Adhesive Interaction of Highly Malignant Hepatoma AH66F Cells with Mesothelial Cells

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We investigated the mechanism of adhesion of highly malignant ascites hepatoma AH66F cells to mesothelial cells. The adhesion rate of AH66F cells to mesentery-derived mesothelial cells (M-cells) was about 46% at 37°C, but it decreased to about 27% at 4°C. The adhesion rate of AH66F cells was about 25% in the presence of leukocyte function-associated antigen 1 (LFA-1) mAb at both 4°C and 37°C. When M-cells were treated with hyaluronidase, the AH66F/M-cell adhesion was decreased to half at 37°C and had nearly disappeared at 4°C. The residual adhesion of AH66F cells to M-cells treated with hyaluronidase almost disappeared in the presence of LFA-1 mAb. AH66F cells strongly adhered to a hyaluronate (HA)-coated plate, but not to a bovine serum albumin-coated plate. AH66F cells expressed a CD44 molecule (a HA receptor) in the plasma membrane, with a molecular size of about 85 to 90 kDa, corresponding to the CD44H isoform.

These results indicated that the adhesion of AH66F cells to mesothelial cells is composed of pathways of CD44/HA and LFA-1/ICAM-1.

Key words: rat hepatoma; leukocyte function-associated antigen 1 (LFA-1); CD44; hyaluronan; mesothelial cell

Malignant tumors are characterized by their unrestrained growth and invasion of surrounding tissues in the host. Pathologic observations and experimental studies have clearly demonstrated that invasion is not only a consequence of tumor growth pressure but also of the active locomotion of tumor cells into and through host tissue barriers.1) The rat ascites hepatoma AH66F cell line is a highly malignant hepatoma line which was induced by dimethylaminoazobenzene and established as transplantable tumor.2) When inoculated intraperitoneally into animals, AH66F cells had high ability to kill the host and they easily metastasized into several organs, such as the lung and liver.3) Interaction with mesothelial cells seems to be an initial step in the invasion of tumor cells into submesothelial tissues and subsequent intravasation. We recently found that AH66F cells have leukocyte function-associated antigen-1 (LFA-1), an adhesion molecule, on the surface of the plasma membrane, and this cell line adheres to mesentery-derived mesothelial cells (M-cells) via intercellular adhesion molecule-1 (ICAM-1), a counter receptor of LFA-1.5,6) However, because the LFA-1/ICAM-1 interaction contributes only about half the adhesion of AH66F cells to M-cells, there appears to be a mechanism other than the LFA-1/ICAM-1 interaction operating in the cell–cell adhesion.

In the case of ovarian or gastric cancer, such as a hyaluronate (HA) receptor, was shown to partially mediate the adhesion of tumor cells to peritoneal mesothelial cells.7,8) Another report has shown that adhesion through CD44 is inhibited by preincubating the mesothelial cells with hyaluronidase, which abolates HA on the cell surface.9) CD44 is an integral cell membrane glycoprotein that exists in a variety of forms with different molecular masses ranging from 85 kDa (CD44H) to 160 kDa (CD44E).10–13) Recent evidence suggests that CD44 participates in the invasion or metastatic stage of cancer. Moreover, it has been reported that there is a significant correlation between CD44 expression and the presence of a vascular invasion of hepatocellular carcinomas.14) CD44 may be related to the adhesion of AH66F cells to M-cells. In this study, we investigated the involvement of the CD44/HA interaction in the adhesion of AH66F cells and mesothelial cells.

MATERIALS AND METHODS

Agents The anti-rat LFA-1α-chain (CD11a), anti-rat LFA-1β-chain (CD18) and anti-rat ICAM-1 were purchased from Seikagaku Kogyo Co., Tokyo. The anti-rat CD44 (Ox-49) and horseradish peroxidase-conjugated anti-mouse IgG were purchased from Pharmigen, San Diego, CA, U.S.A. and Organon Teknika Co., West Chester, PA, U.S.A., respectively. Hyaluronidase and hyaluronic acid were purchased from Wako Pure Chemicals, Osaka. Bovine serum albumin (BSA) was from Sigma Chemical Co., St. Louis, MO, U.S.A. ECL (a light emitting non-radioactive detection system) was from Amersham International, PLC., Little Chalfont, Buckinghamshire, England.

Cells Rat ascites hepatoma AH66F cells were provided by the Department of Experimental Chemotherapeutics, Cancer Research Institute, Kanazawa University, Kanazawa. Cells were passaged weekly through female Donryu rats (Nippon SLC, Hamamatsu) and harvested from tumor-bearing animals 6 to 10 d after transplantation.

Adhesion Assay Tumor cells (4×10^4 per well) were seeded on 24-well culture plates, which had been coated with 0.5 mg/ml of BSA or HA, and were incubated for 1 h in Dulbecco's modified Eagle's medium (DMEM) at 37°C in a CO₂ incubator. After incubation, the plate was stirred for 30 s on a micro-mixer (Taiyo Kagaku Co., Ltd., Tokyo). The medium and washings (twice) of each of the wells were combined in a microtube, and the number of nonadherent cells in the medium was counted under a microscope.

In the experiment to test the adhesion of tumor cells to mesentery-derived mesothelial cells (M-cells), the tumor cells were seeded on an M-cell monolayer and incubated for 1 h in DMEM at 4°C or 37°C. After incubation, the number of nonadherent cells was counted. When treated with hyaluronidase, M-cells or AH66F cells were incubated with 2 mg/ml hyaluronidase for 30 min.

