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All-*trans*-retinoic Acid-dependent Inhibition of E-Cadherin-based Cell Adhesion with Concomitant Dephosphorylation of β-Catenin in Metastatic Human Renal Carcinoma Cells

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We previously described an *in vitro* invasion assay model, using a monolayer of vascular endothelial cells grown on collagen gel, that mimics the metastatic abilities of the highly metastatic human renal carcinoma cell lines, MM-1,3 and 8 and their poorly metastatic counterparts, SN12C and Cl-8. MM-1, 3 and 8 cells were observed to penetrate the monolayer of vascular endothelial cells and grew in a spreading or scattering manner with loose cell-cell contact on collagen gel or on vascular endothelial cells. SN12C and Cl-8 cells failed to penetrate and grew in a clustering manner with tight cell-cell contact. Treatment with all-*trans*-retinoic acid (ATRA) at non-toxic concentrations induced clustering or growth of MM-1, 3 and 8 cells on collagen gel or on vascular endothelial cells with tight cell-cell contact, and inhibited penetration. The clustering induced by ATRA was virtually blocked in the presence of anti-E cadherin antibody. E-Cadherin and β-catenin were each localized mainly at the cell-cell adherent junctions of colonizing cell populations that had been treated with ATRA. While the cellular levels of E-cadherin and β-catenin did not change significantly following ATRA treatment, the tyrosine residue of β-catenin was rapidly dephosphorylated. The concomitant administration of Na vanadate, an inhibitor of tyrosine dephosphorylase, inhibited both the ATRA-induced clustering and the dephosphorylation of β-catenin tyrosine. ATRA-induced clustering of MM-3 cells may be linked to the state of tyrosine phosphorylation of β-catenin.

Key words: ATRA — Invasion — E-Cadherin — β-Catenin

The mechanism underlying tumor metastasis has been studied for more than a decade.[1,2] A loss of expression of E-cadherin and mutation of the gene to an inactive form are now known to occur in cancers of stomach, pancreas, esophagus, lung, bladder, and breast, and in other types of tumors.[3–5] A close interaction between the cytoplasmic domain of cadherin and the actin filaments is required for a strong cell-cell adhesion. The catenins are cytoplasmic anchorage proteins[6]: α-catenin is a homologue to vinculin. Evidence indicates that α-catenin does not bind directly to the cytoplasmic domain of cadherins, but is important in cadherin function. β-Catenin, a homologue of plakoglobin, a component of adherent junctions,[7] binds directly to the cytoplasmic domain of E-cadherin,[8] and is phosphorylated at tyrosine residues in cancer cells that express v-src.[9]

The invasiveness of tumor cells has been studied extensively. Matrigel assays are often used for this purpose.[10] In the Matrigel model, which employs a reconstituted basement membrane, type IV collagenase plays a main role in the invasion of human glioma cells or of HRCC, and the addition of exogenous metalloproteinase inhibitors blocks the invasion.[10,11] However, the metastatic properties of various HRCC lines *in vivo* cannot be correlated with their invasive properties *in vitro* in the Matrigel model.[12] Even when a modified model of Matrigel coated on a porous filter in a Boyden chamber was used,[13] no overall correlation could be established between invasion ability *in vitro* and metastatic potential *in vivo*.[14]

Various tumor cell lines exhibit differing abilities to adhere to vascular endothelial cells.[15] The introduction of vascular endothelial cells into the invasion models should therefore be useful for predicting tumor cell invasiveness *in vivo*. We recently established a modified assay for tumor invasiveness using CPAE cells grown on type I collagen overlaid with type IV collagen. In this model, the metastatic potentials *in vivo* of various HRCC lines are closely associated with their invasive proper-

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[7] The abbreviations used are: HRCC, human renal carcinoma cell; ATRA, all-*trans*-retinoic acid; CPAE, calf pulmonary arterial endothelial; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EGF-R, epidermal growth factor-receptor; mAb, monoclonal antibody; HGF, hepatocyte growth factor; IFN-2α, interferon 2α.

ties.[12] This finding suggests that the interaction of tumor cells with vascular endothelial cells and/or extracellular matrix is necessary for the evaluation of the invasive or metastatic properties of tumor cells. The poorly metastatic HRCC lines were observed to grow in a colonizing or clustering manner, whereas the highly metastatic HRCC lines grew in a scattering or spreading manner on the monolayer of CPAE cells.[12] This observation indicates that the manner of growth of tumor cells, in either a spreading or a clustering style on collagen gel or on vascular endothelial cells, is linked to their invasive or metastatic potential.

We sought to determine whether altered expression of the E-cadherin and β-catenin system was involved in the differences in growth behavior of poorly metastatic HRCC lines, SN12C and Cl-8, and highly metastatic HRCC counterparts, MM-1, 3 and 8. We also evaluated whether ATRA would modulate the growth behavior of these cells on collagen gel or the invasive properties of MM-1, 3 and 8 cells.

