Src Family Kinase Inhibitor PP2 Restores the E-Cadherin/Catenin Cell Adhesion System in Human Cancer Cells and Reduces Cancer Metastasis

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ABSTRACT
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. Restoring this system may enable suppression of the metastatic spread of cancer. This study examined the effect of Src family kinase inhibitor PP2 on E-cadherin-mediated cell-cell adhesion and metastatic potentials. In cell aggregation assays, PP2 stimulated the aggregation of colon, liver, and breast cancer cells. In vitro cultures of cancer cells showed that PP2 induced strong cell-cell contact. Immunoblot analysis showed that PP2 enhanced E-cadherin/catenin expression and that increased E-cadherin/catenin proteins were strongly associated with the actin cytoskeleton. Northern blot studies indicated that the observed increase of E-cadherin/catenin protein content was due to their increased gene expression. After the spleens of severe combined immunodeficient mice were inoculated with cancer cells, treatment with PP2 for 3 weeks markedly reduced the rate of liver metastasis, compared with the control counterparts. Our data demonstrate that PP2 can activate the functioning of the E-cadherin/catenin complex and inhibit metastatic properties. Thus, selective inhibition of Src activation may be potentially useful in the prevention of cancer metastasis.

INTRODUCTION
The cadherins are a family of cell membrane glycoproteins that mediate tight adhesion between cells. E-cadherin is the major cadherin molecule expressed by epithelial cells (1). The cytoplasmic domains of E-cadherin connect with cytoskeletal actin filaments through cytoplasmic proteins called catenins, of which there are three different types (α-, β- and γ-catenin). The E-cadherin/catenin complex plays a critical role in establishing and maintaining the polarity and histological structure of cells. Dysfunction of the E-cadherin-mediated cell adhesion system plays an important role in tumor progression of the relatively benign tumor to invasive, metastatic carcinoma (2). Despite the obvious importance of cadherin function as a determinant of cell behavior and cell fate, the regulation of assembly and disassembly of cadherin-mediated cell-cell adhesions and the recruitment of signaling molecules to complexes at these adhesions are not well understood.

Src is a signal-transducing non-receptor protein kinase that plays central roles in the control of cell growth and differentiation. Overexpression and activation of Src family kinases have been identified in a range of human cancers (3). Previous reports demonstrated that c-Src kinases were enriched at the cell-cell adherens junctions of various types of cells (4). The introduction of the v-Src oncogene in Madin-Darby canine kidney cells led to the disruption of intercellular adhesion and induction of in vitro invasion (5). In addition, E-cadherin dysfunction was induced by c-Src activation from integrin-mediated cell-substratum adhesion in hepatocellular carcinoma cells (6). Furthermore, overexpression of activated c-Src in pancreatic cancer cells resulted in E-cadherin down-regulation and stimulated cell proliferation and migration (7). These findings suggest that the selective inhibition of Src activation could potentially restore E-cadherin-mediated cell adhesion and reduce metastatic tendencies.

We therefore examined whether a specific inhibitor of Src family kinases, PP2, could enhance the adhesive function of the E-cadherin/catenin complex and inhibit metastatic properties. We also examined the effect of PP2 on the components of the E-cadherin/catenin complex. The general purpose of our work is to find agents that activate the E-cadherin-mediated cell adhesion system and are therefore candidates for antimetastatic treatment of cancer.

MATERIALS AND METHODS
Cell Culture and Reagents. Human colon cancer cells (HT29, SW480, and PMCO1), liver cancer cells (PLC/PRF/5, KYN-2, Li7, and HepG2), and breast cancer cells (MCF-7, MDA-MB-468, and BT-474) were used in this study. HT29, SW480, PLC/PRF/5, MCF-7, MDA-MB-468, and BT-474 cells were obtained from the American Type Culture Collection (Manassas, VA) or the Japanese Cancer Research Resources Bank (Tokyo, Japan). PMCO1, KYN-2, and Li7 cell cultures were established as reported previously (8–10). They were maintained in RPMI 1640 (Life Technologies, Inc., 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. PP2 was purchased from Calbiochem (La Jolla, CA). Stock solution of this compound was prepared in 100% DMSO (Sigma, St. Louis, MO) and stored at −70°C.

