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Unstable Expression of E-Cadherin Adhesion Molecules in Metastatic Ovarian Tumor Cells

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E-Cadherin is a member of the cadherin family, which plays a key role in intercellular adhesion in various tumors as well as in normal tissues. Here, we examined the expression of this adhesion molecule in a murine ovarian tumor line OV2944, whose sublines show different degrees of spontaneous metastasis from subcutaneous sites; sublines LM-1 and LM-3 exhibit a low metastatic activity but a variant subline HM-1 has a high metastatic activity. When the expression of E-cadherin in these cells was examined by immunoblot analysis, the highly metastatic HM-1 cells was found to express an extremely small amount of this molecule, as compared with a high level of E-cadherin expression in the weakly metastatic LM-1 and LM-3 cells. Northern blot analysis showed that the amount of tanscripts from the E-cadherin gene is proportional to the amount of proteins detected in these cells. Immunofluorescence staining revealed that cells of the highly metastatic line were heterogeneous, that is, their cultures contained both E-cadherin-positive and negative cells. In contrast, cells of the weakly metastatic lines homogeneously expressed E-cadherin. When the highly metastatic line was subcloned, all the subclones consisted of E-cadherin-positive and negative cells. These results suggest that the expression of E-cadherin gene is not stably controlled in the highly metastatic line.

Key words: Cadherin — Cell adhesion molecule — Ovarian tumor — Metastasis

Metastasis of tumor cells is assumed to be initiated by the detachment of some cells from primary tumor sites. Elucidating the mechanism of the tumor cell detachment, thus, should be important in understanding the initiation process of metastasis. The molecular basis of the detachment of tumor cells, however, remains to be solved.

Generally, cells in normal tissues or tumors are tightly connected to each other via various intercellular adhesion molecules and structures. In such cellular connections, the molecules belonging to the cadherin family appear to play an especially important role. Cadherins are a family of transmembrane glycoproteins, which are subdivided into various types, such as E-, P- and N-types.[1] By their homophilic interactions on the cell surface, cells are firmly connected to each other, and the inhibition of cadherin activity with antibodies results in partial or total disconnection of intercellular adhesion in various cell layers,[1–3] indicating a crucial role of this molecular family in cell adhesion. This notion was further supported by the observation that non-cohesive cells were transformed into cohesive cells by expressing recombinant cadherins exogenously introduced.[4–6]

We can thus put forward the hypothesis that, if a temporary or permanent inactivation of cadherins occurred in some cells in tumors, it might enhance their detachment from the main tumor body. The inactivation of cadherin action could be induced by various mechanisms, such as the suppression of cadherin gene expression, the protease cleavage of cadherin peptides, and the destruction of the cytoplasmic machinery responsible for the cadherin function.[7] To test these possibilities, we studied the expression of E-type cadherin (E-cadherin) in an ovarian tumor line OV2944 and its variants, which exhibit spontaneous metastasis from primary tumor sites to different degrees. The results show that the expression of E-cadherin is unstable at the transcriptional level in highly metastatic cells.

MATERIALS AND METHODS

Establishment and culture of tumor lines An ovarian tumor developed in the right ovary of a female (C57BL/6N × C3H/He) F_1 mouse given a single whole-body neutron irradiation of 2.7 Gy from a ^{252}Cf source at 8 weeks of age. The primary tumor, designated as OV2944, was transplanted into a subcutaneous (sc) site on the back of a syngeneic mouse. A weakly metastatic line was established by serial sc back transplantation of the primary tumor for 11 transplant generations, then tumor cells were transferred to *in vitro* cultivation. The obtained weakly metastatic line was divided into sublines such as OV2944-LM-1 and OV2944-LM-3 with a similar metastatic character. The highly metastatic line, designated as OV2944-HM-1, was established as follows. In the first transplant generation, a metastatic tumor nodule from the sc back tumor was found in the lung.

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Table I. Metastatic Capacity of HM-1, LM-1 and LM-3 Lines

Cell line	Number of mice with metastasis[a]	Mean number of lung nodules ± SD[b]
HM-1	6/6	51 ± 19.9
LM-1[c]	0/3	0
LM-3[c]	0/3	0

a) Mice were killed 1.5 months after transplantation.
b) SD; standard deviation.
c) Five to ten nodules were seen when mice were kept for more than 2.5 months after transplantation.

The metastatic lung nodule was then re-grafted (sc, back). The resultant metastatic lung nodules were again re-grafted (sc, back). The same procedure was repeated 17 times, then tumor cells were transferred to *in vitro* cultivation. These *in vitro* cell lines have been maintained for more than 6 months. The stability of metastatic character of both lines was confirmed by occasional sc back transplantation into syngeneic recipients. In the weakly metastatic lines, no metastatic nodule was detected when mice were killed within 1.5 months after transplantation, while, in the highly metastatic line, numerous metastatic nodules were seen in the lung (Table I).