MATERIALS AND METHODS

Materials Solutions of type I collagen extracted from porcine skin (Cellmatrix I-A) and type IV collagen extracted from bovine lens (Cellmatrix IV) were purchased from Nitta Gelatin Inc. (Osaka). Anti-human E-cadherin mAb (HECD-1) was purchased from Takara (Tokyo) and anti-mouse β-catenin was purchased from Laboratory Transduction (Lexington, KY). Mouse mAb PY-20 to phosphotyrosine was purchased from ICN (Costa Mesa, CA). Anti-human EGF-R polyclonal antibody was purchased from Oncogene Science Inc. (Cambridge, MA). ATRA was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture and *in vitro* invasion assay systems CPAE cells were isolated from the calf pulmonary artery and cultured in DMEM containing 10% FBS, penicillin, 100 U/ml, and kanamycin, 60 μg/ml, as reported.[12] Ten to 15 passages of CPAE cells were used. The poorly metastatic Cl-8 was isolated from parental SN12C by a double limiting dilution technique. Highly metastatic counterparts, MM-1, 3 and 8, were isolated from lung metastases of SN12C cells that had been injected into the renal subcapsule of nude mice.[16] SN12C had been established in cultures of a primary renal cell carcinoma of a 43-year-old man. Its metastatic properties have been described.[12, 16] The *in vitro* invasion assay, with or without endothelial cells, is illustrated in Fig. 1. Type I collagen gel (500 μl), containing 400 μl of Cellmatrix I-A, 50 μl of 10× DMEM, and 50 μl of reconstitution buffer, was placed into each 35 mm culture dish and incubated for 1 h at 37°C. A volume of 1 ml of type IV collagen (Cellmatrix IV) was dissolved in 14 ml of HCl (1×10^{-3} *N*, pH 3.0). A volume of 0.5 ml of this solution was overlaid upon the type I collagen gel and left for 30 min at room temperature. CPAE cells (4×10^5) were seeded onto the collagen gels and incubated at 37°C until they attained confluence. Human renal carcinoma cells (1×10^3 cells each) were seeded onto the confluent CPAE cells (Fig. 1 A) or the collagen gels (Fig. 1B) and incubated at 37°C for 9 days as previously reported.[12] ATRA were added at 1 day after the renal carcinoma cells had been plated. On day 9, the dishes were stained with May-Gruenwald-Giemsa and the number of cancer cells that penetrated the CPAE cell layer and type IV collagen sheet into the type I collagen gel were counted in 20 microscopic fields (×100 magnified).

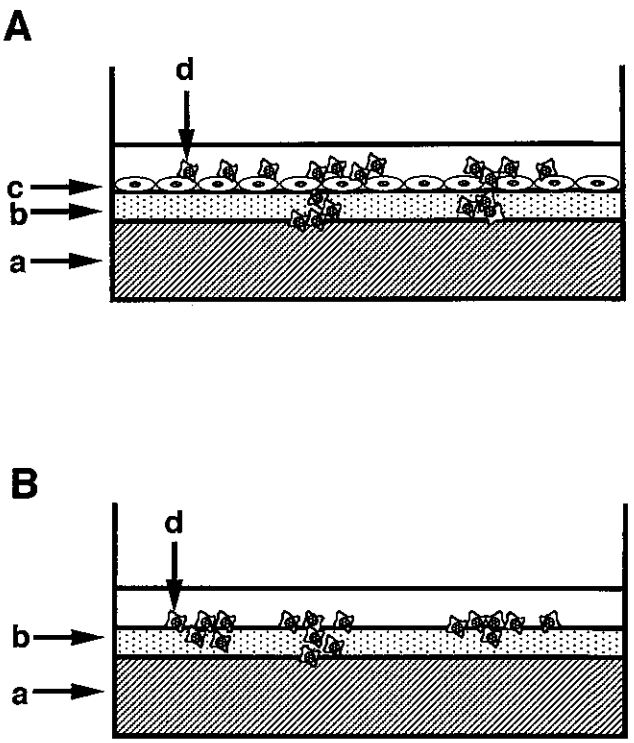

Fig. 1. Depiction of *in vitro* system for assay of invasion with(A) or without(B) CPAE cells used in the present study. (a) type I collagen gel, (b) type IV collagen sheets, (c) monolayer of CPAE cells, (d) carcinoma cells. Type I collagen gel was put into the dishes and overlaid with type IV collagen. CPAE cells were seeded onto the type IV collagen sheets and type I collagen gel, and incubated until they attained confluence. Carcinoma cells were subsequently seeded on the monolayer of CPAE cells (A) or on the type IV-I collagen (B). On day 9, the dishes were stained with May-Gruenwald-Giemsa.

Immunoprecipitation and immunoblotting Cells on collagen-coated dishes were incubated with DMEM con-

taining 10% FBS and treated with ATRA or Na vanadate for various periods. The cells were then lysed with the addition of 0.5 ml of cold immunoprecipitation buffer (1% Triton X-100, 150 m*M* NaCl, 10 m*M* Tris pH 7.4, 1 m*M* EDTA, 0.2 m*M* Na vanadate, 0.2 m*M* PMSF, 0.5% NP-40), scraped, and passed several times through a 26 gauge needle to disperse large aggregates. β-Catenin and other proteins which were immunoprecipitated were examined as described previously.[17]

Immunohistochemistry Type I collagen gel and a type IV collagen sheet were placed upon each chamber slide (Nunc Inc., IL). The cells were then seeded onto the gel. After incubation for 9 days the slide containing the cells was washed briefly in PBS and fixed in 2% paraformaldehyde. For immunohistochemical analysis, the sections were permealyzed for 15 min in TBS (10 m*M* Tris, pH 7.6, 150 m*M* NaCl, 0.1% Triton X-100), then blocked with 1.5% skim milk for 1 h. Next, the primary antibody, a monoclonal mouse antibody against human E-cadherin (diluted 1 : 1000 in TBS) or mouse β-catenin (diluted 1 : 1000 in TBS), was added and allowed to remain for 1 h. After three washings, a secondary antibody, FITC-conjugated Goat antimouse IgG (Organon Teknika, N. V., Tuonhout), diluted 1 : 100, was added for 1 h. The sections were then mounted and examined by confocal laser microscopy.

