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Leukocyte Function-associated Antigen-1-dependent Adhesion of Rat Ascites Hepatoma AH66F to Mesentery-derived Mesothelial Cells

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Rat ascites hepatoma AH66F cells adhered better than AH130 cells to mesentery-derived mesothelial cells (M-cells), though both cells secreted *M*, 92,000 matrix metalloproteinase on a gelatin zymogram with similar activity. AH66F cells expressed leukocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1), a ligand of LFA-1, on the cell surface, while AH130 cells had ICAM-1 alone. The adhesion to M-cells of AH66F cells was inhibited to the adhesion level of AH130 cells by anti-rat LFA-1 α- and/or β-chain monoclonal antibody (mAb) and also by anti-rat ICAM-1 mAb. This is the first report to show the LFA-1-dependent adhesion of cells other than leukocytes, because AH66F cells did not express CD45, T cell-$\alpha\beta$ receptor or CD11b/c (Mac-1/p150,95). These results indicate that a part of the adhesion of AH66F cells to M-cells is due to LFA-1/ICAM-1 interaction, and we suggest that this characteristic feature of AH66F cells may be related to the malignant properties.

Key words: Rat ascites hepatoma — Adhesion — LFA-1 — ICAM-1 — Matrix metalloproteinase

Rat ascites hepatoma (AH) cells have been induced with dimethylaminoazobenzene and established as transplantable tumors.[1] When inoculated intraperitoneally into rats, AH cell lines have a distinct host-killing ability.[2]

Several workers using light and electron microscopy have shown that ascites tumor cells invade through the peritoneum into various abdominal organs.[3–6] AH cells adhere to the peritoneum during the course of growth, invade mostly into the mesentery, and appear in the blood stream.[7,8] Imamura *et al.*[9] have observed that AH cells invade under mesothelial cell layers in the presence of fetal calf serum *in vitro*. We observed that AH66F cell line adhered better than AH130 cell line to mesentery-derived mesothelial cells (M-cells). Tumor cells present a number of adhesive abnormalities which contribute significantly to their ability to invade.[10–12] Therefore, we investigated the adhesion molecules involved in the adhesion to M-cells, and the gelatinase activity.

MATERIALS AND METHODS

Cells Rat ascites hepatoma AH66F and AH130 cells were provided by the Department of Experimental Chemotherapeutics, Cancer Research Institute, Kanazawa University.

Cells were passaged weekly through female Donryu rats (Nippon SLC, Hamamatsu) and harvested from tumor-bearing animals 6 to 10 days after transplantation. The harvested cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Irvine, Scotland) containing 5% fetal bovine serum (FBS) for 48 h before use.

Isolation and culture of mesentery-derived cells Isolation of M-cells was as reported by Akedo *et al.*[13] Briefly, the sheets of mesentery were incubated in 0.2% trypsin (1:250; Difco Laboratories, Detroit, MI) in phosphate-buffered saline (PBS) at 37°C. After digestion, 0.5 volume of DMEM containing 10% FBS was added, then the sheets were filtered, and the filtrate was centrifuged to collect M-cells. The M-cells were inoculated into a 50 ml plastic tissue culture flask (Falcon; Nippon Becton Dickinson Co., Ltd., Tokyo) and cultured in DMEM containing 10% FBS and 100 μg/ml kanamycin at 37°C in a CO_2 incubator. The M-cells grown to subconfluence were redigested with trypsin, washed with PBS, and resuspended in DMEM. The M-cells were inoculated with a density of 10^5 cells/ml in the wells of a 24-well plastic tissue culture plate (Falcon) and cultured in a CO_2 incubator. The M-cells formed monolayers, which were used for the adhesion assay.

