Elevation of α2(I) Collagen, a Suppressor of Ras Transformation, Is Required for Stable Phenotypic Reversion by Farnesyltransferase Inhibitors

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Abstract
Farnesyltransferase inhibitors (FTIs) are a novel class of anticancer drugs that can reverse Ras transformation. One of the intriguing aspects of FTI biology is that continuous drug exposure is not necessary to maintain phenotypic reversion. For example, after a single exposure to FTIs, Ha-Ras-transformed fibroblasts revert to a flat and anchorage-independent phenotype that persists for many days after processed Ras has returned to pretreatment levels. In this study, we show that persistence of the reverted state is mediated by elevated expression of the collagen isofrom α2(I), a suppressor of Ras transformation the transcription of which is repressed by activated Ras and derepressed by FTI treatment. To our knowledge, this is the first report identifying an FTI-regulated gene which is linked to phenotypic reversion. The finding that extracellular matrix alterations can influence the kinetics of reversion supports our assertion that Rho-regulated cell adhesion parameters are a crucial determinant of the cellular response to FTIs.

Introduction
FTIs3 are a new class of anticancer drugs that inhibit the posttranslational farnesylation of Ras and other cellular proteins (reviewed in Refs. 1–4). Protein prenylation is essential in mediating protein-protein interaction and facilitating proper membrane localization (5). This modification involves COOH-terminal addition of a farnesyl protein group; a geranylgeranyl group (20-carbon isoprenoid) or a geranyl group (15-carbon isoprenoid) to a cysteine residue of the COOH-terminal CAAX motif (in which C is cysteine, A is an aliphatic amino acid, and X is any amino acid). Farnesylation of Ras protein is critical for its oncogenic activity (6). Therefore, compounds that specifically inhibit farnesyltransferases were sought as a strategy to block Ras function in cancer cells.

In support of this approach, FTIs have been demonstrated in many laboratories to inhibit malignant cell growth in vitro and in vivo. For example, FTIs reverse the transformed phenotype and inhibit the anchorage-independent growth of Ras-transformed cells (7–11). FTIs also inhibit anchorage-independent growth of a variety of human tumor cells in vitro (12–14). Remarkably, FTIs show little if any detectable toxicity when applied to normal cells, even at doses that completely block the farnesylation of endogenous Ras or lamin that are required for normal cell viability or proliferation (11, 15, 16). For example, FTIs block tumor growth in mouse xenograft models without effects on the host animal (17–19) and, more dramatically, cause tumor regression in H-Ras or N-Ras transgenic mice without discernible side effects (20–22). The exceptionally selective biology exhibited by FTIs has raised many questions about their exact mechanism of action, which goes beyond simply inhibiting farnesylation of oncogenic Ras and instead to altering prenylation of Rho (23).

Two unresolved questions are whether gene activation is essential for reversion and how a single application of FTI induces an unusually stable phenotype (11). Ras-transformed cells typically display a round or spindle morphology, a lack of actin stress fibers, reduced adhesiveness to substrate, and anchorage-independent growth (24). Phenotypic reversion of Ras-transformed cells by FTIs is rapid, characterized by cell enlargement, flattening, and actin cytoskeletal reorganization. Importantly, reversion is closely correlated with loss of the capacity for anchorage-independent growth (11). Remarkably, once induced the reverted phenotype is quite stable, and its maintenance does not require continuous drug exposure. Cells cultured under anchorage-dependent conditions eventually retransform in the absence of drug, but reversion can persist for more than a week after Ras and Rho processing has returned to normal (11).

To explain this effect, we hypothesized that FTIs might elevate expression of a long-lived polypeptide that suppressed Ras transformation and allowed reversion to persist, even after the reappearance of fully processed Ras. A number of genes encoding ECM components that affect morphology and the malignant phenotype are down-regulated in Ras-transformed cells, including α2(I) collagen, lysyl oxidase, collagen type III, and fibronectin (25). α2(I) collagen expression is regulated by epidermal growth factor and p53 and can suppress cell transformation by Ras and other oncogenes (26–28). Because α2(I) collagen is stable and can suppress Ras transformation, if elevated by FTIs it would be a good candidate to mediate reversion persistence. In support of this likelihood, we showed in this study that FTIs elevated expression of α2(I) collagen in Ras-transformed fibroblasts at the level of transcription, and that this event was essential for reversion and its persistence after drug removal (i.e., for maintenance of the reverted phenotype). The finding that FTI biology can be influenced by alterations in ECM supports our assertion that cell adhesion parameters dictate the biological response to FTIs.

Materials and Methods
Plasmid Constructs. A human α2(I) collagen cDNA obtained from American Type Culture Collection (Hp2010) was subcloned into pcDNA3.1zeo (Invitrogen) to generate CMV3-antisense-α2(I) collagen vectors. Orientation was determined by restriction enzyme digest.