RESULTS

Cellular growth behavior of poorly and highly metastatic HRCC lines We first evaluated the morphology of colonies of SN12C, Cl-8, MM-1, 3 and MM-8 cells seeded onto type IV-I collagen gel. The horizontal spreading of colony formation differed greatly in the poorly vs. the highly metastatic cell lines on collagen gel (Figs. 2 and 3). Horizontal spreading as concentric circles with a tight homotypic adhesion was observed in SN12C and Cl-8 cells. MM-1, 3 and 8 showed random horizontal spreading and loose adhesion (Figs. 2 and 3). Treatment with ATRA at 10^{-6} *M* altered the scattering of the untreated MM-1, 3 and 8 cells to horizontal spreading as concentric circles with tight adhesion (Fig. 3). The morphology of Cl-8 and SN12C on CPAE cells was the same as that on type IV-I collagen gel, and was not altered by ATRA treatment (Fig. 3). Highly metastatic cells showed a scattered growth pattern, which was altered to concentric circles after treatment with ATRA (Fig. 3).

Treatment with ATRA, 10^{-8} *M*, inhibited the penetration of MM-3 cells through the CPAE cells by 50% in the invasion assay model system. Proliferation of MM-3 cells was unaffected by ATRA up to 10^{-5} *M*, whereas a concentration of 10^{-4} *M* inhibited cell growth by more than 50% of the control value (data not shown). ATRA

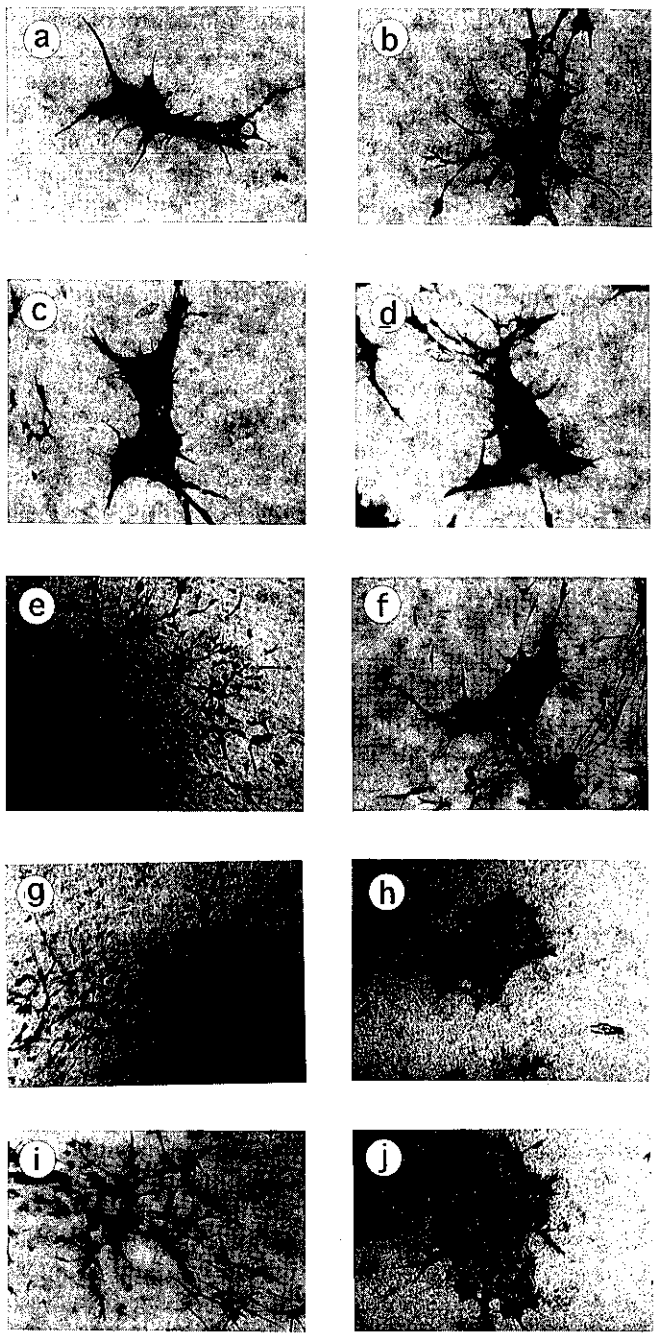

Fig. 2. Growth pattern of poorly and highly metastatic renal cancer cell lines on type IV-I collagen gel. Poorly metastatic cell lines, SN12C (a and b) and Cl-8 (c and d), highly metastatic cell lines, MM-1 (e and f), MM-3 (g and h) and MM-8 (i and j), and non-treated cells (a, c, e, g and i) and ATRA-treated cells(b, d, f, h and j) were seeded on type IV-I collagen sheets and incubated for up to 9 days. ATRA (1×10^{-6} *M*) was added 1 d after the cells had been plated. The samples were stained with May-Gruenwald-Giemsa and observed under a light microscope ($\times$100 magnification).

