absence of L-739,749. In the absence of drug, both Rat1/ras/hyg and Rat1/ras/bclxl lines proliferated as suspension cultures. With the addition of L-739,749, the BCL-XL-overexpressing cells showed significant resistance to FTI-induced apoptosis, with ~80% survival compared to <5% viability in the parental and vector control cell lines (Fig. 3). This indicates that BCL-XL renders cells resistant to FTI-induced apoptosis but not reversion.

**L-739,749-induced Apoptosis Is p53-independent**

Because p53 plays a significant role in many human cancers as well as in apoptosis, researchers sought to determine if p53 function was required for FTI-induced apoptosis. Stable Rat1/ras lines (Rat1/ras/p53^135) were made that express the murine temperature-sensitive mutant allele p53^135 which assumes a dominant negative mutant conformation at 38.5°C and a wild-type conformation at 32°C. Expression of p53^135 in Rat1/ras lines was confirmed via Western analysis. Under these conditions, growth suppression was evident due to overexpression of p53 in its wild-type conformation. Lines that showed high expression and biological activity of p53 were tested for susceptibility to FTI-induced apoptosis at 38.5°C, the temperature at which p53^135 assumes the dominant negative mutant conformation. Rat1/ras/p53^135 lines underwent apoptosis in a manner similar to that of parental and vector control cell lines (Fig. 4B). We saw no significant resistance of Rat1/ras/p53^135 lines as compared to control lines.

**A dose-response experiment** was performed to verify that FTI-induced apoptosis corresponded with FT inhibition. This experiment indicated that L-739,749-induced apoptosis occurs with an IC50 of 2–5 μM (Fig. 2B), similar to the IC50 for inhibition of v-Ha-Ras farnesylation (12) and suppression of Rat1/ras cell growth in soft agar culture (14). L-739,749 does not affect the activity of geranylgeranyltransferase, an enzyme closely related to FT, at these concentrations (12, 14). We concluded that L-739,749 induces apoptosis by specifically inhibiting FT activity.

A time-course experiment indicated that the kinetics of FTI-mediated cell death and FTI-induced morphological reversion are similar. L-739,749 induced apoptosis in 50% of a population of Rat1/ras cells cultured on polyHEMA within 24 h of treatment (data not shown). This period corresponded well with the 20–24-h period required by L-739,749 to cause morphological reversion of cells cultured on plastic (12). This finding argued that the mechanisms of FTI-induced apoptosis and reversion are similar or related and that the ability of cells to attach to substratum determines the response to drug treatment.

**BCL-XL Blocks L-739,749-induced Apoptosis**

BCL-2 family members suppress many types of apoptosis (21, 22). One member of this gene family, BCL-XL, has been reported to be frequently overexpressed in certain carcinomas (23). To begin to characterize the FTI-induced apoptotic response, researchers generated stable Rat1/ras cell lines that overexpress BCL-XL; expression was confirmed by Northern analysis (data not shown). The apoptotic response of these cells (Rat1/ras/bclxl) was tested by seeding cells on polyHEMA in the presence or absence of 20 μM L-739,749. In the absence of drug, both Rat1/ras/hyg and Rat1/ras/bclxl lines proliferated as suspension cultures. With the addition of L-739,749, the BCL-XL-overexpressing cells showed significant resistance to FTI-induced apoptosis, with ~80% survival compared to <5% viability in the parental and vector control cell lines (Fig. 3). Significantly, although BCL-XL blocked FTI-induced apoptosis, FTI-induced reversion still occurred on adherent substrates (data not shown). We concluded that BCL-XL rendered cells resistant to FTI-induced apoptosis but not reversion.

**L-739,749-induced Apoptosis Is p53-independent**

Because of the prominent role p53 plays in a large percentage of human cancers as well as in apoptosis (24), researchers sought to determine if p53 function was required for FTI-induced apoptosis. Stable Rat1/ras lines (Rat1/ras/p53^135) were made that express the murine temperature-sensitive mutant allele p53^135 which assumes a dominant negative mutant conformation at 38.5°C and a wild-type conformation at 32°C (17). Previous studies indicate that p53^135 blocks endogenous p53 activity in rodent cells (18). Expression of p53^135 in Rat1/ras lines was confirmed via Western analysis (Fig. 4A). In addition, the biological activity of p53 in the stable lines was assayed by incubation for 2 days at 32°C. Under these conditions, growth suppression was evident due to overexpression of p53^135 in its wild-type conformation (data not shown). Lines that showed high expression and biological activity of p53 were tested for susceptibility to FTI-induced apoptosis at 38.5°C, the temperature at which p53^135 assumes the dominant negative mutant conformation. Rat1/ras/p53^135 lines underwent apoptosis in a manner similar to that of parental and vector control cell lines (Fig. 4B). We saw no significant resistance of Rat1/ras/p53^135 lines as compared to control lines.
To investigate whether Myr-rhoB^V14 also confers resistance to FTI-induced apoptosis, we seeded Rat1/ras/myr-rhoB^V14 cells on polyHEMA-coated dishes and treated the cells with L-739,749. Myr-rhoB^V14-expressing cells exhibited considerable resistance to FTI-induced apoptosis (Fig. 5), maintaining ~60% viability compared to <5% viability in control cells in the presence of 15 μM L-739,749. We concluded that Myr-rhoB^V14 conferred resistance to L-739,749-induced apoptosis.