The cell lines were maintained in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum.

Immunoblot analysis Immunoblot analysis was carried out as described previously.[8] Briefly, 1×10^6 cells were dissolved in 100 μl of a sample buffer containing 2% SDS for SDS-PAGE. After boiling for 5 min in the presence of 5% 2-mercaptoethanol, the samples were subjected to 7.5% SDS-PAGE, and then the fractionated proteins were transferred onto nitrocellulose sheets. For detection of E-cadherin peptides, the nitrocellulose sheets were incubated with a rabbit anti-E-cadherin serum[4] diluted 1 to 500, and incubated with ^{125}I-labeled Protein A (Amersham).

Northern blot analysis Northern blot analysis was performed using total cellular RNA from tumor cells. RNA was isolated from *in vitro*-cultured tumor cells by sedimentation through cesium chloride as described.[9] RNA thus purified was denatured, electrophoresed on 1.0% agarose gel, transferred onto a nitrocellulose filter and used for Northern analysis as described previously.[10]

The recombinant plasmid, pSTEM-1, carrying cDNA of the mouse E-cadherin gene was described previously.[4] A recombinant plasmid carrying a 0.4 kb *Hin*fI fragment from the human β-actin gene was kindly provided by Dr. K. Kakunaga.[11] These plasmids were labeled with ^{32}P and used as probes.

Immunofluorescence staining Immunofluorescence staining for E-cadherin on cultured cells were performed as previously described.[12] Briefly, cells were cultured on coverslips, fixed with 3.5% paraformaldehyde in Hanks' solution for 30 min at 4°C and then with methanol for 10 min at −20°C. The fixed cells were incubated with 5% skim milk in 50 m*M* Tris-buffered saline (pH 7.6) containing 1 m*M* $CaCl_2$ (TBS-Ca) for 30 min at room temperature, with the rabbit anti-E-cadherin serum diluted 1 to 500 for 60 min and then with rhodamine-labeled anti-rabbit Ig antibody (Dakopatts, Copenhagen). After several washes with TBS-Ca, they were mounted with 90% glycerol-10% TBS-Ca containing 0.1% paraphenylenediamine. The samples were examined using a Zeiss Axiophoto fluorescence microscope.

Subcloning of cell lines HM-1 cells were treated with saline containing 0.05% trypsin and 1 m*M* EDTA, and dissociated completely into single cells by thorough pipetting. The dissociated cells were counted, diluted with culture medium and distributed into 96-well plates (Falcon 3072) by a limiting dilution method. After incubation for 2 weeks, 12 clones were isolated and transferred into 3.5 cm culture dishes, and immunofluorescence-stained for E-cadherin.

RESULTS

Immunoblot analyses Preliminary studies showed that the OV2944 tumor line expressed E-type cadherin. We then examined the amount of E-cadherin expressed in the weakly metastatic lines OV2944-LM-1 and -LM-3 as well as in the highly metastatic variant line OV2944-HM-1, by immunoblot analysis. The results showed that the weakly metastatic lines express abundant E-cadherin peptides (Fig. 1, lanes 1 and 2), whereas the highly metastatic variant expressed a very little of these peptides (Fig. 1, lane 3).

Northern blot analyses In order to determine the amount of mRNA encoding E-cadherin in the above cell lines, we performed Northern blot analysis. Figure 2A shows that a 4.5 kb band corresponding to E-cadherin mRNA in size[4] is detected in RNA from LM-1 (lane L) and LM-3 (data not shown), but this band is not detected in RNA from HM-1 at the present level of sensitivity for the detection (lane H).

Analyses at individual cellular level Figure 3A shows the result of immunofluorescence staining for E-cadherin on LM-1 cells. All the cells in the culture were strongly stained at a roughly constant level. LM-3 cells showed a similar staining pattern.