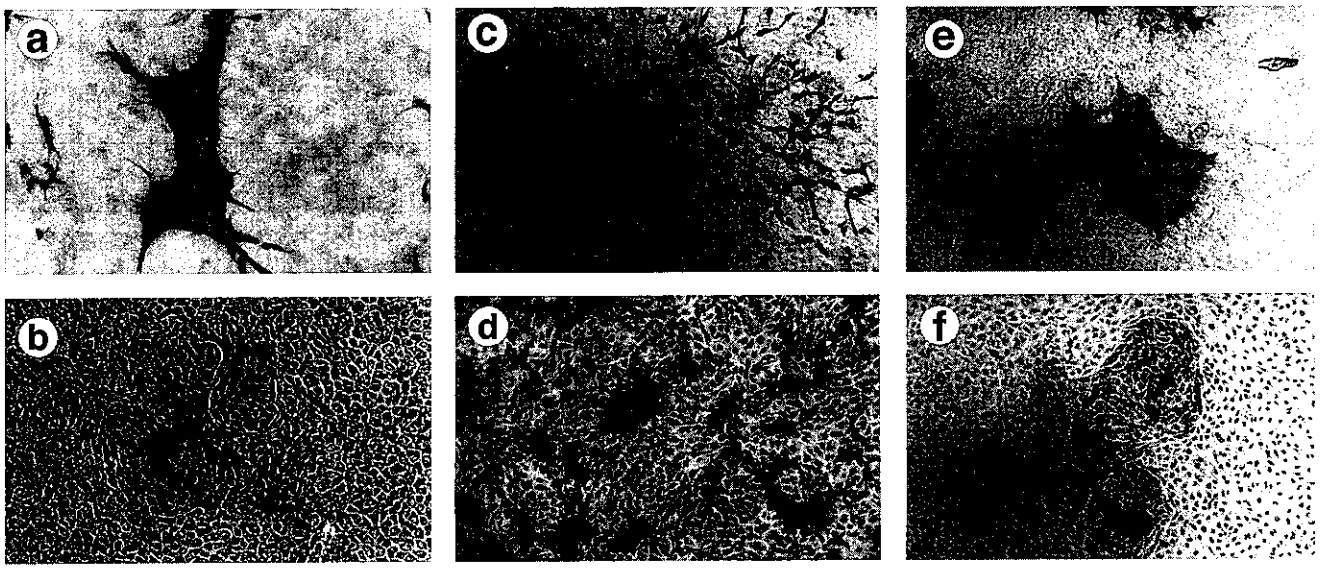

Fig. 3. Growth pattern of poorly and highly metastatic renal cancer cell lines. Poorly metastatic cell line Cl-8 (a and b), highly metastatic cell line MM-3 (c and d), and ATRA-treated MM-3 (e and f) were seeded on type IV-I collagen sheets (a, c and e) or on CPAE cells (b, d and f) and incubated for up to 9 days. ATRA (1×10^{-6} *M*) was added 1 d after the MM-3 cells had been plated (e and f). The samples were stained with May-Gruenwald-Giemsa and observed under a light microscope (× 100 magnification).

thus appeared to modulate specifically the invasion or penetration of the HRCC rather than cell proliferation in general.

Involvement of E-cadherin/β-catenin in morphological changes induced by ATRA We first sought to determine whether E-cadherin was involved in the ATRA-induced changes in the horizontal spread of MM-3. Administration of anti-E-cadherin antibody inhibited almost completely the ATRA-induced concentric circles with tight adhesion of MM-3 cells on collagen gel (Fig. 4). The growth behavior of MM-3 cells treated with 10^{-5} or 10^{-6} *M* ATRA in the presence of anti-E-cadherin antibody resembled that of untreated cells, suggesting an involvement of E-cadherin in the cell clustering with tight adhesion. We next compared the distribution of E-cadherin and β-catenin in MM-3 cells in the absence and presence of ATRA. Both E-cadherin and β-catenin were localized mainly on the cell surface at the adherent junctions of colonizing cells in the presence of ATRA, 10^{-6} *M* (Fig. 5, c and d). By contrast, both E-cadherin and β-catenin were diffusely distributed in an unorientated manner in untreated MM-3 cells grown on collagen gel (Fig. 5, a and b). We did not observe any marked difference between untreated and treated MM-3 cells in the organization of β-actin (unpublished result).

Changes in β-catenin phosphorylation and morphology induced by ATRA Using western blot analysis, we examined the cellular level of E-cadherin during treatment with ATRA. There were no apparent changes in cell level of E-cadherin of MW 120 kDa in MM-3 cells treated with ATRA at 10^{-6} *M*, although a slight increase occurred 3 h after treatment (Fig. 6A). When we examined the expression of β-catenin by western blot analysis, we found no apparent change in the cellular β-catenin level during treatment with ATRA (Fig. 6C). Phosphorylation of β-catenin tyrosine was observed in untreated MM-3 cells (Fig. 6C). Phosphorylation of β-catenin tyrosine was decreased to 20% of that of untreated controls at 1 h after the addition of ATRA, and was almost completely abolished at 3 h (Fig. 6C). Phosphorylation of β-catenin tyrosine was almost completely abolished at day 3 (unpublished results). Unlike MM-3 cells, Cl-8 cells grew in a clustering manner on endothelial cells or on collagen gels (Fig. 2). We compared β-catenin phosphorylation in Cl-8 and MM-3 cells in the absence or presence of ATRA. The phosphorylation of β-catenin tyrosine was less in Cl-8 cells than in MM-3 cells, and there appeared to be no inhibition of phosphorylation of β-catenin tyrosine by ATRA in Cl-8 cells (Fig. 7B). Phosphorylation of β-catenin tyrosine was almost completely inhibited by ATRA in MM-3 cells. Tyrosine phosphorylation of the EGF receptor was not affected by ATRA in either Cl-8 or MM-3 cell lines (Fig. 7C). The ATRA-induced dephosphorylation appeared to be rather specific for β-catenin in the HRCC line.

