Ras Farnesylation Inhibitor FTI-277 Restores the E-Cadherin/Catenin Cell Adhesion System in Human Cancer Cells and Reduces Cancer Metastasis

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The E-cadherin/catenin cell adhesion system is often down-regulated in epithelial tumors. This is thought to play an important role in cancer invasion and metastasis, and restoration of this system may suppress metastatic spread of cancer. In this study, the effects of a Ras farnesylation inhibitor (FTI-277) on E-cadherin-mediated cell-cell adhesion and metastatic potential were examined. In cell aggregation assays, FTI-277 stimulated aggregation of colon, liver and breast cancer cells. In vitro cultures of cancer cells showed that FTI-277 induced strong cell-cell contact. Immunoblotting analysis showed that FTI-277 increased E-cadherin/catenin (α, β and γ) expression and strongly stabilized E-cadherin/catenin with the actin cytoskeleton. Northern blotting studies indicated that the observed increase in the E-cadherin/catenin protein content was due to increased expression of their genes. After inoculation of the spleens of mice with severe combined immunodeficiency (SCID) with cancer cells, FTI-277 treatment for 3 weeks markedly reduced splenic primary tumor growth and the rate of liver metastasis compared with control counterparts. Our data demonstrate that FTI-277 can activate functioning of the E-cadherin-mediated cell adhesion system, which is associated with suppression of cancer cell metastasis. Therefore, selective inhibition of Ras activation may be useful for preventing cancer metastasis.

Key words: E-Cadherin — Catenin — Ras — FTI-277 — Metastasis

Cadherin-mediated cell-cell adhesion plays a critical role in the establishment and maintenance of cell polarity and cell society. The transmembrane glycoprotein E-cadherin represents the predominant cadherin in epithelial cells and is responsible for cell-cell contact by complexing with the actin cytoskeleton via three major cytoplasmic catenins (α-, β- and γ-catenin). Alterations in the expression or function of the E-cadherin/catenin cell adhesion system occur frequently in a wide variety of human carcinomas. Indeed, a causal role for the loss of E-cadherin during the transition from adenoma to invasive carcinoma has recently been demonstrated. However, the molecular mechanisms underlying the loss of expression or functionality of individual components of the E-cadherin/catenin complex in tumorigenesis are still poorly understood.

Ras is a signal-transducing, guanine nucleotide-binding protein for various membrane receptors and it participates in the regulation of cell proliferation, differentiation and morphology. Activated Ras oncogenes have been identified in various forms of human cancer, and several lines of evidence suggest that activation of Ras induces dys-function of the E-cadherin system. For example, microinjection of Ras oncogenes into Madin-Darby canine kidney (MDCK) cells induced adherens junction disassembly. In addition, introduction of the Ras oncogene into keratinocytes promoted loss of E-cadherin and α-catenin and relocation of β-catenin to the cytoplasm and nucleus.

A recent study demonstrated that down-regulation of the mitogen-activated protein kinase (MAPK) pathway resulted in restoration of epithelial morphology and assembly of tight junctions in Ras-transformed epithelial cells. These findings suggest that selective inhibition of Ras activation may restore E-cadherin-mediated cell adhesion and reduce the tendency for metastasis.

All nascent Ras proteins undergo a series of post-translational modifications in order to associate with the inner plasma membrane and thus attain biological activity. The first of these modifications is catalyzed by the enzyme farnesyltransferase (FTase). The farnesylation step alone has been shown to be sufficient for Ras-dependent transformation in some systems. These findings have led to the development of FTase inhibitors designed to prevent isoprenylation and, therefore, translocation of oncogenic Ras to the plasma membrane, thus inhibiting its constitutive activity.

Therefore, we examined whether a Ras farnesylation inhibitor (FTI-277) could enhance the adhesive function of the E-cadherin/catenin complex and inhibit metastatic properties of cancer cells. We also examined the effects of FTI-277 on the components of the E-cadherin/catenin complex. The general purpose of our work was to find agents that activate the E-cadherin-mediated cell adhesion system and which may, therefore, be candidates for anti-metastatic treatment of cancer.

