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Inverse Relation of Autocrine Motility Factor Receptor and E-Cadherin Expression following MDCK Epithelial Cell Transformation

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Autocrine motility factor interacts with its cell surface receptor (AMF-R) to stimulate tumor cell motility. To study AMF-R expression following transformation of polarized epithelial MDCK cells, we have used the invasive Moloney sarcoma virus transformed MDCK (MSV-MDCK) cell population. Decreased E-cadherin expression of the transformed MSV-MDCK clones is associated with both increased cellular motility and increased AMF-R expression. Increased AMF-R expression is due to MSV transformation as differentially motile MSV-MDCK clones, which either retain low E-cadherin levels or express no E-cadherin, exhibit equivalent high levels of AMF-R. Loss of the polarized epithelial phenotype and increased cellular motility following transformation of MDCK cells is thus associated with a shift from a high E-cadherin/low AMF-R to a low E-cadherin/high AMF-R phenotype. © 1996 Academic Press, Inc.

Pathological studies of tumors of epithelial origin show that increasing malignancy is associated with a progressive loss of polarity and an increasingly fibroblastoid phenotype (1,2). E-cadherin mediated epithelial cell adhesion is crucial to the establishment of epithelial polarity (3,4) and the loss of E-cadherin expression is directly related to the acquisition of motile and invasive properties by transformed epithelial cells (5,6,7,8). E-cadherin has thus been identified as a tumor suppressor molecule and the absence of its expression serves as a marker for tumor cell malignancy (9). However, malignancy of cells of epithelial origin also requires the expression of molecules which confer motile and invasive capabilities.

Autocrine motility factor (AMF) was originally identified in the conditioned medium of human A2058 metastatic melanoma cells and shown to be able to stimulate the motility of the secreting cells (10). Interaction of AMF with its receptor, AMF-R (or gp78), stimulates cellular motility via a pertussis toxin sensitive G-protein mediated pathway (11,12,13). A role for AMF-R in the acquisition of motile capability by malignant tumor cells has been demonstrated by the direct correlation of AMF-R expression with malignancy of human bladder and colorectal tumor (14,15). In particular, increased expression of AMF-R in malignant bladder carcinomas is associated with the concomitant decreased expression of E-cadherin (15). Our previous characterization of the tubular localization of AMF-R in the polarized MDCK epithelial cell (16) led us to study AMF-R expression following epithelial transformation in vitro in the highly invasive Moloney sarcoma virus transformed MDCK (MSV-MDCK) cell line (17). We found that decreased E-cadherin expression and increased cellular motility of MSV-MDCK cells is associated with significantly increased AMF-R expression levels. The acquisition of molecules which stimulate and the loss of those which restrict cellular motility are thus both associated with the increased cellular motility and invasiveness of MSV transformed MDCK epithelial cells.

MATERIALS AND METHODS

Cell and antibodies. Moloney sarcoma virus transformed MDCK cells (MSV-MDCK) (18) were obtained from the American Type Culture Collection (cell line $DoCl_1$; Rockville, MD). MSV-MDCK and MDCK II cells were grown in

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Abbreviations: AMF, Autocrine motility factor; AMF-R, autocrine motility factor receptor; MSV, Moloney sarcoma virus.

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Dulbecco's minimum essential medium (DMEM), supplemented with 10% fetal calf serum (FCS), glutamine, non-essential amino acids, vitamins, penicillin and streptomycin (Gibco, Burlington, Ontario). MSV-MDCK cells were cloned by limiting dilution and the resultant clones were characterized for E-cadherin expression using epifluorescence microscopy. E-cadherin positive epithelioid (MSV-MDCK-E) clones and E-cadherin negative fibroblastoid (MSV-MDCK-F) clones were obtained. To maintain a consistent phenotype, MSV-MDCK-F clones were passaged at high density (1.5×10^6 cells per 100 mm tissue culture dish) and maintained in culture for less than 6 passages. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Monoclonal antibody against AMF-R was used in the form of concentrated hybridoma supernatant (11). Mouse anti-E-cadherin (rr1) monoclonal antibody (19) was obtained from the Developmental Studies Hybridoma Bank, maintained by a contract from the NICHD (NO1-HD-2-3144). Rat anti-ZO-1 monoclonal antibody (20) and mouse anti-desmoplakin monoclonal antibody (21) were as previously described. Secondary anti-mouse and anti-rat IgM antibodies conjugated to Texas Red or horseradish peroxidase were purchased from Jackson Laboratories (West Grove, Pennsylvania). Except where otherwise indicated all chemicals were purchased from ICN.