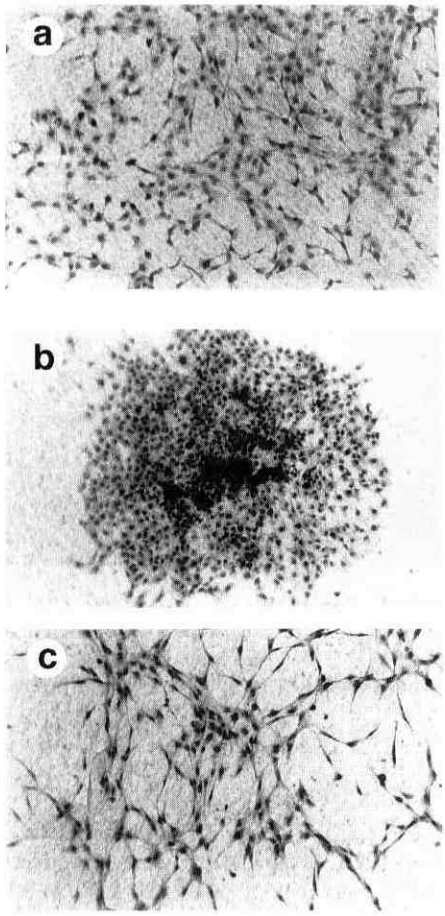

Fig. 4. Inhibition of the clustering of the ATRA-treated MM-3 cells by E-cadherin mAb. ATRA (1×10^{-6} *M*) and E-cadherin mAb were added 1 d after MM-3 cells had been plated. (a) Untreated MM-3 cells, (b) MM-3 cells treated with 10^{-6} *M* ATRA, (c) MM-3 cells treated with 10^{-6} *M* ATRA and E-cadherin mAb at 1 μg/ml.

To test further for a possible correlation between the state of β-catenin phosphorylation and morphological change in the horizontal spread of ATRA-treated MM-3 cells, we examined the effects of Na vanadate, an inhibitor of tyrosine dephosphorylase. As is shown in Fig. 8, treatment with 10^{-6} *M* ATRA almost completely blocked phosphorylation of β-catenin tyrosine. However, the concomitant administration of 10^{-6} or 10^{-5} *M* Na vanadate almost completely inhibited the ATRA-induced dephosphorylation (Fig. 8). Treatment with 10^{-6} *M* ATRA again induced colonies of MM-3 cells with cell-cell tight contact, but the concomitant administration of ATRA with 10^{-6} *M* Na vanadate completely blocked the ATRA-induced colonizing appearance of MM-3 cells, and instead, spreading colonies with a small number of

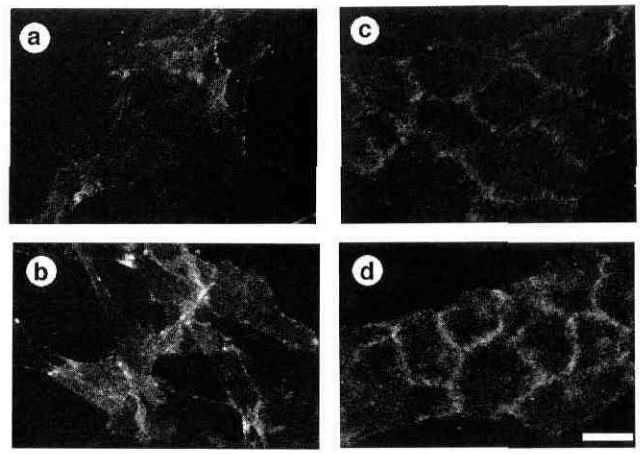

Fig. 5. Expression and localization of E-cadherin and β-catenin of MM-3 cells treated with or without ATRA. Untreated (a and b) and ATRA-treated (c and d) MM-3 cells were cultured on type IV-I collagen sheets for 9 d, then fixed, permeabilized and stained with antibodies for E-cadherin (a and c) or β-catenin (b and d). bar, 10 μm.

cells appeared (Fig. 9). It appeared likely that phosphorylation of β-catenin tyrosine is linked to the ATRA-induced change in cell-cell contact of MM-3 cells.

DISCUSSION

We recently found that an *in vitro* invasion assay system using CPAE cells mimics the vascular metastatic situation between cancer cells and vascular endothelial cells: highly metastatic HRCC such as MM-1, 3 and 8 are more invasive, and poorly metastatic HRCC such as SN12C and Cl-8, are less invasive.[12] Nakayama *et al.*[12] also demonstrated that, on CPAE cells in monolayer, SN12C and Cl-8 evinced horizontal spreading of colonies with many cells as concentric circles with tight junctions, and MM-1, 3 and 8 evinced a scattered growth pattern.