Cell Culture. The rat fibroblast cell lines Rat1 and Rat1/ras have been described previously (11). Rat1/ras is a v-H-ras-transformed derivative of Rat1 fibroblasts. Cells were cultured in DMEM containing 10% fetal bovine serum (Life Technologies, Inc.) and 10 units/ml of penicillin-streptomycin (Life Technologies, Inc.). Rat1/ras cell lines expressing sense or antisense α2(I) collagen were generated by transfection with pcDNA3.1zeo, CMV3-antisense-α2(I) collagen, and CMV3-sense-α2(I) collagen into Rat1/ras cell via a standard modified calcium phosphate transfection. Cells were then selected in 200 μg/ml zeocin (Invitrogen) for 2 weeks, ring cloned, and expanded into mass culture. Expression levels of α2(I) collagen in cell lines was determined by
ELISA. In drug treatment experiments, FTI L-744,832 (20) was added to a final concentration of 10 μM unless otherwise indicated.

ELISA. The amount of α2(I) collagen secreted into the culture medium was measured by ELISA as described (29). Cells (3 x 10^6) were seeded in a 60-mm dish, and then medium was collected 48 h later and clarified by centrifugation. Serial 1:2 dilutions were made in DMEM containing 1% FCS, and 50 μl of each dilution were added to wells of a 96-well PVC ELISA plate in quadruplicate. Dilutions of rat tail collagen was used as a standard. The plate was incubated for 2 h at room temperature in a humid container to permit collagen binding to the plastic substratum. Wells were washed four times with PBS, followed by 3% BSA/PBS blocking overnight at 4°C. Fifty μl of 1:100 diluted rabbit anti-rat collagen type I polyclonal antibody (Chemicon) were added and incubated at room temperature for 2 h in a humid container (antibody was diluted in 3% BSA/PBS). Unbound antibody was removed by four washes with PBS, followed by incubation with a horse radish peroxidase-conjugated anti-rabbit IgG (Cappel) for an additional 2 h at room temperature. After extensive washing with PBS, color detection was carried out using a chemiluminescence kit (Kirkegaard & Perry Laboratories). Data were collected by spectroscopy at 405 nm and analyzed with DeltasoftII software (Biometallics, Inc.).

Northern Analysis. α2(I) RNA was examined by Northern analysis of 10 μg total cytoplasmic RNA prepared as described previously (30). Hybridization probes were 32P-labeled human α2(I) cDNA (American Type Culture Collection) or GADPH, the latter to normalize for RNA loading.

Results

We have previously demonstrated that the peptidomimetic FTI L-739,749 induces phenotypic reversion of Rat1/ras, a Rat1 fibroblast cell line transformed by v-H-Ras, to a flattened, enlarged phenotype that resembles normal Rat1 cells. To explore the requirement for gene activation in this process, experiments were conducted in which the protein synthesis inhibitor cycloheximide was added with or without FTIs. Incubation of Rat1/ras cells with 10 μg/ml cycloheximide for periods up to 6 h did not produce any discernible cytotoxic effects or gross morphological changes. In contrast, when added during the first 6 h of cell exposure to FTIs, the kinetics of reversion were dramatically lengthened (Fig. 1). These results supported the hypothesis that gene induction is part of the cellular response to FTIs. To examine whether reversion was associated with changes in the expression of α2(I) collagen, which is suppressed in Ras-transformed cells (26, 28), we performed Northern analysis to examine α2(I) collagen levels in FTI-treated cells. As shown in Fig. 2a, the level of α2(I) collagen mRNA was low in Rat1/ras cells relative to normal Rat1 fibroblasts, and its level increased following FTI exposure, reaching the highest level after 16 h of drug treatment. In contrast, the levels of α2(I) collagen RNA were not affected by FTIs in either normal Rat1 or v-Raf-transformed Rat1 cells, the latter of which have been shown to be resistant to FTI-induced reversion (11). The kinetics of message elevation in Rat1/ras were well correlated with the kinetics of FTI-induced reversion in those cells. In addition, the final level of α2(I) collagen message in FTI-treated Rat1/ras cells was similar to that in

**Fig. 2.** FTIs derepress transcription of α2(I) collagen, which is suppressed by oncogenic Ras. A, Northern analysis. Rat1, Rat1/ras, or Rat1/raf cells (9, 11) were treated with 10 μM FTI L-744,832 for the times indicated (hr). Cytoplasmic RNA was isolated and examined by Northern analysis with α2(I) collagen and GADPH cDNA probes. Upper panel, α2(I) collagen mRNA level; middle panel, GADPH mRNA level as an internal control to normalize RNA loading; bottom panel, ethidium bromide-stained gel to confirm RNA integrity and loading. B, transient promoter assay. NIH 3T3 cells were transfected with v-Ha-Ras or empty vector plus a murine α2(I) collagen promoter-CAT reporter gene (a kind gift of B. de Crombrugghe, M.D. Anderson Cancer Center, Houston, TX). Cells were treated the next day with FTI or vehicle and processed the following day for normalized CAT activity.