**DISCUSSION**

In this study, we have identified and investigated the apoptotic response induced by FTIs in Rat1/ras cells that are prevented from attaching to substratum. Our analysis indicated that FTI-induced apoptosis is blocked by BCL-X_L, is independent of p53 status, and may involve an alteration of Rho activity. This work provides support for a link between FTI effects on transformed cells and Rho, which has been implicated in focal adhesion functions involved in substratum attachment. Furthermore, the work suggests a mechanism for FTI-induced tumor regression and may explain the basis for tumor resistance and persistence observed in animals.

Characterization of FTI-induced apoptosis is particularly relevant to the observations of tumor resistance and tumor persistence in the v-Ha-Ras mouse model (16). Our results suggest two explanations for these phenomena: (a) our in vitro model suggests that a lack of appropriate substratum attachment in vivo might facilitate FTI-induced apoptosis of tumor cells, thereby causing tumor regression. Tumor cell persistence might occur at privileged locations, perhaps in the periphery of the tumor, where normal anchorage cues may allow tumor cells to survive; and (b) a second explanation for persistence and resistance is suggested by the BCL-X_L experiment. Genetic alterations that block the apoptotic response, such as BCL-X_L overexpression, might arise in a percentage of tumor cells, thereby allowing them to survive drug treatment and regrow upon FTI removal. The recurrent tumor might then be resistant to tumor regression when FTI treatment is reimplemented. The ability of BCL-X_L to defeat FTI-induced death raises serious clinical issues because BCL-X_L has been observed to be overexpressed in human carcinomas (23).

The similar kinetics and dose response for FTI-mediated reversion and apoptosis suggests that the mechanism underlying each response is similar and/or overlapping, with the outcome determined by the substratum availability. If cells can attach, they revert to a state in which proliferation is possible; otherwise, a cell death program is initiated. Our observations argue that FTIs revert transformed cells to a more normal physiological state in which they require substratum attachment for survival. Detachment-mediated apoptosis, or “anoikis,” has been analyzed by others (20) who have reported that many nontransformed cells require attachment for survival. The ability of BCL-X_L to defeat FTI-induced death raises serious clinical issues because BCL-X_L has been observed to be overexpressed in human carcinomas (23).

Resistance to FTI-induced apoptosis was also observed in cells where RhoB-dependent pathways were rendered insensitive to drug treatment by Myr-rhoB^V14 expression. Previously, we have reported that Rat1/ras/myr-rhoB^V14 cells have altered susceptibility to FTIs.

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5 P. F. Lebowitz, unpublished observations.
inhibition (26), suggesting complexity in the mechanism by which Myr-RhoB^{V14} confers drug resistance. Nevertheless, alteration of Rho protein function seems critical to the drug mechanism because Myr-RhoB^{V14} can block both FTI-induced apoptosis and phenotypic reversion (9).

The finding that FTIs induce a requirement for substratum attachment suggests that they affect cellular adhesion pathways. Interestingly, Rho proteins play a key role in adhesion; they have previously been shown to be essential for integrin function and focal adhesion complex formation (27–29). Thus, alteration of Rho activity and subsequent changes in integrin function may mediate FTI-induced apoptosis in the absence of appropriate substratum and morphological reversion in its presence. This speculative model is consistent with other observations. First, FTIs induce cell flattening and spreading, morphological changes known to be controlled by integrins (30). Second, by inhibiting Rho activity with Clostridium botulinum C3 exoenzyme, it has been shown that a Rho-integrin pathway plays a role in regulation of tumor cell growth (31). Finally, FTIs induce actin stress fibers (12), cellular structures that are associated with integrins and Rho activity (32).

Given the prevailing model that Rho proteins cause stress fiber formation, it might be expected that alteration of RhoB function by FTIs would lead to the disappearance rather than the appearance of actin stress fibers. However, there are a growing number of reports suggesting that Rho regulation of actin is more complex than this model would imply: (a) despite the fact that Ras transformation is accompanied by stress fiber dissolution, it is clear that Rho proteins are necessary for transformation by Ras (25, 33, 34); (b) several different Rho proteins have been identified, some of which act at different cellular locations (35, 36), suggesting the possibility of differences in function; and (c) there are cases in which Rho inhibition has actually been shown to have opposite effects in different cell types (27). Given that this work suggests the involvement of Rho-adhesion pathways in FTI actions, further analysis of the Rho hypothesis should prove valuable to understanding drug actions.

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Fig. 5. Myr-rhoB confers resistance to FTI-induced apoptosis. Stable Rat1/ras transfectants overexpressing a myristylated form of RhoB and vector control lines were cultured on polyHEMA in the presence and absence of 15 μM L-739,749. The flow cytometry analysis of PI-stained cells was performed to quantitate apoptosis. Results represent the average of three trials.


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