***In vitro* adhesion assay** Cells were washed twice with PBS and suspended in DMEM containing 5% FBS. Cells (4×10^4 per well) were seeded on an M-cell monolayer and incubated for 1 h at 37°C in a CO_2 incubator. After the incubation, the plate was stirred for 30 s on a micromixer (Taiyo Kagaku Co., Ltd., Tokyo). The medium and washings (twice) of each well were combined in a

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microtube (Treff AG, Degersheim, Switzerland), and the number of nonadherent cells in the medium was counted under a microscope. The adhesion rate (%) was calculated from the following equation:

$$\text{Adhesion rate } (\%) = (1 - N/T) \times 100$$

where N and T represent the mean number of nonadherent cells and the mean number of seeded cells, respectively.

No dissociation of M-cell layers was detected by this measurement method.

Monoclonal antibodies (mAbs) The anti-leukocyte function-associated antigen-1 (LFA-1) α-chain, anti-LFA-1 β-chain, and anti-intercellular adhesion molecule-1 (ICAM-1) were purchased from Seikagaku Corporation (Tokyo). Anti-CD45, anti-T cell-$\alpha\beta$ receptor (TCR-$\alpha\beta$) and anti-CD11b/c (Mac-1/p150,95) were purchased from Pharmingen (San Diego, CA).

Flow cytometry Cells were incubated on ice in a volume of 250 μl with mAb (20 μg/ml) for 45 min and stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG,A,M (H+L) (The Binding Site Ltd., Birmingham, England). The cells were washed twice, and the fluorescence intensity was measured using an EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL).

Immunoblotting The plasma membrane preparation (50 μg protein)[14] was electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide 7.5% gel and transferred to a nitrocellulose membrane filter (Schleicher & Schuell, Dassel, West Germany). This was blocked with 5% skim milk, and incubated overnight with 1 μg/ml mAb, and with horseradish peroxidase-conjugated anti-mouse IgG (Organon Teknika Co., West Chester, PA) for 1 h. Following each incubation, the membrane was washed extensively with PBS containing 0.1% Tween-20. The immunopositive band was detected by a light-emitting non-radioactive detection system (ECL; Amersham International plc, Little Chalfont, Buckinghamshire, England) and by using Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY).

Zymography Cells (10^6 cells/ml) were washed twice with serum-free DMEM, and incubated for 24 h in the absence of serum. After centrifugation (2,000g, 5 min, 4°C), the medium was concentrated (100-fold) with saturated ammonium sulfate, as described by Koshikawa *et al.*,[15] and electrophoresed on 10% (w/v) polyacrylamide gel containing 0.1% gelatin by the method of DeClerck *et al.*[16] Samples were not heated before electrophoresis. Gels were run at 20 mA/gel, incubated for 24 h in 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM $CaCl_2$, stained with Coomassie brilliant blue (0.25%, w/v), and destained in methanol:acetic acid:water (20:10:70). Clear zones indicated the presence of proteinases with gelatinolytic activity.