**In Vitro Cell Proliferation Assay.** Cell viability was determined using an *in vitro* toxicity 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 PP2 (10⁻⁶, 10⁻⁵, and 10⁻⁴ M). At the end of this period, cell proliferation was evaluated by a colorimetric assay based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by mitochondria dehydrogenase in viable cells, leading to formazan formation. This experiment was repeated three times with 10 determinations/tested concentration.

**Ca²⁺-dependent Aggregation.** Cell-cell adhesion was evaluated numerically in an aggregation assay, as described previously (11). In brief, cultures were rinsed with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered Ca²⁺/Mg²⁺-free Hanks’ solution (HCMF) and 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 HCMF containing 1% BSA and 1.25 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 PMSF, and a tablet of protease inhibitor mixture and referred to as the TI pool. The supernatant was considered the TS 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 mixture and referred to as the TI pool.

**In Vitro Kinase Assay.** Cells were lysed and immunoprecipitated with anti-Src antibodies as described previously (6). The tyrosine kinase activity of immune complexes containing Src was determined with a Takara tyrosine kinase assay kit (Taka Shuzo, Shiga, Japan) according to the manufacturer’s instructions. This experiment was repeated three times.

**RNA Isolation and Northern Blotting.** RNA from human cancer cells was isolated with Trizol reagent (Life Technologies, Inc.) following the manufacturer’s instructions. 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 (14). Human G3PDH cDNA control hybridization probes were purchased from Clontech (Palo Alto, CA). The probes for E-cadherin and catenins (α, β, and γ) were prepared as reported previously (15–18) and 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, and then exposed to enhanced chemiluminescence film (Amerham). The signal intensities were quantified with a computerized imaging densitometer (model GS-700; Bio-Rad). The G3PDH signal was used to verify equal loading and blotting of RNA.

**In Vivo Metastasis Study.** Male SCID mice (C.B.-17/Icr Crj-scid/scid) were purchased from Charles River (Tokyo, Japan) and maintained in a specific pathogen-free environment. The animals received human care, and the studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Five- to 6-week-old mice were used in this experiment. To assay metastatic abilities, viable cancer cells were suspended in serum-free medium, and 15 μl of the cell suspension containing 1.5 × 10⁶ cells were 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). PP2 (1 and 5 mg/kg/day) was administered daily for 3 weeks via the i.p. route in a 1% DMSO vehicle. Administration was initiated 1 day after cell inoculation. The mice were killed 3 weeks after inoculation, and autopsies were performed immediately. The relative organ weight (spleen and liver) was measured using the following formula: organ weight/body weight × 100 (%). The splenic primary tumor and liver metastases were measured using the tumor volume calculation formula: length/2 × width/2 × height/2 × 4/3 × π (19).

**Statistical Analysis.** Parametric and nonparametric data were statistically analyzed by Dunnett’s *t* test and Nemenyi-Kruskal-Wallis multiple comparisons. Significance was defined at the level of *P* < 0.05.

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3 The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; SCID, severe combined immunodeficient; DIG, digoxigenin; TS, Triton-soluble; TI, Triton-insoluble.
RESULTS

Effect of PP2 on Cancer Cell Growth and Src Kinase Activity in Vitro. As shown in Fig. 1, PP2 caused a dose-dependent growth inhibition of all cancer cell lines. In vitro kinase assay revealed that PP2 decreased the Src activity of HT29 cells in a dose-dependent manner (Fig. 2A). PP2 induced 40–50% growth inhibition at 10^{-6}, 10^{-5}, and 10^{-4} M. This concentration reduced the Src activity as early as 1 h and maintained a 35% inhibition of Src activity for 2 days (Fig. 2B). This concentration was chosen to further study the effect of PP2 on the regulation of the cadherin system.