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MATERIALS AND METHODS

Cell culture and treatments Human colon cancer cells (HT29, SW480, PMCO1), liver cancer cells (PLC/PRF/5, KYN-2, Li7, HepG2), and breast cancer cells (MCF-7, MDA-MB-468, BT-474) were used in this study. HT29, SW480, PLC/PRF/5, HepG2, MCF-7, MDA-MB-468 and BT-474 cells were obtained from the American Type Culture Collection (Rockville, MD) or the Japanese Cancer Research Resources Bank (Tokyo). PMCO1, KYN-2 and Li7 cell cultures were established as reported previously.13–15 They were maintained in RPMI-1640 (Gibco Laboratory, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2. FTI-277 was purchased from Calbiochem Co. (La Jolla, CA). Stock solution of this compound was prepared in 100% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) and stored at −70°C.

In vitro cell proliferation assay Cell viability was determined using an In Vitro Toxicology Assay Kit (Sigma) following the manufacturer’s instructions. Cells were seeded in 96-well plates at day 0. Starting at day 1, cells were treated for 2 days with each of a series of increasing concentrations of FTI-277 (10−7, 5×10−6 and 5×10−5 M). At the end of this period, cell proliferation was evaluated by a colorimetric assay based on cleavage of the 3-(4,5-dimethyldihydroxyphenyl) thiazol-2-yliumtetrazolium bromide (MTT) by mitochondrial dehydrogenase in viable cells, leading to formazan formation. This experiment was repeated three times with 10 determinations per tested concentration.

Ca2+-dependent aggregation Cell-cell adhesion was numerically evaluated in an aggregation assay, as described previously.16 In brief, cultures were rinsed with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered Ca2+-, Mg2+-free Hanks’ solution (HCFM), then dissociated into single cell suspensions under E-cadherin-saving conditions. They were then incubated under gyratory shaking at 80 rpm for 30 min in HCFM containing 1% bovine serum albumin and 1.25 mM Ca2+. The aggregation index was expressed as 1−(N0/Np), where N0 and Np indicate the initial number of particles and the number of particles after 30 min of aggregation, respectively, as measured by a hemocytometer. All N0 and Np measurements were done in triplicate and the experiments were repeated at least three times.

Cell extraction and immunoblotting For protein detection, cells were rinsed with a washing buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM sodium orthovanadate, and 0.02% sodium azide. The cells were then pelleted and lysed in a lysis buffer containing 1% Triton X-100, washing buffer, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and a tablet of protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany), and centrifuged at 15000g for 10 min at 4°C. Protein amounts were determined using a BioRad DC Assay Kit (BioRad, Hercules, CA). The proteins (2 µg) were separated on an 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, Massachusetts). Membranes were blocked with 5% skimmed milk, incubated with recommended dilution primary antibodies against E-cadherin and α-, β- and γ-catenin (Transduction Laboratories, Lexington, KY), then incubated with secondary antibodies after having been extensively washed. Antibody reaction was revealed using an enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL), used as instructed by the manufacturer. Equal protein loading of blotting membrane was confirmed by reprobing with anti-actin antibodies (Sigma) and Coomassie staining (BioRad).

Soluble and cytoskeletal fractions were prepared essentially as described previously.17 Cells were first lysed in 0.5% Triton X-100 containing washing buffer, 1 mM EGTA, 1 mM PMSF and a tablet of protease inhibitor cocktail, then centrifuged at 15000g for 30 min at 4°C. The supernant was considered the Triton-soluble pool. The pellet was solubilized in 1% SDS lysis buffer containing 25 mM Tris-HCl (pH 6.8), 1 mM PMSF and a tablet of protease inhibitor cocktail, and referred to as the Triton-insoluble (TI) pool.