RESULTS

***In vitro* adhesion assay to M-cell layers** These cultured AH cells did not adhere to the substrate and grew in

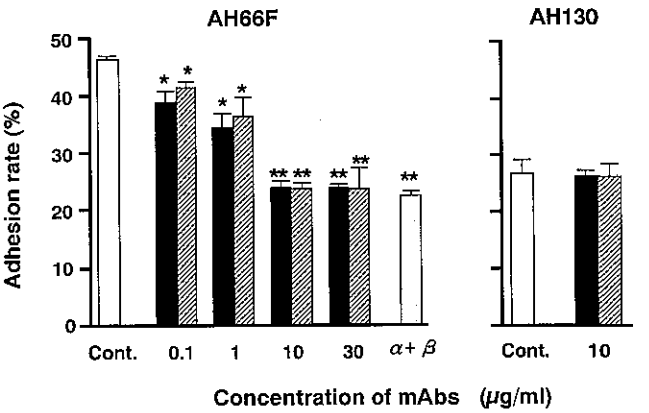

Fig. 1. Effects of anti-LFA-1 α- and β-chain mAbs on adhesion of AH66F or AH130 cells to M-cells. AH66F or AH130 cells were added to a confluent layer of M-cells in the presence of varying concentrations of mAbs. The open column (Cont.) indicates adhesion of cells in the presence of 30 μg/ml anti-TCR-$\alpha\beta$ mAb, and the closed column and the oblique column, adhesion in the presence of anti-LFA-1 α- and β-chain mAbs, respectively. The speckled column ($\alpha+\beta$) indicates adhesion of cells in the presence of 10 μg/ml of both anti-LFA-1 α- and β-chain mAbs. Data are the mean±SD of five measurements. *, ** Significantly different from the Cont. at $P<0.05$ and $P<0.01$, respectively.

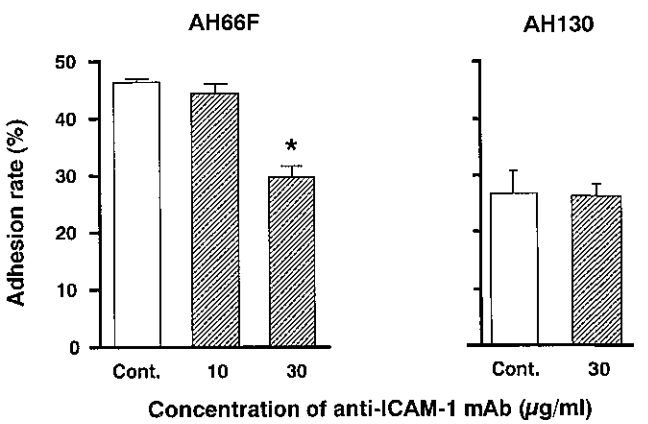

Fig. 2. Effects of anti-ICAM-1 mAb on adhesion of AH66F or AH130 cells to M-cells. AH66F or AH130 cells were added to a confluent layer of M-cells in the presence of 10 or 30 μg/ml mAbs. The open column (Cont.) indicates adhesion of cells in the presence of 30 μg/ml anti-TCR-$\alpha\beta$ mAb, and the oblique column, adhesion in the presence of anti-ICAM-1 mAb. Data are the mean±SD of five measurements. * Significantly different from the Cont. at $P<0.01$.

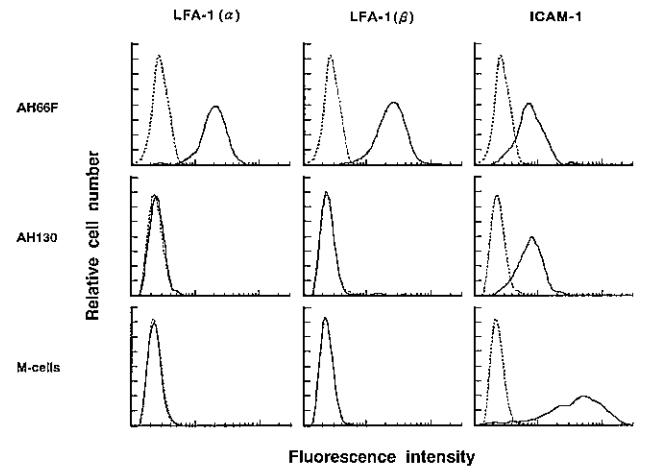

Fig. 3. Expression of LFA-1 (α-, β-chain) and ICAM-1 on AH66F, AH130 and M-cells. Background reactivity (dotted line) is that of cells stained with FITC-conjugated second antibody only.

