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# Leukocyte Function-Associated Antigen 1-Dependent Adhesion of Rat Hepatoma AH66F Cells and Inhibition by Protein Kinase C Inhibitors

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**ABSTRACT.** When rat ascites hepatoma AH66F cells were incubated on a mesothelial cell (M-cell) layer for 1 hr, the adhesion rate of the cells to M-cells was *ca.* 46%. The protein kinase C (PKC) inhibitors, *N*-(2-methylpiperazyl)-5-isoquinolinesulfonamide (H-7) and *N*-ethoxycarbonyl-7-oxostaurosporine (NA-382), inhibited the adhesion of AH66F cells in a concentration-dependent manner, and the effect of NA-382 appeared after a treatment of more than 24 hr. The decreased adhesion rate after treatment with NA-382 for 48 hr was not further inhibited by addition of monoclonal antibodies of leukocyte function-associated antigen-1 (LFA-1) α- and β-chains and intercellular adhesion molecule-1 (ICAM-1) (WT.1, WT.3, and 1A29, respectively). The expression of LFA-1 α- and β-chains on the surface of the plasma membrane of AH66F cells was decreased after treatment with NA-382 for 48 hr; treatment with a potent inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), did not affect the cell adhesion and the expression of LFA-1 molecules on AH66F cells. These results suggest that the expression of LFA-1 molecules on AH66F cells is regulated through the PKC pathway. BIOCHEM PHARMACOL 53;9:1333–1337, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** rat ascites hepatoma; AH66F cells; adhesion; LFA-1; PKC; NA-382

Adhesive interactions between cells or between cells and extracellular matrix proteins are important in the altered invasive and metastatic behavior of malignant cells [1–3]. It is clear that many of these interactions are mediated by the integrin family as cell surface receptors [1, 4, 5]. Phosphorylation of protein receptors often induces conformational changes in receptors than can affect binding characteristics of the ligands [6], and integrins have been shown to be substrates for both protein tyrosine kinase [7] and PKC§ [8, 9]. The ability of the integrins to adhere to specific cell or protein targets has been shown in lymphocytes and some malignant cells to be related to the phosphorylation state of the subunits [9–11].

We have reported that the rat ascites hepatoma AH66F cell line is a unique tumor expressing LFA-1 (CD11a/CD18) molecule on the outer surface of the plasma membrane, and that this property may be related to its high malignancy and metastatic ability [12, 13]. The adhesion rate of AH66F cells to mesothelial cells (M-cells), which express ICAM-1, was much higher than with other tumor cells without stimuli and was inhibited by PKC inhibitors, but not by other inhibitors of PKA, calmodulin-dependent protein kinase or tyrosine kinase [13]. In this study, we suggest that the inhibition by PKC inhibitors of the adhesion of AH66F cells to M-cells through the LFA-1/ICAM-1 interaction is due to inhibition of LFA-1 expression.

## MATERIALS AND METHODS

### *Agents*

H-7 and H-89 were purchased from Seikagaku Kogyo Co., Tokyo, Japan. NA-382 was kindly provided by the Pharmaceutical Research Center, Meiji Seika Kaisha Ltd., Yokohama, Japan.

These protein kinase inhibitors, NA-382, H-7, and H-89, were dissolved in DMSO and used after 1000-fold dilution with the culture medium.

Monoclonal antibodies (mAbs) of WT.1 (mouse IgG2a class), WT.3 (mouse IgG1 class), and 1A29 (mouse IgG1

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§ *Abbreviations:* PKC, $Ca^{2+}$/phospholipid-dependent protein kinase; PKA, cyclic AMP-dependent protein kinase; M-cells, mesothelial cells; LFA-1, leukocyte function-associated antigen-1, ICAM-1, intercellular adhesion molecule-1; H-7, *N*-(2-methylpiperazyl)-5-isoquinolinesulfonamide; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; NA-382, *N*-ethoxycarbonyl-7-oxostaurosporine; mAb, monoclonal antibody; WT.1, anti-LFA-1 α-chain mAb; WT.3, anti-LFA-1 β-chain mAb; 1A29, anti-ICAM-1 mAb.

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class) were purchased from Seikagaku Kogyo Co., Tokyo, Japan.

### *Cells*

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

AH66F cells were passaged weekly through female Donryu rats (Nippon SLC, Hamamatsu, Japan) and harvested from tumor-bearing animals 6 to 10 days after transplantation.