RNA isolation and northern blotting RNA from human cancer cells was isolated with TRIZOL reagent (Gibco Laboratory) following the manufacturer’s instruction. Total RNA (20 µg) was separated on 1% agarose formaldehyde denaturing gel, followed by transfer to Hybond-N* (Amersham) by capillary blotting. Northern blot analysis was performed as described previously.18 Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA control hybridization probes were purchased from Clontech (Palo Alto, CA). The probes for E-cadherin and catenins (α, β and γ) were established as reported previously.19–23 and digoxigenin (DIG)-labeled with CDP-Star (Boehringer Mannheim) before use. Membranes were hybridized with the DIG-labeled probes in DIG Easy Hyb hybridization solution (Boehringer Mannheim), washed, then exposed to ECL film (Amersham). The signal intensities were quantified with a computerized imaging densitometer (Model GS-700, BioRad). The G3PDH signal was used to verify equal loading and blotting of RNA.

In vivo metastasis study Male mice (C.B-17/Icr Crj/Scid/scid) with severe combined immunodeficiency (SCID) were purchased from Charles River (Tokyo), and maintained in a specific pathogen-free environment. The animals received humane care and the studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Five- to six-week-old mice were used in this experiment. To
assay metastatic abilities, viable cancer cells were sus-
pended in serum-free medium, and 15 µl of the cell sus-
pension containing 1.5×10⁶ cells was inoculated into the
spleen of SCID mice under anesthesia. After inoculation,
the mice were randomized into two treatment groups
(n=6) and one control group (n=6). FTI-277 (1 and 5 mg/
kg/day) was administered daily for 3 weeks via the i.p.
route in a 1% DMSO vehicle as a volume of 0.10 ml of
solution/10 g body weight. The control group received
the same dosage/volume of the 1% DMSO vehicle. Adminis-
tration was initiated 1 day after cell inoculation. The mice
were killed 3 weeks after inoculation and autopsy was per-
formed immediately. The relative organ weight (spleen
and liver) was measured using the calculation formula:
organ weight/body weight ×100 (%). The splenic primary
tumor and liver metastases measured using the tumor vol-
ume calculation formula: length/2×width/2×height/2×4/
3×π. Statistically analyzed by means of Dunnett’s t test
and Nemenyi-Kruskal-Wallis multiple comparisons. Sig-
nificance was defined at the level of P<0.05.

RESULTS

Effects of FTI-277 on growth of cancer cell lines
As shown in Fig. 1, FTI-277 caused dose-dependent growth
inhibition of all the cancer cell lines studied. FTI-277 was
expected to induce 35–45% growth inhibition at 2×10⁻³
M. This concentration was chosen to study further the
effects of FTI-277 on cancer cell aggregation and expres-
sion of the E-cadherin/catenin complex.

Effects of FTI-277 on Ca²⁺-dependent cell aggregation
To examine whether down-regulation of the Ras signaling
pathway restores E-cadherin function, cells were cultured
with FTI-277 for 2 days and an aggregation assay was per-
formed. FTI-277 significantly increased aggregation of
most of the cancer cells (HT29, SW480, PMCO1, PLC/
PRF/5, KYN-2, Li7, MCF-7 and MDA-MB-468) com-
pared with the control counterparts, whereas although it
enhanced aggregation of HepG2 and BT-474 cells, the
increases did not reach significance (Fig. 2, A, B and C).
The increase in cell aggregation was E-cadherin dependent
because inclusion of an anti-E-cadherin antibody (HECD-
1) or the absence of Ca²⁺ abolished FTI-277-induced
aggregation (data not shown). Furthermore, FTI-277
induced morphological changes of cancer cells; the treated
cells showed much stronger cell-cell contacts than their
control counterparts (Fig. 2, D and E). Control data of Fig.
2 were shared with the article that we previously reported.