Effect of PP2 on Ca^{2+}-dependent Cell Aggregation. To examine whether inhibition of Src activity restores the functioning of E-cadherin, cells were cultured with PP2 for 2 days, and an aggregation assay was performed. PP2 significantly increased aggregation in most of the cancer cells (HT29, SW480, PMCO1, PLC/PRF/5, KYN-2, Li7, MCF-7, and MDA-MB-468); it enhanced aggregation in HepG2 and BT-474 cells, but the increase was not significantly different from the control counterparts (Fig. 3, A–C). The increase in cell aggregation was E-cadherin dependent because the inclusion of anti-E-cadherin antibody (HECD-1) or the absence of Ca^{2+} abolished PP2-induced aggregation (data not shown). In addition, PP2 induced morphological changes in cancer cells; the treated cells were strongly compacted with their control counterparts, representing a tightening of cell adhesion (Fig. 3, D and E).

Effect of PP2 on E-Cadherin Protein Expression. To test whether the influence of PP2 on E-cadherin function is correlated with expression of E-cadherin in epithelial cancer cells, E-cadherin expression and protein localization were examined by immunoblot analysis. As shown in Fig. 4, immunoblot analysis of total protein extracts showed that total E-cadherin expression increased in cancer cells exposed to PP2 for 2 days.

Analysis of the distribution of E-cadherin in the TS and TI fractions of cancer cells showed that PP2 markedly increased the concentration in both TS and TI fractions. These results confirm that PP2 enhances E-cadherin expression and also strongly increases E-cadherin’s association with the actin cytoskeleton.
Effect of PP2 on Protein Expression of the Catenin Complex. Three cancer cell lines (HT29, PLC/PRF/5, and MCF-7) were chosen to further study the effect of PP2 on expression of the catenin complex. Fig. 5 shows examples of catenin (α-, β-, and γ-catenin) immunoblot analyses for cancer cells treated with PP2. After treatment with PP2 for 2 days, total expression of α-, β-, and γ-catenin increased in HT29 cells, whereas in PLC/PRF/5 and MCF-7 cells, the total protein level of α-catenin did not change, but the levels of β- and γ-catenin increased slightly.

In analysis of the distribution of catenin complex in the TS and TI fractions, PP2 induced localization of α-, β-, and γ-catenin in both the TS and TI fractions in HT29 cells. In PLC/PRF/5 and MCF-7 cells, PP2 did not increase the concentration of α-catenin in either the TS or TI fraction, but it did increase the concentration of β- and γ-catenin in both fractions. In a manner similar to that of E-cadherin, PP2 enhanced catenin complex expression and strongly increased catenin complex association with the actin cytoskeleton.

Effect of PP2 on E-Cadherin/Catenin mRNA Expression. To investigate whether the observed increase of E-cadherin/catenin protein content was attributable to altered E-cadherin/catenin gene expression, we carried out Northern blot analysis of total RNA from HT29 cells after PP2 treatment for 2 days. PP2 increased the mRNA concentration of E-cadherin/catenin, as compared with the control counterparts (Fig. 6).

Effect of PP2 on In Vivo Metastasis after Orthotopic Implantation. PP2 effectively restored E-cadherin/catenin complex expression in various human cancer cells and also up-regulated E-cadherin-mediated cell-cell adhesion in vitro. We therefore evaluated whether these activities of PP2 could also regulate metastasis of HT29 cells in SCID mice. The relative spleen weight (Fig. 7A) and splenic primary tumor volume (Fig. 7C) were smaller in PP2-treated groups compared with the control group, although the differences were not statistically significant. Accordingly, these results show that inhibition of Src family kinases induces some slowing in the growth rate of the primary tumors relative to the control treated with vehicle. On the other hand, PP2 treatment significantly reduced the relative liver weight and liver metastasis volume compared with the controls (Fig. 7B and D). Macroscopic findings also showed that PP2 (Fig. 7F) markedly reduced liver metastasis as compared with the control (Fig. 7E). Accordingly, these results reveal that inhibition of Src family kinases suppresses liver metastasis in this experimental model.
DISCUSSION