### *Adhesion Assay*

Isolation of M-cells and the adhesion assay were performed as reported previously [12]. Reversible growth of AH66F cells treated with protein kinase inhibitors was confirmed after agents were washed out [13]. Cell viability was determined by the Trypan-Blue dye-exclusion method. The viable cells ($4 \times 10^4$ per well) were seeded on an M-cell monolayer and incubated for 1 hr in the presence or absence of mAb(s) in Dulbecco's modified Eagle's medium at 37°C in a $CO_2$ incubator. After incubation, the plate was stirred for 30 sec on a micromixer (Taiyo Kagaku Co., Ltd., Tokyo, Japan). The medium and washings of each well were combined in a microtube, and the number of nonadherent cells in the medium was counted under a microscope.

The dissociation of M-cell layers by this measurement method was not observed.

### *Flow Cytometry Analysis*

Flow cytometry was done as previously described [12]. The viable cells, after treatment with or without protein kinase inhibitors, were incubated on ice in a volume of 250 μL with WT.1 or WT.3 (20 μg/mL) for 45 min and stained with Texas Red conjugated antimouse IgG (H+L) (Caltag Laboratories, San Francisco, CA). The cells were washed twice, and the fluorescence intensity was measured using an EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL) and MDADS II (Coulter).

### *Immunoblotting*

The plasma membrane of viable cells was prepared by a Percoll sedimentation method as previously reported [14]. The membrane protein (50 μg protein) was electrophoresed on SDS-polyacrylamide 10% gel and transferred onto nitrocellulose membrane filters (Schleicher & Schuell, Dassel, West Germany). After it was blocked with 5% skim milk, the membrane was incubated overnight with 1 μg/mL WT.3 and with horseradish peroxidase-conjugated antimouse IgG (Organon Teknika Co., West Chester, PA) for 1 hr. Following each incubation, the membrane was washed extensively with phosphate-buffered saline containing 0.1% Tween-20. The immunopositive band was detected by a light-emitting nonradioactive detection system (Amersham International plc, Little Chalfont, Buckinghamshire, England) and exposure to a Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY).

## RESULTS

### *Effects of Protein Kinase Inhibitors on Adhesion*

We previously reported that the adhesion of AH66F cells to an M-cell layer was specifically inhibited by PKC inhibitors, H-7 [15] and NA-382 [16], among others [13]. Figure 1A,B shows the adhesion ability of AH66F cells to the M-cell layer after treatment with NA-382. NA-382 inhibited the cell adhesion in a concentration- and time-dependent manner. Another PKC inhibitor, H-7, also inhibited the adhesion rate in a concentration-dependent manner, but the PKA inhibitor H-89 [17] had little effect even at 6 μM, which is the 50% growth inhibitory concentration [13] (Fig. 2).

The adhesion rate of AH66F cells to M-cells was clearly decreased from *ca.* 46% to 23–25% in the presence of each of the mAbs of the adhesion molecules WT.1, WT.3, and 1A29, and the cell adhesion was not changed even when 2 of these mAbs were combined (Fig. 3). These results indicate that a half fraction of adhesion of AH66F cells to M-cells is dependent upon the LFA-1/ICAM-1 system.

To confirm the involvement of PKC in the LFA-1-dependent adhesion of AH66F cells, we investigated the effects of LFA-1 mAbs on the adhesion of AH66F cells treated with NA-382 for 48 hr. The adhesion rate of AH66F cells treated with 0.5 μM NA-382, 50% growth inhibitory concentration, for 48 hr was the same as that inhibited by an mAb or a combination of two of the mAbs,

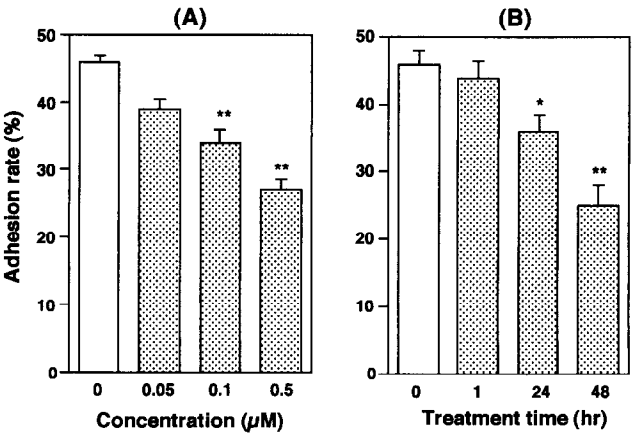


**FIG. 1. Effect of NA-382 on adhesion of AH66F cells to M-cells. AH66F cells treated with varying concentrations of NA-382 for 48 hr (A), or treated with 0.5 μM NA-382 for the indicated periods (B) were added to the M-cell layer, and the adhesion rate (%) was measured as described in Materials and Methods. Data are the means ± SE (bar) of at least 5 experiments. Significantly different from the untreated control at *$P < 0.05$ and **$P < 0.01$, respectively.**