Effects of FTI-277 on E-cadherin protein expression
To test whether the influence of FTI-277 on E-cadherin
function correlated with E-cadherin expression in epithelial
cancer cells, immunoblotting analysis of E-cadherin
expression and protein localization was performed. As
shown in Fig. 3, immunoblotting analysis of total protein
extracts showed that total E-cadherin expression was
increased in cancer cells exposed to FTI-277 for 2 days.
Analysis of association with the cytoskeleton of E-cad-
herin showed that FTI-277 induced marked increases in
the E-cadherin level of the TI fraction. These results confirm that FTI-277 induces E-cadherin expression and strongly stabilizes the complexes of E-cadherin with the actin cytoskeleton.

**Effects of FTI-277 on catenin complex protein expression**  Three cancer cell lines (HT29, PLC/PRF/5 and MCF-7) were chosen for further study of the effects of FTI-277 on expression of the catenin complex. Fig. 4 shows examples of catenin (α, β and γ) immunoblotting analyses of cancer cells treated with FTI-277. After treatment with FTI-277 for 2 days, the total expression levels of catenins α, β and γ in HT29 cells were increased,
whereas in PLC/PRF/5 and MCF-7 cells, the total protein level of α-catenin was unchanged, but the β- and γ-catenin protein levels were slightly increased.

Analysis of association with the cytoskeleton of catenin complex showed that FTI-277 markedly increased catenin α, β, and γ levels in the TI fraction of HT29 cells. In PLC/PRF/5 and MCF-7 cells, however, it did not increase the α-catenin concentration, but did increase the β- and γ-catenin concentrations, in the TI fraction. Although the effect of FTI-277 on catenin complex expression differed according to the type of cancer cell analyzed, FTI-277 significantly stabilized the catenin complex with the actin cytoskeleton in a similar manner to that observed with E-cadherin.

Effects of FTI-277 on E-cadherin/catenin mRNA expression To investigate whether the observed increase in the E-cadherin/catenin protein content was attributable to altered E-cadherin/catenin gene expression, we carried out northern blotting analysis of total RNA from HT29 cells treated with FTI-277 for 2 days. FTI-277 increased the E-cadherin/catenin complex mRNA concentration compared with that of the control (Fig. 5).

Effects of FTI-277 on in vivo metastasis after splenic implantation FTI-277 effectively restored E-cadherin/
catenin complex expression in various human cancer cells and also up-regulated E-cadherin-mediated cell-cell adhesion in vitro. Therefore, we evaluated whether FTI-277 could also regulate metastasis of HT29 cells in SCID mice. We detected no toxicity, such as diarrhea or loss of body weight, in the FTI-277 treatment groups (Fig. 6E). Compared with control mice, FTI-277 treatment significantly reduced relative spleen weight and splenic primary tumor volume (Fig. 6, A and C) and also significantly reduced relative liver weight and liver metastasis volume (Fig. 6, B and D). Macroscopic examination also demonstrated that FTI-277 (Fig. 6G) dramatically reduced splenic primary tumor growth and liver metastasis compared with the control group (Fig. 6F). Control data of Fig. 6 were shared with the article that we previously reported.25)

Fig. 6. Effects of FTI-277 on splenic primary tumor and liver metastasis. HT29 cells (1.5×10⁶ cells) were inoculated into the spleen of SCID mice. After inoculation, either FTI-277 (1 and 5 mg/kg/day) or carrier solution (1% DMSO, control) was administered daily for 3 weeks i.p. to SCID mice (No. of mice/group=6). A and C show parameters of the splenic primary tumors. B and D show parameters of the liver metastatic tumor. E shows body weight change during the administration of FTI-277 (E: □ 0 mg/kg/day, ▲ 1 mg/kg/day, ● 5 mg/kg/day). Values represent mean±SD. * Significantly different from the control (P<0.05). F and G show the macroscopic appearance of splenic primary tumor and liver metastasis in the control group (0 mg/kg/day) and FTI-277 treatment group (1 mg/kg/day), respectively.