The staining pattern of the highly metastatic HM-1 cells was quite different. The intensity of the staining for E-cadherin differs from cell to cell; some cells were strongly stained but some others were completely nega-

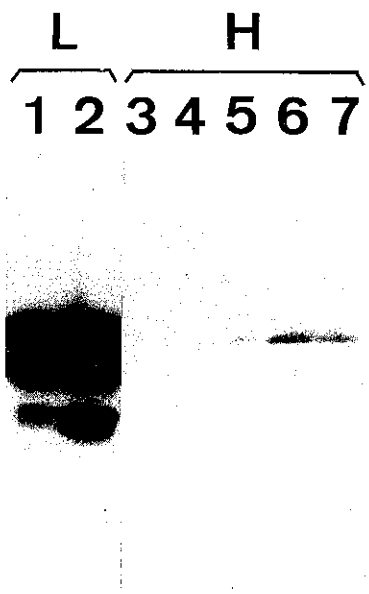

Fig. 1. Immunoblot analyses of the expression of E-cadherin. Lane 1, LM-1; lane 2, LM-3; lane 3, HM-1; lanes 4 to 7, independent subclones isolated from HM-1. Total cellular proteins applied to each lane were adjusted to be equal. The diffused appearance of bands in lanes 1 and 2 is due to the overexposure of the autoradiogram. Bands with lower molecular weight represent degradation products. L, weakly metastatic lines; H, highly metastatic lines.

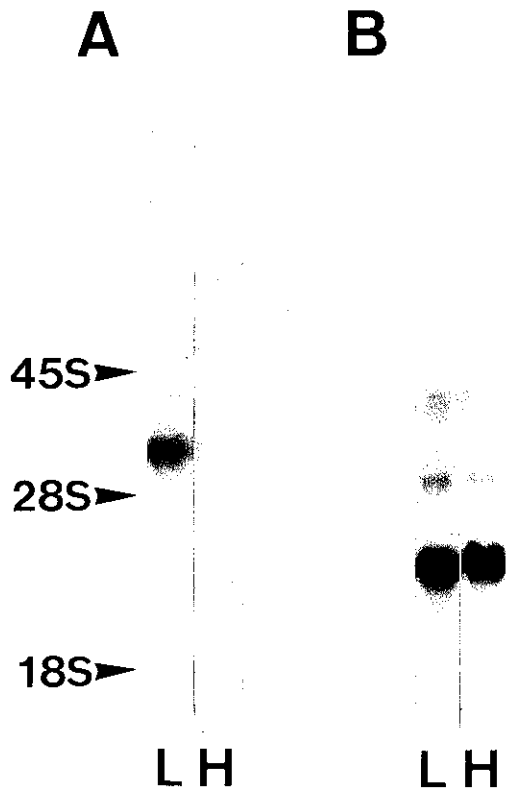

Fig. 2. Northern blot analyses of E-cadherin transcripts. (A) Detection with an E-cadherin cDNA probe, and (B) detection with a β-actin probe as a control. Lane L, RNA from LM-1; lane H, RNA from HM-1.

tive (Fig. 3C). Thus, the cell line HM-1 is heterogeneous with respect to E-cadherin expression. Since it is possible that the HM-1 line is a mixture of heterogeneous cell types, we randomly isolated a number of clones from this line and examined the expression of E-cadherin in each clone by immunoblotting as well as by immunostaining analyses. Figure 1 (lanes 4–7) shows the results of the immunoblot experiments, demonstrating that all clones express E-cadherin at low levels. Immunostaining analysis of these clones revealed that each of them contained both E-cadherin-positive and negative cells (Fig. 3E). The ratio of the two cell populations was not constant and varied from culture to culture in each clone, possibly responding to some unknown culture conditions. The expression of E-cadherin, thus, fluctuates unstably in HM-1 cells.

DISCUSSION

Loss of cadherin activity is not a general property for tumor cells. Many tumor cell lines express as much cadherins as normal cells, and cells of such lines are firmly connected to each other in a cadherin-dependent manner (see Takeichi[1] for a review). The expression of cadherins thus appears to be rather stable in many cell types and is not always affected by malignant transformation. In this context, it should be noted that most solid tumor lines maintained in experimental animals do not spontaneously metastasize from the primary tumor sites.

The present results, however, indicated that the cadherin expression could be perturbed as a result of malignant transformation in some tumor cells. Generally, cadherins are stably expressed in normal tissues as well as in solid tumor lines through their cell cycle (M. Takeichi, unpublished observations). Exceptionally, these molecules are never expressed in blood cells such as lymphocytes and their malignant transformants or in many ascites tumor lines. Obviously, there must be a mechanism for supporting the stable expression or the stable suppression of cadherin genes. In HM-1 cells, some conditions associated with malignant transformation probably led the regulatory mechanism of cadherin gene