**Fig. 1.** Protein synthesis inhibitors block reversion of the Ras-transformed phenotype by FTIs. Where indicated, Rat1/ras cells were treated 6 h with 10 μg/ml cycloheximide (which inhibits protein synthesis >95%) in the presence or absence of 20 μM FTI L-739,749. After this treatment, cells were washed and replenished with normal growth media or growth media containing 20 μM FTI L-739,749. Cell morphology was documented by photography 30 h later. CX, cycloheximide.
normal Rat1 cells, consistent with a role in phenotypic reversion. To determine whether derepression of α2(I) collagen by FTIs was transcriptional, we performed a set of transient promoter activity experiments in mouse NIH3T3 fibroblasts using a CAT reporter gene driven by 2 kb of 5’ flanking promoter sequences from the mouse α2(I) collagen gene (31). Cells were cotransfected with activated v-Ha-Ras or vector plasmids and the reporter gene, treated with FTI or vehicle only, and normalized CAT activity was determined 24 h later. As predicted, v-Ha-Ras repressed promoter activity, and FTI treatment derepressed this effect (Fig. 2b). These data supported the conclusion that FTIs up-regulated α2(I) collagen in Ras-transformed cells by reversing the transcriptional repression caused by oncogenic Ras.

To determine whether elevation of α2(I) collagen had a role in phenotypic reversion by FTIs, we generated antisense α2(I) collagen-expressing Rat1/ras cell lines or vector-only control cell lines and compared their response to drug treatment. Posttranscriptional effects are reported to contribute to suppression of type I collagen by oncogenic Ras (33, 34). Therefore, we determined levels of α2(I) collagen secreted by the cell lines by ELISA to verify the expected attenuation of endogenous expression by the antisense approach. Several vector and antisense lines were generated, the latter of which showed various reductions of endogenous expression by the antisense approach. Several vector and antisense lines were generated, the latter of which showed various reductions of endogenous expression by the antisense approach.

Because higher concentrations of FTI and longer exposure times were observed to promote a more flattened morphology (data not shown), similar to the case in parental Rat1/ras cells (11) and likely due to the fact that the antisense gene only partially inhibited expression of α2(I) collagen. Strikingly, at longer times after FTI addition, there was a dramatic difference in the phenotype of the vector and antisense cell lines (Fig. 5). Vector cells cultured in the absence of additional FTIs and passed while still subconfluent every 3 days continued to display a flat phenotype for up to 14 days after drug addition, as documented previously in Rat1/Ras cells (11). However, antisense-expressing cells displayed a transformed morphology at long times after FTI addition, a period within which farnesyltransferase activity and Ras farnesylation return to normal without additional drug treatment (11). We concluded that elevation of α2(I) collagen was necessary for FTIs to initiate reversion and also an essential event for stable persistence of the reverted phenotype.

Discussion

In this study, we provide the first evidence that gene activation is an important component of the mechanism by which FTIs cause reversion of Ras-transformed cells.

![Figure 3](image-url)

Fig. 3. Reduced expression of α2(I) collagen in Rat1/ras expressing an antisense gene. A, basal expression, protein. ELISA was performed using an anti-collagen α2(I) antibody and identical amounts of media taken from culture dishes to compare the level of α2(I) collagen secretion. Antisense clones 5 and 6 showing reduced α2(I) collagen levels relative to vector control cell lines, and the parental Rat1/ras cell line is shown. B, basal expression, message. Northern analysis was performed to compare steady-state levels of α2(I) message. Antisense clone 5 exhibited a reduction relative to the control line vector clone 1. Equivalent loading of RNAs was confirmed by ethidium bromide staining of the Northern gel. C, FTI-induced expression, protein. Relative level of α2(I) collagen secreted by vector clone 1 and antisense clone 5 cells into growth media treated 24 h with vehicle or FTI is shown.

![Figure 4](image-url)

Fig. 4. α2(I) collagen suppression blunts FTI-induced reversion. Rat1/ras cells expressing antisense α2(I) collagen or vector only were treated with 1 μM FTI L-744,832. Cells were photographed at the time of drug addition (t = 0) and 48 h later.
is initiated and maintained. The exact basis for the action of α2(I) collagen is unclear. However, as a long-lived protein, its deposition might help the reverted phenotype persist by suppressing the emergence of the transformed phenotype. To our knowledge, α2(I) collagen is the first FTI-regulated gene to be shown to be associated with phenotypic response to the drug. The α2(I) collagen promoter should therefore offer a sound model to dissect the Ras signaling pathways that are specifically disrupted in transformed and malignant cells by FTIs.

**Acknowledgments**

We thank B. de Crombrugghe for providing our laboratory with a subclone of the mouse collagen α2(I) promoter and A. Oliff for providing FTIs L-739, 749 and L-744-832.

**References**


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