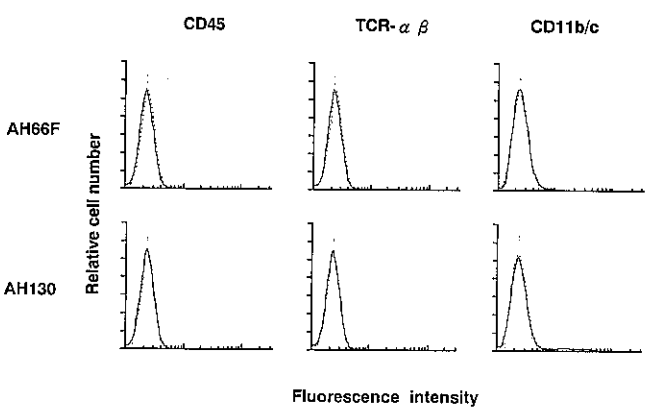

Fig. 4. Flow cytometric analysis of leukocyte antigens on AH66F and AH130 cells. Background reactivity (dotted line) is that of cells stained with FITC-conjugated second antibody only.

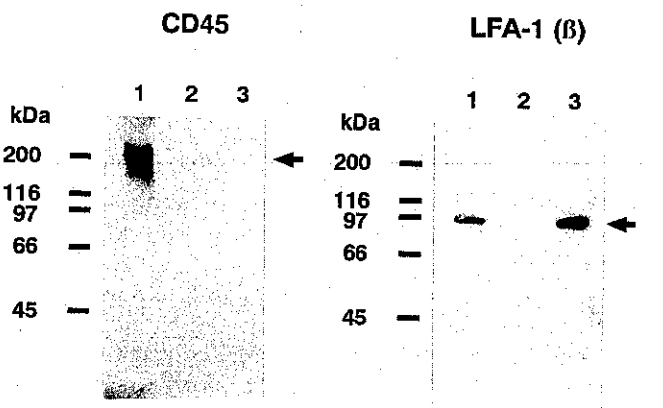

Fig. 5. Immunoblot analysis of LFA-1 β-chain and CD45 in the plasma membrane from spleen, AH130 and AH66F cells. Lanes 1, 2 and 3 indicate the plasma membrane from spleen, AH130 and AH66F cells, respectively.

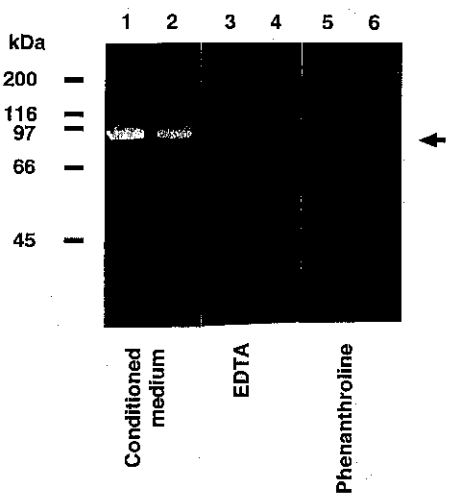

Fig. 6. Gelatin zymography of conditioned media of AH66F and AH130 cells. The serum-free conditioned media of AH66F and AH130 cells were electrophoresed on 10% polyacrylamide gel containing 0.1% gelatin, and the gels were incubated without (conditioned medium) or with 5 mM EDTA (EDTA) or 1 mM 1,10-phenanthroline (phenanthroline) for 24 h. Left, molecular weight markers. Arrow, gelatinolytic activity of M_r 90,000 MMP. Lanes 1, 3, 5, medium from AH66F cells; Lanes 2, 4, 6, medium from AH130 cells.

suspension form in plastic culture dishes. When cells were incubated on M-cell monolayers for 1 h, the adhesion rates (%) of AH66F and AH130 were about 46% and 27%, respectively. The adhesion of AH66F was inhibited to the adhesion level of AH130 by both LFA-1 α- and β-chain mAbs in a concentration-dependent manner (Fig. 1), while that of AH66F was also inhibited by 30 μg/ml ICAM-1 mAb (Fig. 2). These mAbs have no influence on the adhesion of AH130 (Figs. 1 and 2).

Flow cytometric analysis As shown in Fig. 3, AH66F expressed LFA-1 α-, β-chains and ICAM-1, while AH130 expressed only ICAM-1. The expression level of LFA-1 on AH66F was not different for α- and β-chains. M-cells expressed ICAM-1 but not LFA-1 α-, β-chains. The expression level of ICAM-1 on M-cells was higher than that on AH cells.