Immunofluorescence. Immunofluorescent labeling was essentially as previously described (16). Briefly, cells plated on glass cover slips were fixed by the addition of cold (−80°C) methanol directly to the cover slip and then placed at −20°C for 15 minutes. The cells were extensively washed with phosphate buffered saline (pH 7.4) supplemented with 0.1 mM Ca^{2+} and 1 mM Mg^{2+} (PBS/CM) and then incubated in PBS/CM supplemented with 1% BSA as a blocking agent (PBS/CM/BSA). All washings and sequential incubations with primary antibodies and Texas Red conjugated secondary antibodies were done using PBS/CM/BSA. To visualize junctional expression, the cells were plated at high density such that they reached confluence after 2 days of culture. After labeling, the coverslips were mounted in Airvol (Air Products and Chemicals Inc., Allentown, PA) and observed with the 63X Plan Apochromat objective of a Zeiss Axiophot epifluorescent microscope.

Phagokinetic track motility assay. Cell motility was quantified using a phagokinetic track assay essentially as previously described (11). Briefly, 5,000 cells were plated on 22 mm coverslips coated with gold particles as described by Albrecht-Buehler (22) and fixed after 24 hours with 3% paraformaldehyde for 15 minutes and then mounted in Airvol. The cells were visualized using dark field illumination and a 10X objective and the area cleared of gold particles by cellular displacement was quantified using a Sigma-Scan measurement system (Jandel Scientific, Corte Madera, California). Only uninterrupted particle-free tracks containing a single cell were quantified and the area calculated included that of the cell. At least 50 cells were quantified per experiment and the mean of three experiments is presented. Error bars represent a 95% confidence interval.

Immunoblot. Cells cultured at approximately 50% confluency, such that the cells maintained a spread morphology, were scraped, lysed and sonicated in Tris pH 8 containing 1% sodium dodecyl sulfate and protease inhibitors (1 mM PMSF and 0.05 mM p-nitrophenyl p′-guanidino benzoate, 10 μg/ml leupeptin, pepstatin A and aprotinin) on ice. Protein content was assayed using the BCA protein assay (Pierce, Rockford, Illinois) and 25 μg of protein were separated by SDS-PAGE and blotted to nitrocellulose. Nitrocellulose blots were blocked with 20% skim milk in PBS/CM, incubated with primary antibodies to E-cadherin and AMF-R, species specific secondary antibodies coupled to horseradish peroxidase (Jackson, West Grove, Pennsylvania) and visualized by chemiluminescence (ECL, Amersham, Oakville, Ontario) using preflashed Kodak XRP X-ray film. Band intensities, within the linear range of the densitometer, were quantified by densitometry (Molecular Dynamics). Prestained molecular weight markers were from Sigma (St. Louis, Missouri).

RESULTS

We have previously characterized the tubular expression of AMF-R in the Madin-Darby canine kidney (MDCK) cell line (16). The MDCK cell line exhibits functional polarity in vitro and thus constitutes, for our purposes, a ''normal'' epithelial cell. In order to assess the expression of AMF-R during epithelial transformation, we obtained a population of Moloney sarcoma virus transformed MDCK cells from the American Type Culture Collection (18). The MSV-MDCK cell population has been previously shown to exhibit significantly decreased E-cadherin expression levels compared to MDCK cells and to be highly invasive (8). Immunofluorescence analysis of the MSV-MDCK cell population revealed the presence of E-cadherin expressing and non-expressing cells. The parental MSV-MDCK cell population was cloned by limiting dilution and selected clones were analyzed by immunofluorescence for E-cadherin expression (Figure 1). Compared to the highly lateral E-cadherin distribution in MDCK cells (Figure 1 a), E-cadherin expressing clones exhibited a diffuse labeling which contained both a lateral and cytoplasmic component (Figure 1 d) as previously described for the E-cadherin expressing epithelioid clones obtained from Ha-ras transformed MDCK cells (8). E-cadherin was not detected in the fibroblastoid clones (Figure 1 g). Both E-cadherin expressing and non-expressing MSV-MDCK clones exhibited a similar loss of expres-