FTI-277 Prevents Cancer Metastasis
DISCUSSION

Dysfunction of the E-cadherin system plays an important role in tumor progression from relatively benign tumor to invasive, metastatic carcinoma. Therefore, E-cadherin-mediated cell adhesion could be a target for anticancer therapy. We previously reported that a Src family kinase inhibitor could be an anti-cancer candidate drug to restore the E-cadherin system in human cancer cells and to suppress cancer metastasis.25

In this study we examined whether the Ras farnesylation inhibitor FTI-277 could be a candidate. Our studies demonstrated that FTI-277 increased both E-cadherin/catenin protein and mRNA levels in human cancer cells. We speculated that FTI-277 might regulate the transcriptional active complex and induce the observed up-regulation of E-cadherin/catenin expression. However, the effects of FTI-277 on the extent of catenin expression varied in different cancer cells. It is possible that differences between the cell lines account for the variability in response to FTI-277.

The Ras-induced signaling pathway is mediated by multiple effector proteins and crosstalks with various factors including other small GTPases.4) Raf serine/threonine kinase is the best-characterized effector for Ras. Activated Raf phosphorylates and activates MEK (MAPK/ERK kinase). Subsequently, the activated MEK phosphorylates and activates MAPK and activated MAPK is translocated to the nucleus where it activates several transcription factors (AP-2, CAAT box and SP-1). Therefore, Ras signaling to the nucleus may regulate transcription factors via the Raf/MEK pathway and affect promoter elements of E-cadherin/catenin.26–28 However, whether one of the transcription factors is involved in the response to Ras inhibition remains to be elucidated.

In this study, in vitro cultures of cancer cells showed strong cell-cell contact after FTI-277 treatment. FTI-277 strongly stabilized the complex between E-cadherin/catenin and the actin cytoskeleton in human cancer cells and this effect correlated with increased cellular aggregation. Although Raf may play critical roles by regulating the mitogenic signaling involved in E-cadherin/catenin expression, several lines of evidence indicate that Raf alone is not sufficient to regulate the E-cadherin/catenin cell adhesion system.29, 30 For example, phosphatidylinositol 3-kinase (PI 3-kinase) interacts with activated Ras and is itself activated. PI 3-kinase is involved in actin cytoskeleton rearrangement, which regulates E-cadherin mediated cell-cell adhesion.31, 32 Furthermore, a critical role for Rho family GTPases in cell-cell adhesion in response to Ras activation is supported by a number of experimental observations.33, 34 Therefore, Ras may regulate cell-cell adhesion by effects on multiple effector proteins of Ras. Further studies will lead to a better understanding of how blockade of Ras signaling induces restoration of the down-regulated E-cadherin/catenin complex adhesion system in cancer cells.

It has been proposed that Ras antagonists could inhibit tumorigenicity.35, 36 Our in vivo study demonstrated that, compared with the control group, administration of FTI-277 markedly retarded splenic primary tumor growth, thus validating this suggestion. In addition, our present study also demonstrated that FTI-277 dramatically reduced the rate of metastasis in vivo compared with the control group. This result is, to the best of our knowledge, the first demonstration of antimitastatic activity of a Ras inhibitor in a liver metastasis model. However, we cannot conclude that Ras inhibition directly reduced metastatic potential by activating the cadherin system, because the Ras signaling pathway has also been shown to play critical roles in apoptosis and angiogenesis.37) Therefore, FTI-277-induced restoration of the cadherin system may be one of multiple mechanisms involved in the suppression of human cancer metastasis.

In summary, our studies suggest that when Ras is activated in certain cancer cells, inhibition of Ras activation might restore the E-cadherin-mediated cell-cell interaction and suppress tumor metastasis. Our work further predicts that if clinically useful anticancer drugs that specifically target Ras signaling are developed, they will be of particular benefit in the treatment of potentially metastatic tumors.

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