No expression of CD45, leukocyte common antigen, TCR-$\alpha\beta$, Mac-1 and/or p150,95 (CD11b/c) on these AH cells was observed (Fig. 4).

Immunoblot analysis Fig. 5 shows the immunoblot results. The splenic cell membrane was immunopositive to the mAbs against CD45 and LFA-1 β-chain. The positions of the immunopositive bands to the CD45 and LFA-1 β-chain mAbs corresponded to molecular weights of 200 and 95 kDa, respectively. The LFA-1 β-chain, but not CD45, was detected in AH66F cells. The position of the immunopositive band to the LFA-1 β-chain mAb in AH66F cells was the same as that from spleen cells. Neither CD45 nor LFA-1 β-chain was detected in AH130 cells.

Secretion of proteinase from AH cells Gelatinolytic activity at a band with a molecular weight of 92 kDa was observed in the conditioned media of both AH66F and AH130, to the same extent (Fig. 6). This activity of the M_r 92,000 band was completely inhibited by 1,10-phenanthroline (1 m*M*) and EDTA (5 m*M*), showing that the gelatinolytic enzyme was a matrix metalloproteinase (MMP), gelatinase B.

DISCUSSION

We found that AH66F cells adhered much more than AH130 cells to M-cell layers, while both AH cells secreted an M_r 92,000 MMP, gelatinase B, with the same activity. We detected LFA-1 α- and β-chains, but not Mac-1 and/or p150,95, on the membrane surface of AH66F cells, but not AH130 cells; namely, AH66F cells express the LFA-1 molecule. Most tissues and cells express ICAM-1 as an LFA-1 ligand on their cell surface, including M-cells. It is thus considered that when inoculated into the abdominal cavity, AH66F cells have a growth advantage due to the ability to adhere to various abdominal tissues. Anti-LFA-1 and ICAM-1 mAbs inhibited the adhesion of AH66F cells to M-cells to the adhesion level of AH130 cells, which was unaffected by these mAbs.

The adhesion mechanism of AH130 cells to M-cells and that of the residual AH66F-M-cell adhesion in the presence of anti-LFA-1 and anti-ICAM-1 mAbs are unclear. There appear to be other mechanisms than the LFA-1/ICAM-1 interaction operating in the cell-cell adhesion, e.g., involving selectins, other integrins, etc. LFA-1 is also known to have other ligands; ICAM-2 and ICAM-3.[17] However, these adhesion molecules and antibodies are not yet available from rat. The other adhesion molecules of rat cells remain to be identified.

The adhesion through LFA-1 is known to be important in various cell-cell interactions in the immune system, including leukocyte binding to the endothelium, subsequent emigration of leukocytes into inflammatory sites, and antigen-specific immune responses.[18–22] In addition, accumulating evidence suggests that integrins, including LFA-1, play important roles not only in intercellular adhesion but also in signal transduction.[23] Triggering of LFA-1 by the ligands, ICAM-1, -2 or -3, provides the co-stimulus for the activation, i.e., production of interleukin-2 and proliferative response, by stimulation of the antigen receptor (TCR/CD3) in T cells.[24–28] Our results indicate that AH66F cells do not have CD45, a common antigen of leukocytes, TCR-$\alpha\beta$ and CD11b/c (Mac-1/p150,95). Fukumoto *et al.*[29] also reported that AH66F cells have the antigen(s) recognized by mAbs against hepatocytes, but do not have the specific antigens of leukocytes. This is the first report to describe the LFA-1-dependent adhesion of hepatoma cells.

We consider that AH66F cells adhere to the abdominal tissues via the LFA-1/ICAM-1 pathway, and this interaction may also stimulate tumor cell proliferation, resulting in the high host-killing ability.

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