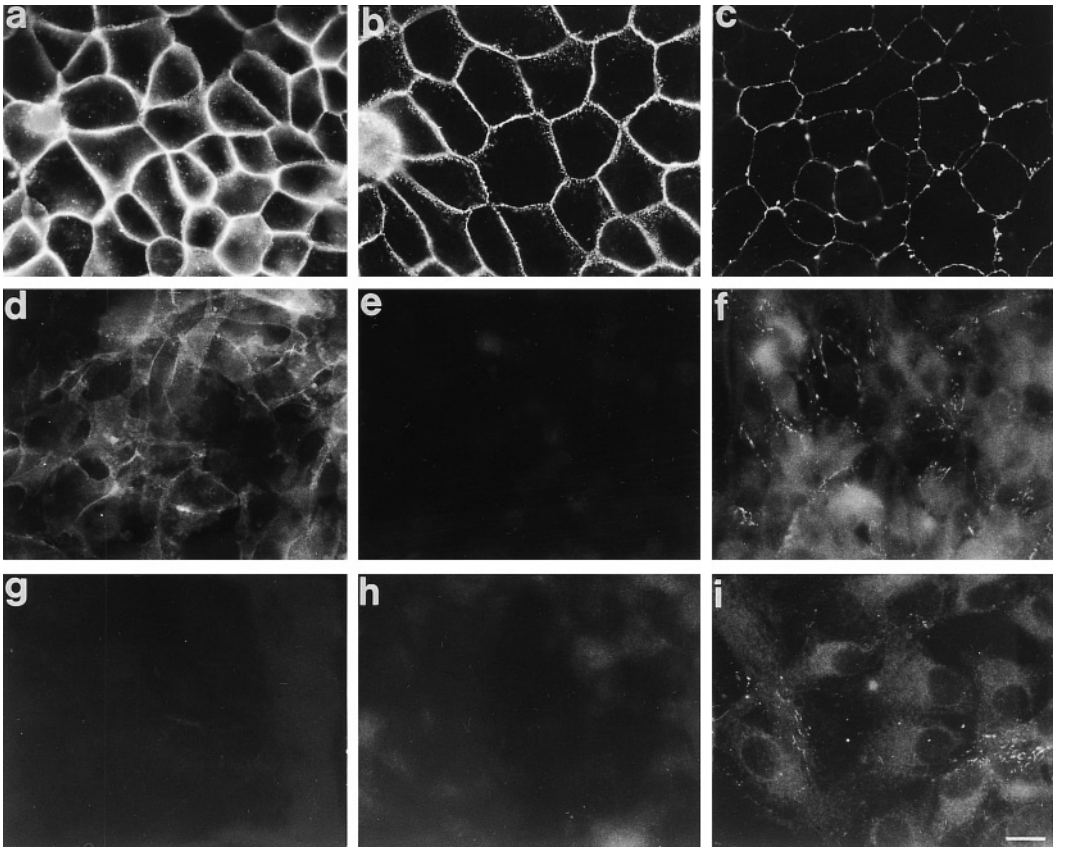

FIG. 1. Expression of epithelial junctional proteins following MSV transformation of MDCK cells. Confluent cultures of MDCK (a,b,c), MSV-MDCK-E (d,e,f) and MSV-MDCK-F (g,h,i) cells were immunofluorescently labeled with antibodies to E-cadherin (a, d, g), desmoplakin (b, e, h) and ZO-1 (c, f, i). In the well polarized MDCK cells E-cadherin (a), desmoplakin (b) and ZO-1 (c) exhibit a typical annular localization to the lateral cell membrane. In contrast to MSV-MDCK-F clones (g), MSV-MDCK-E (d) clones have retained expression of E-cadherin but both MSV-MDCK-E and -F clones have lost the organized annular expression of desmoplakin (e,h) and ZO-1 (f,i). Bar, 20 μm.