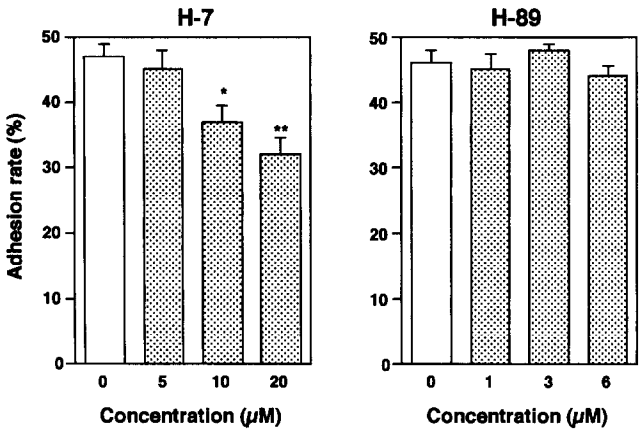


**FIG. 2. Effects of H-7 and H-89 on adhesion of AH66F cells to M-cells. AH66F cells treated with varying concentrations of H-7 or H-89 for 48 hr were added to the M-cell layer, and the adhesion rate (%) was measured. Data are the means ± SE (bar) of at least 5 experiments. Significantly different from the untreated control at *$P < 0.05$ and **$P < 0.01$, respectively.**

WT.1, WT.3, and 1A29. The decreased adhesion by NA-382 was not further influenced by addition of WT.1 or WT.3 (Fig. 3).

### *Effects of Protein Kinase Inhibitors on Expression of LFA-1*

When AH66F cells were treated with protein kinase inhibitors for 48 hr, the expression of both the LFA-1 α and β chains was decreased by NA-382 in a concentration-dependent manner but was not influenced by H-89 (Fig. 4).

Figure 5 shows the immunoblot results. The molecular weight of the immunopositive band to the LFA-1 β chain was 95 kDa. The decrease in LFA-1 β chain expression in AH66F cells by NA-382 was also confirmed by immunoblot analysis. LFA-1 β chain expression was not changed in the cells treated with H-89 for 48 hr.

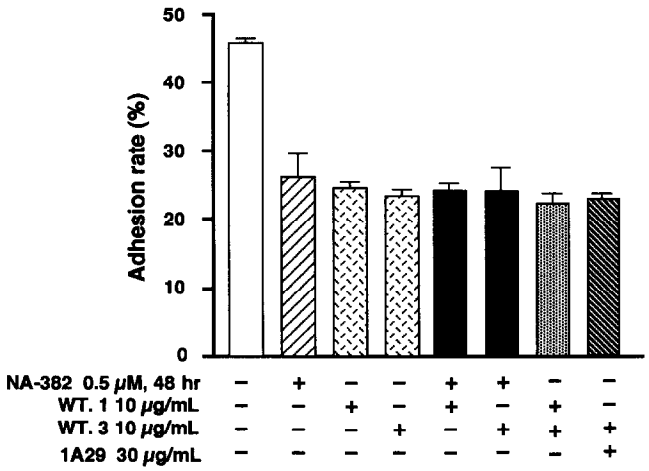


**FIG. 3. Effects of anti-LFA-1 mAbs on adhesion of AH66F cells treated with NA-382 for 48 hr. AH66F cells were treated with or without 0.5 μM NA-382 for 48 hr and added to the M-cells in the absence or presence of the indicated concentrations of mAb(s). Data are the means ± SE (bar) of at least 5 experiments.**

## DISCUSSION

In this paper, we have shown that the PKC inhibitors, but not the PKA inhibitor, reduced LFA-1 expression on the cell surface of AH66F cells, and that the decreased fraction by PKC inhibitors completely agreed with those inhibited by mAbs of LFA-1 molecules and ICAM-1 molecule. On the other hand, the fraction of AH66F/M-cell adhesion, which was not inhibited by mAbs of LFA-1 and ICAM-1 and remained after treatment with NA-382, could not be clarified because most adhesion molecules and antibodies are not yet available from rats. More studies on adhesion molecules will be required.