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Table 1. Adhesion Rates of AH66F Cells to M-Cells under Various Conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adhesion rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
</tr>
<tr>
<td>Untreated</td>
<td>46.3±2.1</td>
</tr>
<tr>
<td>LFA-1β mAb (L)</td>
<td>25.4±2.1*</td>
</tr>
<tr>
<td>Hyaluronidase (AH)</td>
<td>43.8±2.3</td>
</tr>
<tr>
<td>Hyaluronidase (M)</td>
<td>23.8±1.0*</td>
</tr>
<tr>
<td>Hyaluronidase (M) + LFA-1β mAb (L)</td>
<td>7.2±0.8**</td>
</tr>
</tbody>
</table>

Adhesion assay of AH66F cells to M-cells was done for 60 min at 37°C or 4°C. a) Adhesion assay in the presence of LFA-1β mAb (10 μg/ml). b) Adhesion assay after the treatment of AH66F cells (AH) or M-cells (M) with hyaluronidase (2 mg/ml). c) Adhesion assay in the presence of LFA-1β mAb (10 μg/ml) after the treatment of M-cells with hyaluronidase (2 mg/ml). Each value represents the mean±S.E. of at least five experiments. * Significantly different from the untreated control at p<0.01 and p<0.001, respectively. ** Significantly different from the adhesion rate at 37°C at p<0.01.

No dissociation of M-cell layers in this assay system was observed.

Immunoblotting The plasma membrane of AH66F cells was prepared by a Percoll sedimentation method as previously reported. The membrane protein (10 μg protein) was electrophoresed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto nitrocellulose membrane filters (Schleicher & Schuell, Dassel, Germany). After being blocked with 5% skim milk, the membrane was incubated overnight with 1 μg/ml LFA-1β-chain or CD44 mAb and with horseradish peroxidase-conjugated anti-mouse IgG for 1 h. Following each incubation, the membrane was washed extensively with phosphate buffered saline containing 0.1% Tween-20. The immunopositive band was detected by ECL, and by exposure to a Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY, U.S.A.).

RESULTS AND DISCUSSION

We investigated the mechanism of adhesion of highly malignant ascites hepatoma AH66F cells to mesothelial cells. As shown in Table 1, the adhesion rate of untreated AH66F cells to M-cells was about 46% at 37°C, but it decreased to about 27% at 4°C, at which temperature the adhesion of integrin molecules diminished. AH66F cell binding to M-cells was about 25% in the presence of LFA-1 mAb at both 37°C and 4°C, indicating that the decreased adhesion at 4°C was due to the diminution of an LFA-1-dependent interaction. On the other hand, the adhesion rate of AH66F cells to M-cells was not changed, even after treatment of the tumor cells with hyaluronidase. When M-cells were treated with hyaluronidase, the adhesion rate of AH66F cells was decreased to about 24% at 37°C and nearly disappeared at 4°C. The adhesion almost disappeared in the presence of LFA-1 mAb after treatment of M-cells with hyaluronidase. AH66F cells hardly adhered to the BSA-coated plate, but adhered strongly to the HA-coated plate (Fig. 1). The adhesion was disappeared when the HA-coated plate was treated with hyaluronidase.

Cellular binding of HA has been identified with CD44, which is generally considered to be a molecule involved in intercellular adhesion. As expected, the CD44 molecule was detected in the plasma membrane of AH66F cells together with the LFA-1 molecule (Fig. 2). The position of an immunopositive band to CD44 mAb was at about 85 to 90 kDa, corresponding to the molecular mass of the CD44H isoform.

The different CD44 isoforms are generated by alternative splicing and/or by posttranslational modification. Most diversity is produced by the incorporation of amino acid stretches encoded by 10 alternatively spliced exons into one particular part of the extracellular portion of the CD44 proteins, close to the transmembrane domain. The most common and widely expressed 85-kDa isoform does not include any of these variant exons (CD44H). The expression of variant isoforms of CD44 containing sequences encoded by the variant exons (CD44V) is restricted mainly to a limited selection of epithelia, in addition to certain tumor cells. Many studies have suggested links between the expression of CD44V to tumor progression and metastatic capability in malignant cancer, but there are also several reports that the interaction of CD44H with HA on mesothelial cells mediates peritoneal dissemination. Therefore, the
CD44H of AH66F cells was considered to mediate the adhesion to HA. Indeed, AH66F cells almost completely adhered to a HA-coated plate (Fig. 1), but only about a half adhered with an M-cell layer through HA-mediated adhesion (Table 1). These results suggest that M-cells are a heterogenous population which may or may not express HA on the plasma membrane.

Tumor cells exhibit a number of adhesive abnormalities which contribute significantly to their ability to invade.22,23 AH66F cells adhered better than low malignant AH130 cells line to M-cells.5 It is thus considered that the high malignancy of AH66F cells is due to this adhesive advantage. In this study, we indicated that the adhesion of AH66F cells to M-cells is accomplished by at least two pathways: CD44H/HA and LFA-1/ICAM-1.

Acknowledgment This work was supported in part by a Grant-in-Aid from the Ministry for Education, Science, Sports and Culture, Japan.

REFERENCES