sion of the desmosome specific protein desmoplakin (Figure 1 e,h) and a highly dispersed expression of the tight junction specific protein ZO-1 (Figure 1 f,i). Comparison with the continuous lateral and apicolateral localization of these two junctional proteins in polarized epithelial MDCK cells (Figure 1 b,c) clearly demonstrates that MSV-MDCK cells express neither functional desmosomes nor tight junctions and are no longer polarized. Using the nomenclature of Behrens et al. (8), E-cadherin expressing MSV-MDCK clones will subsequently be referred to as epithelioid or MSV-MDCK-E clones and clones which do not express E-cadherin will be referred to as fibroblastoid or MSV-MDCK-F clones.

To determine whether an increasingly fibroblastoid phenotype is associated with increased cell motility, we assessed the cellular motility of the cells using a phagokinetic track assay (11,22). The motility of MDCK cells, two representative E-clones and two representative F-clones is shown in Figure 2. MDCK cells are the least motile cells. The area cleared of gold particles by MDCK cells was limited to the region around the cell and may be due solely to membrane activity at the cell periphery and not directional motility. In contrast to the MDCK cells, both E and F clones left distinct phagokinetic tracks significantly larger than the cell itself and therefore necessarily formed by directional displacement of the cell. Compared to the basal phagokinetic displacement of MDCK cells, both the E-clones ($\sim$3X) and F-clones ($\sim$6X) exhibit significantly increased cellular

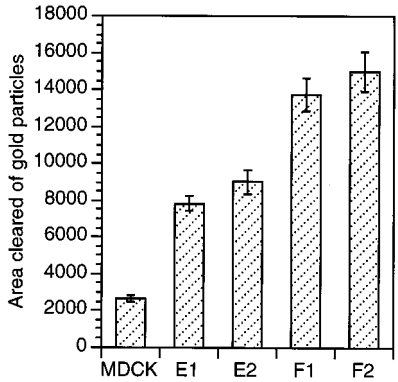

FIG. 2. Motility of MDCK, MSV-MDCK-E and -F clones. Cell motility (μm^2/24 hours) of the indicated cells was measured using a phagokinetic track assay.

motility. E-cadherin expression levels therefore correlate inversely with the phagokinetic motility of the cells. Since the phagokinetic track assay measures the motility of individual cells, the increased motility of the MSV-MDCK clones is not due to the loss of E-cadherin mediated intercellular contacts but rather reflects the intrinsic motility of the cells. The increased invasivity of the MSV-MDCK cell population (8) is also associated with increased intrinsic cellular motility.

Quantitative immunoblot of E-cadherin expression in MDCK cells, two E-clones and two F-clones (Figure 3), plated at 50% confluency, confirmed the differential expression levels of E-cadherin as previously indicated by immunofluorescence. Relative to equivalent protein levels in the SDS extracts, E-cadherin expression in MDCK cells was significantly greater than in the MSV-MDCK cells. E-cadherin in the MSV-MDCK-E clones varied from 5-5–15% of that in MDCK cells and in the F-clones was undetectable. The E-cadherin expression levels of MSV-MDCK-E clones, relative to MDCK cells, are significantly reduced compared to the epithelioid revertants obtained from Harvey sarcoma virus transformed MDCK cells which express E-cadherin at levels equivalent to and greater than that of MDCK cells (8). The same SDS cell extracts blotted with antibodies to E-cadherin were blotted with antibodies to AMF-R. AMF-R expression levels were significantly higher (~8-fold) in both E-clones and F-clones, which exhibit essentially equivalent high levels of AMF-R, relative to MDCK cells (Figure 3). MSV transformation of MDCK cells is therefore associated with a shift from a high E-cadherin/low AMF-R to a low-E-cadherin/high AMF-R phenotype.