It has been reported that, in lymphatic cells, integrins are phosphorylated by tyrosine kinase and PKC and get high affinity for their ligands [7–9]. In T lymphocytes, the stimulation of 12-O-tetradecanoylphorbol-13-acetate and the triggering of CD2 or T cell receptor (CD3) strongly activated LFA-1-dependent adhesion [18–20]. Triggering by T cell receptor cross-linking induces PKC-mediated rearrangement of the cytoskeleton, actin polymerization, and LFA-1 association with the cytoskeleton [19, 21, 22]. In particular, because the cytoskeletal association and adhesive ability of activated LFA-1 are lost by the truncation of the LFA-1 β chain cytoplasmic domain, the phosphorylation of the β chain has been suggested to be an important factor in regulation of LFA-1-dependent adhesion [23, 24]. This evidence suggests that stimulation of protein kinases and phosphorylation of β chains are important to LFA-1-dependent cell adhesion in lymphocytes. On the other hand, the ability of AH66F cells to adhere to an M-cell layer was increased after treatment with 12-O-tetradecanoylphorbol-13-acetate, but it could not be determined if the increased adhesion resulted from stimulation of LFA-1-dependent adhesion (data not shown). Nonetheless, AH66F cells display a high avidity for adhesion to M-cells without any stimuli, that is not affected by protein kinase inhibitors, except for PKC inhibitors [13]. In this study, although a short-term (1-hr) treatment with NA-382 hardly influenced the adhesion, a long-term (more than 24-hr) treatment with the agent significantly decreased the LFA-1/ICAM-1-mediated adhesion and the expression of LFA-1 molecules on the cell surface. Therefore, the effect of PKC inhibitors in AH66F cells seems to be based on the inhibition of LFA-1 expression, rather than on the inhibition of phosphorylation of the molecule(s).

LFA-1 plays an important role in the metastasis of lymphoma cells [3, 25], but the involvement of LFA-1 in the metastasis of malignant cells other than malignant leukocytes has not been reported. In this paper and in our pre-

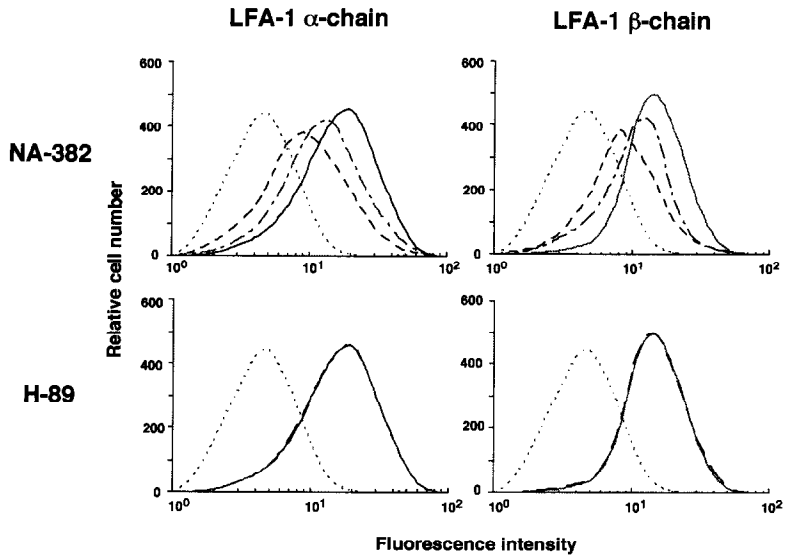


**FIG. 4. Effects of NA-382 and H-89 on expression of LFA-1 molecules on AH66F cells. AH66F cells were treated without (———) or with 6 μM H-89 (— —), or with 0.1 μM (— · —) or 0.5 μM NA-382 (- - -) for 48 hr. Background reactivity (····) of cells was stained with Texas Red conjugated second antibody only.**

vious paper [12], we indicated that part of the adhesion of AH66F cells to M-cells is mediated through LFA-1. Therefore, it is possible that LFA-1 is involved in the metastatic events of high-malignant tumor cells. Further study on the adhesive mechanism of AH66F cells may provide important information on tumor metastasis.

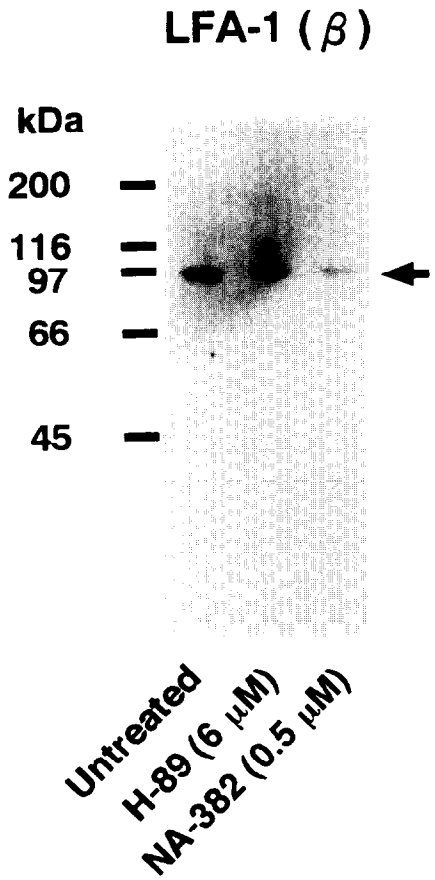


**FIG. 5. Immunoblot analysis on expression of LFA-1 (β chain) in plasma membranes from AH66F cells treated without or with 6 μM H-89 or 0.5 μM NA-382 for 48 hr.**

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