The invasive potentials of five HRCC lines appear to be associated with their scattered growth pattern on vascular endothelial cells or on collagen gel.[12] In this study, SN12C and Cl-8 cells tended to form colonies, and MM-1, 3 and 8 cells tended to be scattered with loose cell-cell contact, in association with extracellular collagens or vascular endothelial cells (Fig. 2). As MM-3 was the most highly metastatic cell line *in vivo*[16] and *in vitro*,[12] we used MM-3 in this study. Retinoic acid modulates transformed phenotype and differentiation in many tumor cell types in culture and *in vivo*.[18] It has been evaluated for potential use in prevention and treatment of human cancer.[19] Saiki *et al.*[20] reported that the collagenolytic activity of low-metastatic cell lines was very

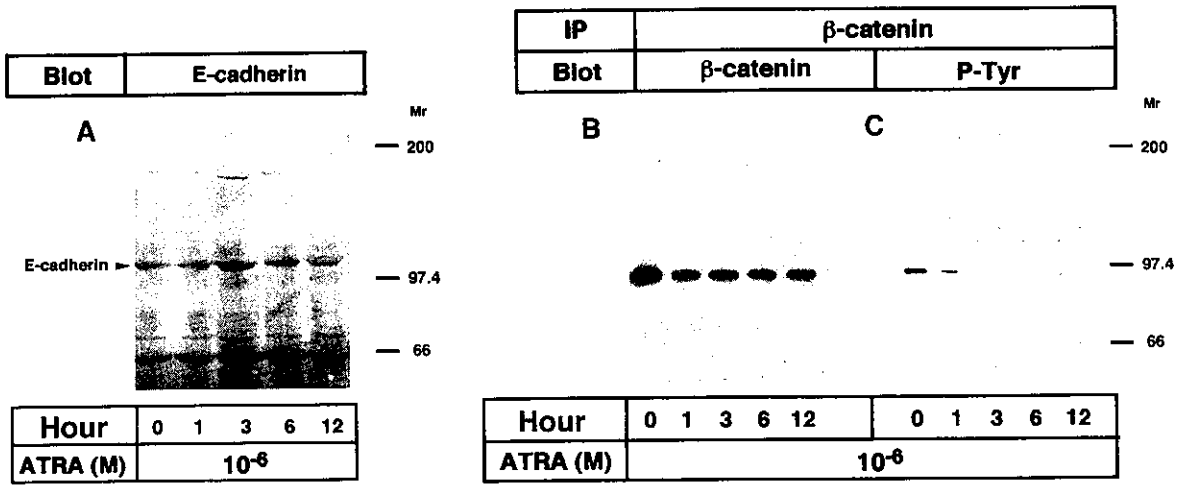

Fig. 6. Expression of E-cadherin and inhibition of phosphorylation of β-catenin tyrosine of MM-3 cells treated with ATRA. MM-3 cells on a collagen-coated dish were incubated with 10^{-6} *M* ATRA for 1, 3, 6, and 12 h. The molecular weight of E-cadherin was 120 kDa, and E-cadherin of MM-3 cells had no deletions (A). Immunoprecipitates of β-catenin obtained from equal aliquots of cell extract. These immunoprecipitates were resolved by SDS-PAGE and subjected to immunoblot analysis of β-catenin (B) and phosphotyrosine (C). Molecular weight markers of 200, 97.4 and 66 kDa are indicated.

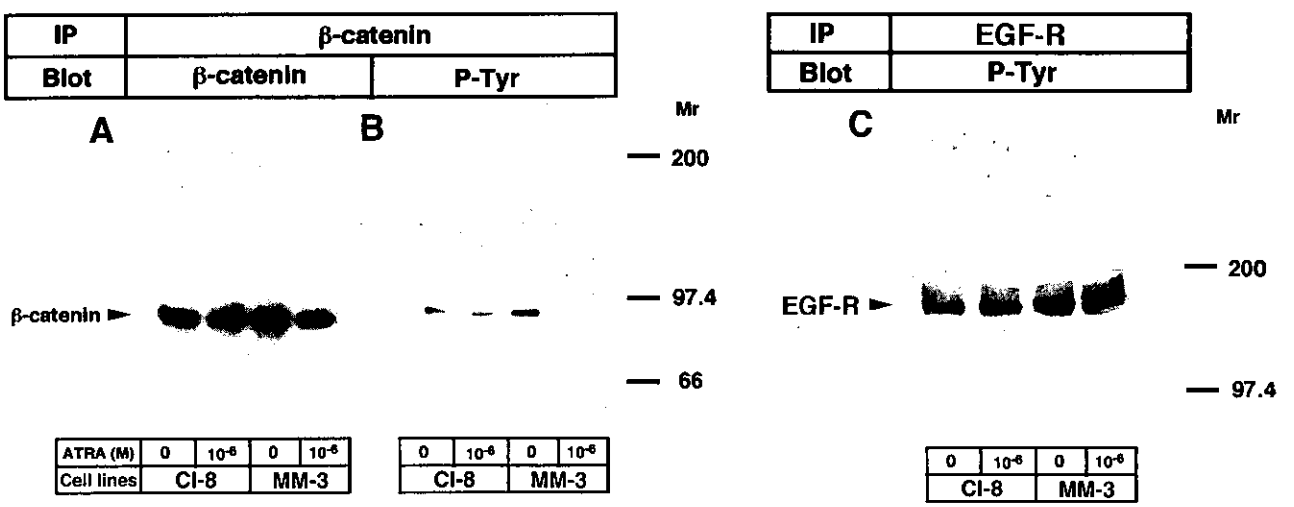

Fig. 7. Levels of phosphorylation of β-catenin and EGF-R tyrosine in Cl-8 or MM-3 cells. A and B, Cl-8 and MM-3 cells on collagen-coated dishes were incubated with or without ATRA for 12 h in DMEM with 10% FBS. Immunoprecipitates of β-catenin were obtained from equal aliquots of cell extract. C, Cl-8 and MM-3 cells on collagen-coated dishes were incubated with 10 ng/ml of EGF and ATRA for 3 h in DMEM with 10% FBS. Immunoprecipitates of EGF-R were obtained from equal aliquots of cell extract. These immunoprecipitates were resolved by SDS-PAGE and subjected to immunoblot analysis of phosphotyrosine (A and C) and β-catenin (B).