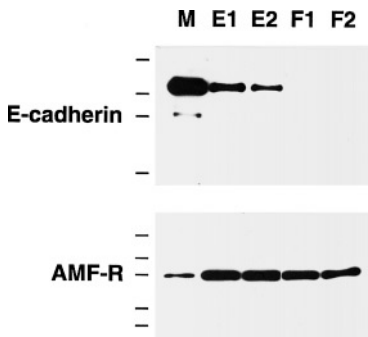

FIG. 3. E-cadherin and AMF-R expression of MDCK and MSV-MDCK transformants. SDS cell lysates were prepared from MDCK, MSV-MDCK-E1, -E2, -F1 and -F2 cells and equal quantities of protein (25 μg) were immunoblotted with antibodies to E-cadherin and AMF-R. Molecular mass markers from top to bottom: 180, 116, 84, 58 and 46 kDa.

DISCUSSION

The role of AMF-R in tumor cell motility in vitro and metastasis in vivo is well-established (23). More recently, AMF-R has been shown to be a marker for tumor malignancy in human bladder and colorectal tumors (14,15). Decreased levels of AMF-R have been detected following contact inhibition of A31/3T3 fibroblasts and in the contact inhibited FHs738BL bladder cell line but not the transformed J82 bladder carcinoma cell line at confluency (24,25). Of particular relevance to epithelial transformation, the increased expression of AMF-R in malignant bladder carcinoma correlated directly with the loss of E-cadherin expression (15). In order to address whether increased AMF-R expression was associated with loss of a 'normal' epithelial phenotype in culture, we studied AMF-R and E-cadherin expression following transformation of the polarized epithelial MDCK kidney cell line.

MDCK cells form a polarized monolayer in culture and are well-established as a cellular model for the study of the biogenesis of epithelial polarity. Oncogene induced transformation of MDCK cells is associated to differing degrees with the loss of the polarized epithelial phenotype (8,26,27). The MSV-MDCK cell population had been previously shown to be highly invasive (8), has lost the organized expression of protein components of desmosomes and the tight junction and is therefore not polarized. In our hands, it proved to be heterogenous and generated two types of subclones, epithelioid (E) clones which express low levels of E-cadherin, relative to MDCK cells, and fibroblastoid (F) clones which do not express E-cadherin. As expected for highly invasive cells, the motility of both MSV-MDCK-E and -F clones measured using a phagokinetic track assay was significantly greater than that of untransfected MDCK cells and inversely proportional to E-cadherin expression levels. Retention of certain aspects of the epithelial phenotype, including low levels of E-cadherin as well as keratin expression (not shown), in epithelioid clones of the parental MSV-MDCK population apparently restricts their increased intrinsic cellular motility subsequent to MSV transformation.

The significantly increased levels of AMF-R in MSV-MDCK clones relative to MDCK cells plated at equivalent levels of 50% confluency indicates that epithelial transformation and decreased E-cadherin expression is also associated in vitro with increased AMF-R expression. The intermediate cellular motility of epithelioid MSV-MDCK clones suggests that the interplay between the loss of molecular parameters, such as E-cadherin, which constrain epithelial cell displacement, and the acquisition of those which stimulate cell movement, such as AMF-R, may determine the motility of the transformed cell. The equivalent high AMF-R levels in the E and F clones indicates that the increased expression of AMF-R is due to the MSV transformation event. The proximity of the genes for AMF-R and E-cadherin on chromosome 16, 16q21 and 16q22.1 respectively, is suggestive of the coordinate regulation of these two genes following epithelial transformation (25,28). The inverse expression levels of E-cadherin and AMF-R observed following MDCK transformation in vitro correspond to that observed in vivo for malignant human bladder carcinomas (15). Malignancy of cells of epithelial origin may be associated not only with the disruption of the polarized epithelial phenotype but also with the expression of the molecular attributes of the motile, invasive tumor cell, such as AMF-R.