In this study, we demonstrated that the Src inhibitor PP2 enhanced cell-cell adhesiveness in a Ca\(^{2+}\)-dependent cell aggregation assay and by morphological findings. Additionally, PP2 caused concurrent up-regulation of E-cadherin/catenin protein and mRNA in human cancer cells. Menke et al. (7) proposed that cell-substratum adhesion could down-regulate E-cadherin gene expression in a Src kinase-dependent manner. Our results validated this suggestion. Accordingly, we speculated that Src inhibition might regulate the transcriptional activation complex and induce the observed up-regulation of E-cadherin/catenin expression. However, the effects of PP2 on the extent of expression of catenin complex showed variation in cancer cells. It is possible that differences between the cell lines may account for the variability in response to PP2.

Until now, there has been limited information about the mechanism of Src regulation of gene expression. It appears that Src is able to enhance binding of the SP-1 transcription factor to the promoter of the urokinase receptor (20). In addition, Src induces binding of transcription factors to the CCAAT box, such as nuclear factor \(\gamma\), which was found to be necessary to promote expression of the osteopontin and bone sialoprotein promoter (21, 22). Promoter activity of E-cadherin appears to be driven mainly by the presence of constitutive transcription factors (CCAAT-binding proteins, SP-1, and AP2) and the absence of repressors [Ets factors and Snail (23)]. In future studies, we hope to be able to show which transcription factors regulate gene expression of E-cadherin/catenin in response to Src activity.

Our results showed that blockade of Src activation up-regulated E-cadherin/catenin gene expression, but we cannot exclude the possibility that an additional mechanism might regulate function of the E-cadherin/catenin adhesion complex. It has been reported that Src activation could change the phosphorylation status of the E-cadherin/catenin complex and perturb cadherin-mediated cell adhesion (5, 24). In addition, the elevation of Src kinase activity appears to induce the dysfunction of cadherin through tyrosine phosphorylation of the other junctional proteins, ERM, ZO-1, or unidentified proteins (25). Src
has also been shown to interact with downstream effectors of Rho and induce cytoskeleton remodeling, suggesting that these effects influence E-cadherin-mediated cell-cell adhesion (26, 27). With respect to these observations, additional studies will lead to a better understanding of how blockade of Src activation induces restoration of the E-cadherin/catenin cell adhesion system.

Decreased Src activity has been shown to inhibit the rate of xenograft tumor growth in a nude mouse model (28, 29). Our in vivo study also showed a tendency for administration of PP2 to retard primary tumor growth relative to the control, although the difference was not statistically significant.

It has been proposed that Src activation can induce metastatic potentials by inactivation of the cadherin system (6, 30). Our present study demonstrated that PP2 restored E-cadherin-mediated cell adhesion in vitro and markedly reduced the rate of metastasis in vivo. Our results thus validated this thinking. However, activated Src has also been shown to decrease apoptosis and stimulate motility and angiogenesis (31, 32). Furthermore, He et al. (33) showed that a Src family kinase inhibitor, PP1, inhibited Ras-induced p21-Cdc42/Rac-activated kinase activation and malignant transformation both in vitro and in vivo. Accordingly, PP2 could inhibit cancer metastasis through alternative mechanisms. With respect to these observations, additional studies are essential to elucidate the mechanism by which Src inhibition regulates cancer metastasis.

In summary, our studies suggest that in certain cancer cells, when Src is activated, its inhibition could restore the E-cadherin/catenin cell adhesion system. Moreover, because we detected no toxicity such as diarrhea or loss of body weight in the PP2-treated groups, long-term administration of the Src inhibitor at therapeutic doses or in combination with other approaches such as surgical resection may be useful for the prevention of metastasis in cancer patients.

REFERENCES


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