low. Retinoic acids also inhibit tumor cell invasion by suppressing the production of type IV collagenase.[21] But collagenolytic activity was roughly the same in ATRA-treated or untreated MM-3 cells (unpublished results). Retinoic acids induce their pleiotropic and biological effects through retinoic acid receptors.[22, 23] In this study, we observed inhibition by retinoic acids such as ATRA of MM-1, 3 and 8 cell invasion in the model system (unpublished results) and also horizontal spreading of MM-1, 3 and 8 cells with weaker cell-cell adhesion. We

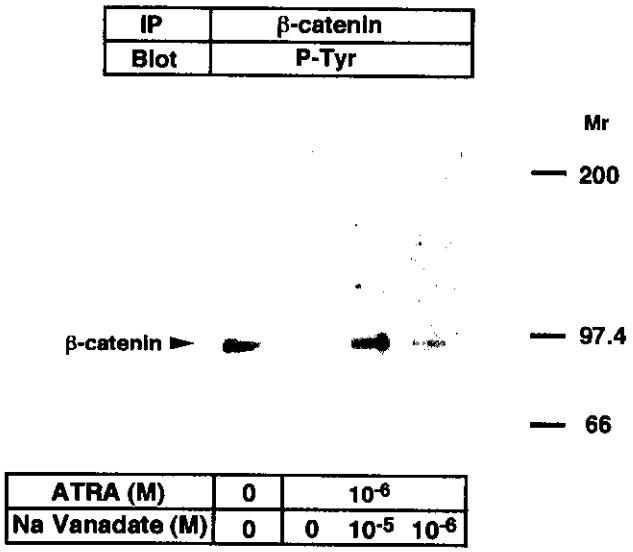

Fig. 8. Recovery of ATRA-induced dephosphorylation of β-catenin tyrosine of MM-3 cells by Na vanadate. MM-3 cells on collagen-coated dishes were incubated with 10^{-6} *M* ATRA and Na vanadate for 12 h. Immunoprecipitates of β-catenin were obtained from equal aliquots of cell extract. These immunoprecipitates were resolved by SDS-PAGE, and subjected to immunoblot analysis of phosphotyrosine.

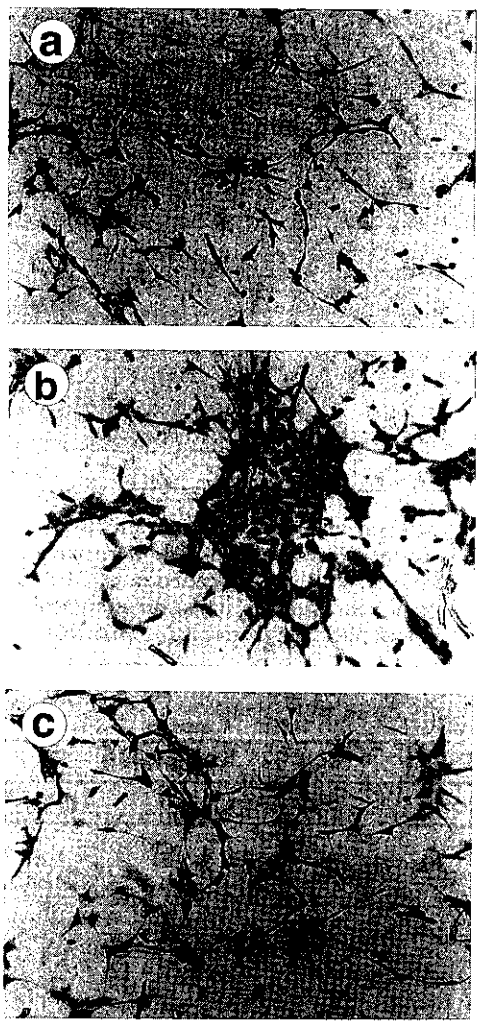

Fig. 9. Inhibition of clustering of ATRA-treated MM-3 cells by Na vanadate. ATRA (1×10^{-6} *M*) and Na vanadate were added 1 d after MM-3 cells had been plated. a, untreated MM-3 cells, b, MM-3 cells treated with ATRA, c, MM-3 cells treated with ATRA and Na vanadate at 10^{-6} *M*.

also observed that treatment of SN12C and Cl-8 cells with ATRA at 10^{-6} *M* did not affect their clustering growth behavior (unpublished results). Scattering of human renal cancer cells might be necessary for invasion in the model system *in vitro*.

In the present study, ATRA induced dramatic morphologic changes of the highly metastatic cells towards the appearance of the poorly metastatic cells (Fig. 2). This ATRA-induced change of growth patterns was inhibited when anti-E-cadherin was present (Fig. 4), suggesting that it might occur via an E-cadherin pathway. Vermeulen *et al.*[24] reported that ATRA induced aggregation of human MCF-7 breast cancer cells and this effect was abolished by antibodies against E-cadherin, but ATRA did not change cellular levels of E-cadherin in breast cancer cells. Down-regulation of E-cadherin occurs in various carcinomas[3-5] and is often associated with tumor invasion or metastasis.[4] Mutation of the E-cadherin gene is also observed in human gastric cancer cell lines,[3] but E-cadherin mutation rates appear to be relatively low in other human metastatic cancers.[3,25] Anzano *et al.*[26] reported that the expression of the E-cadherin gene is enhanced by retinoic acid. Consistent with this observation, we found about a two-fold increase in E-cadherin level at 3 h following ATRA treatment. However, we found no significant increase of E-cadherin thereafter during treatment with ATRA for 9 days (unpublished data). The expression of E-cadherin in the presence of ATRA appeared to be constant in our HRCC line during morphogenesis. Enhanced levels of E-cadherin are unlikely to be involved in the morphologic changes induced by ATRA.