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REFERENCES

1. Gabbert, H., Wagner, R., Moll, B., and Gerharz, C.-D. (1985) *Exp. Metastasis* **3,** 257–279.
2. Weinstein, R. S., Merk, F. B., and Alroy, J. (1976) *Adv. Cancer Res.* **23,** 23–89.

3. Takeichi, M. (1991) *Science* **251,** 1451–1455.
4. Rodriguez-Boulan, E., and Nelson, W. J. (1989) *Science* **245,** 718–725.
5. Vleminckx, K., Vakaet, L., Mareel, M., Fiers, W., and Van Roy, F. (1991) *Cell* **66,** 107–119.
6. Frixen, U. H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Löchner, D., and Birchmeier, W. (1991) *J. Cell Biol.* **113,** 173–185.
7. Chen, W., and Öbrink, B. (1991) *J. Cell Biol.* **114,** 319–327.
8. Behrens, J., Mareel, M. M., Van Roy, F. M., and Birchmeier, W. (1989) *J. Cell Biol.* **108,** 2435–2447.
9. Birchmeier, W., and Behrens, J. (1994) *Biochim. Biophys. Acta Rev. Cancer* **1198,** 11–26.
10. Liotta, L. A., Mandler, R., Murano, G., Katz, D. A., Gordon, R. K., Chiang, P. K., and Schiffman, E. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83,** 3302–3306.
11. Nabi, I. R., Watanabe, H., and Raz, A. (1990) *Cancer Res.* **50,** 409–414.
12. Silletti, S., Watanabe, H., Hogan, V., Nabi, I. R., and Raz, A. (1991) *Cancer Res.* **51,** 3301–3311.
13. Stracke, M. L., Guirguis, R., Liotta, L. A., and Schiffman, E. (1987) *Biochem. Biophys. Res. Comm.* **146,** 339–345.
14. Nakamori, S., Watanabe, W., Kameyama, M., Imaoka, S., Furukawa, H., Ishikawa, O., Sasaki, Y., Kabuto, T., and Raz, A. (1994) Cancer **74,** 1855–1862.
15. Otto, T., Birchmeier, W., Schmidt, U., Hinke, A., Schipper, J., Rübben, H., and Raz, A. (1994) *Cancer Res.* **54,** 3120–3123.
16. Benlimame, N., Simard, D., and Nabi, I. R. (1995) *J. Cell Biol.* **129,** 459–471.
17. Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M. M., and Birchmeier, W. (1993) *J. Cell Biol.* **120,** 757–766.
18. Peebles, P. T., Gerwin, B. I., Papageorge, A. G., and Smith, S. G. (1975) *Virol.* **67,** 344–355.
19. Gumbiner, B., and Simons, K. (1986) *J. Cell Biol.* **102,** 457–468.
20. Stevenson, B. R., Siliciano, J. D., Mooseker, M. S., and Goodenough, D. A. (1986) *J. Cell Biol.* **103,** 755–766.
21. Cowin, P., Kapprell, H.-P., and Franke, W. W. (1985) *J. Cell Biol.* **101,** 1442–1454.
22. Albrecht-Buehler, G. (1977) *Cell* **11,** 395–404.
23. Nabi, I. R., Watanabe, H., and Raz, A. (1992) *Cancer Met. Rev.* **11,** 5–20.
24. Silletti, S., and Raz, A. (1993) *Biochem. Biophys. Res. Comm.* **194,** 446–457.
25. Silletti, S., Yao, J., Sanford, J., Mohammed, A. N., Otto, T., Wolman, S. R., and Raz, A. (1993) *Int. J. Oncol.* **3,** 801–807.
26. Schoenenberger, C.-A., Zuk, A., Kendall, D., and Matlin, K. S. (1991) *J. Cell Biol.* **112,** 873–889.
27. Warren, S. L., and Nelson, W. J. (1987) *Mol. Cell Biol.* **7,** 1326–1337.
28. Mansouri, A., Spurr, N., Goodfellow, P. N., and Kemler, R. (1988) *Differentiation* **38,** 67–71.