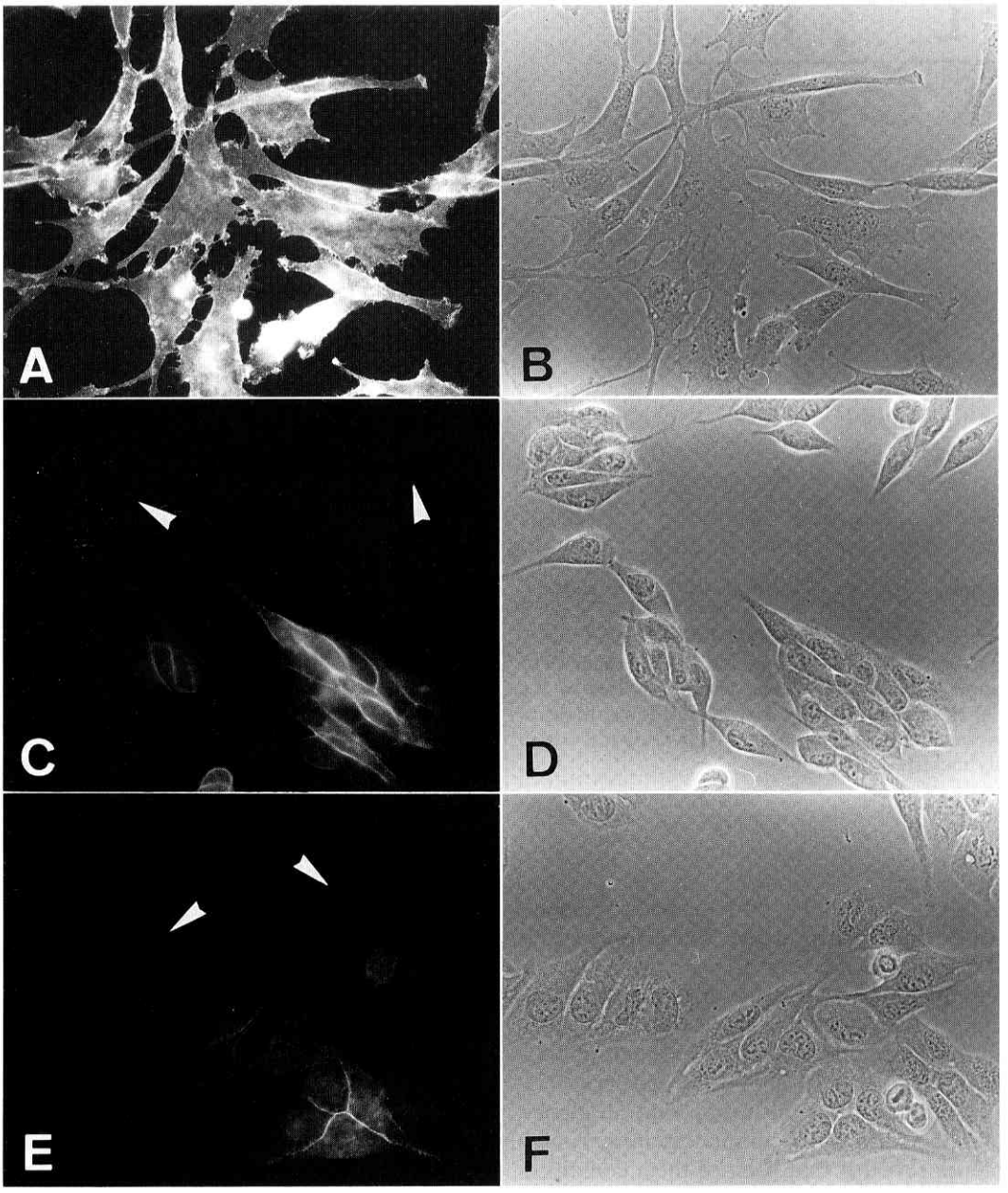

Fig. 3. Immunofluorescence staining for E-cadherin. (A, B) LM-1 cells. (C, D) HM-1 cells. (E, F) A subclone of HM-1, the same clone as used for lane 7 in Fig. 1. (A, C, E) Immunofluorescence photomicrographs; (B, D, F) phase-contrast photomicrographs of the corresponding field. Note the presence of E-cadherin-negative cells as indicated by arrows in (C) and (E). ×300.

transcription into an unstable state. This kind of perturbation of cadherin gene expression could take place in many kinds of tumors, though not in all the cells composing the tumors.

Whatever the mechanism of the unstable expression of cadherin is, the present results demonstrated a clear correlation between the metastatic activity of tumor cells and the level of cadherin expression. This finding is

consistent with the idea that the inhibition of cadherin action may enhance the release of cells from the primary tumor sites. In order to test the validity of this idea, however, we must examine more tumor samples, especially naturally occurring tumors. It should also be emphasized that the loss of cadherin expression might be a necessary condition for metastasis but probably not a sufficient condition. Various cellular properties such as motility[13, 14] and responsiveness to local growth factors[15] may also be important in the establishment of metastasis. The metastasis of cancer cells is likely to depend upon multiple factors.

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