The cytoplasmic domain of E-cadherin interacts with catenins, including α, β and γ-catenin,[6,27] and phosphorylation of β-catenin is known to abolish cell adhesion of cancer cells in association with the v-*src* oncogene.[9] A deletion of β-catenin also disrupts the interaction of E-cadherin with α-catenin associated with actin filaments, resulting in a loss of intercellular adhesiveness of gastric

cancer cell lines.[28] The expressions of α, β-catenin and E-cadherin mRNAs were similar in Cl-8 and MM-3 cells (unpublished data). In the present study, ATRA was found to inhibit phosphorylation of β-catenin tyrosine. This ATRA-induced inhibition of β-catenin phosphorylation was completely blocked by Na vanadate, a dephosphorylase inhibitor. Na vanadate also blocked the ATRA-induced colonization of HRCC with tight cell-cell contact. These data suggest that the morphologic changes in highly metastatic HRCC are closely related to the state of tyrosine phosphorylation of β-catenin. Concerning the effect of ATRA on other cancer cell types, we examined whether pancreas carcinoma cells with high metastatic potential could alter their morphological changes on collagen gel. A highly metastatic pancreatic carcinoma cell line also showed induction of clustering morphology by ATRA, but it remains to be determined whether ATRA modulates the phosphorylation status of β-catenin in this cell line (Jimi, S., unpublished data).

Upregulation of tyrosine phosphorylation is often observed in many cancer cell lines that show a high scatter or spread on plastic dishes in the absence of any matrix proteins.[29] Shibamoto *et al.*[30] reported that EGF or HGF induces an upregulation of β-catenin and morphologic changes in human colon and gastric cancer cells. Our previous study indicated the HRCC line, KPK13, showed high invasive and spreading activities in response to HGF via the HGF receptor, c-*met*.[11] Both the cellular level and phosphorylation of c-*met* tyrosine were similar in Cl-8 and MM-3 cells (Nakayama, Y., unpublished data), so it is unlikely that the involvement of the HGF-c-*met* pathway accounts for the difference in scattering potential of the two cell lines.

MM-3 cells, which had a higher scattering activity than the poorly metastatic counterpart, Cl-8, also exhibited a greater phosphorylation of β-catenin tyrosine than did Cl-8 cells (Fig. 7). This observation supports the hypothesis that tyrosine phosphorylation, rather than cellular level of β-catenin, is associated with the scattering potential of HRCC. The elevation of tyrosine phosphorylation of β-catenin plays a key role in E-cadherin function.[31] Kinch *et al.*[32] reported that tyrosine phosphorylation of β-catenin regulates the adhesion in *ras*-transformed breast epithelia. They indicated that the function of E-cadherin was inhibited when β-catenin was diffusely distributed in an oriented manner. Both E-cadherin and β-catenin were localized mainly on the cell surface at the adherent junctions of colonizing cells in the presence of ATRA, 10^{-6} *M* (Fig. 5, c and d). By contrast, both E-cadherin and β-catenin were diffusely distributed in an unorientated manner in untreated MM-3 cells grown on collagen gel (Fig. 5, a and b). The localization of β-catenin appeared to be important in the E-cadherin-dependent cell adhesion in our assay system. In contrast, Takeda *et al.*[33] reported that tyrosine phosphorylation of β-catenin, but not α-catenin, is elevated in the weak adhesion state of cultured mammalian cells, and also that the tyrosine phosphorylation of β-catenin is not required for the cadherin-based cell adhesion. In our present study, ATRA was found to alter the phosphorylation of β-catenin in the highly metastatic cell line. The phosphorylation state of β-catenin appeared to be associated at least with retinoic acid-induced shifts from a weak to a strong adhesion state of highly metastatic cancer cells, but the involvement of E-cadherin in the process remains unknown.

The poor response of Cl-8 cells to retinoic acid might be due to a loss of retinoic acid receptor(s). MM-3 expressed RAR-β, γ and RXR-α, β and γ mRNA as highly as Cl-8 cells (unpublished data). Further study is needed to determine whether or not ATRA differentially alters the interaction of E-cadherin and catenin in both cell lines, and also whether ATRA alters the expression or phosphorylation of other cadherin-associated proteins.

In conclusion, we observed that (1) ATRA preferentially inhibited the invasion of highly metastatic HRCC through vascular endothelial cells in a model system; (2) ATRA induced marked clustering of HRCC; and (3) ATRA induced the dephosphorylation of β-catenin tyrosine. The concomitant administration of Na vanadate completely inhibited both the morphological changes and the dephosphorylation of β-catenin. ATRA and its derivatives may modulate β-catenin, resulting in the invasion or metastasis of cancer cells. Motzer *et al.*[34] have reported that a combination of IFN-2α and 13-*cis*-retinoic acid gave a higher response proportion in patients with renal cell carcinoma than IFN-2α alone in a phase II trial. The clinical use of retinoic acid or its derivatives may modulate cell-cell attachment or cell invasion in renal cell carcinoma or other cancer types, resulting in antimetastatic activity *in vivo*. Further study is needed to determine whether retinoic acid and its derivatives inhibit cancer metastasis.

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