Jpn. J. Cancer Res. 88, 267–272, March 1997

Inhibition by Protein Kinase C Inhibitor of Expression of Leukocyte Function-associated Antigen-1 Molecules in Rat Hepatoma AH66F Cells

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To examine the mechanism of inhibition by protein kinase C (PKC) inhibitors of the adhesion of highly malignant hepatoma AH66F cells to the mesentery-derived mesothelial cell (M-cell) layer through leukocyte function-associated antigen-1 (LFA-1)/intercellular adhesion molecule-1, the effects of a PKC inhibitor, NA-382, on the expression of LFA-1 molecules in AH66F cells were examined and compared with those in thymocytes from normal rats. NA-382 inhibited the adhesion of AH66F cells to the M-cell layer and the expression of LFA-1 on the membrane of the hepatoma cells after treatment for more than 24 h. It was confirmed that AH66F cells express similar mRNAs for LFA-1 subunits to those of thymocytes, and their levels were also decreased after treatment with NA-382. On the other hand, the LFA-1-mediated adhesion and the expression of both protein and mRNA for LFA-1 subunits in thymocytes were not changed by the PKC inhibitor. These results suggest that the expression of LFA-1 molecules in AH66F cells may be regulated by PKC via quite different mechanisms from those in normal lymphocytes.

Key words: Rat hepatoma AH66F cell — LFA-1 — mRNA — Expression — Protein kinase C inhibitor

Leukocyte function-associated antigen-1 (LFA-1) is a member of the β_2 integrin family and mediates adhesive interactions, including priming of T cells by antigen-presenting cells, migration of leukocytes across the endothelium, and binding of effector cells (e.g., cytotoxic T cells) to their targets.[1-3] Until now it has been believed that LFA-1 molecules are expressed exclusively on the membrane of leukocytes. We recently found that the highly malignant rat ascites hepatoma cell line AH66F has LFA-1 molecules on the surface of the plasma membrane and adheres to mesentery-derived mesothelial cells (M-cells) through LFA-1/intercellular adhesion molecule-1 (ICAM-1) interaction, but the low-malignant AH130 line does not.[4] Moreover, we have indicated that the adhesion of AH66F cells to M-cells was partially inhibited by protein kinase C (PKC) inhibitors.[5] Several investigators have suggested that phosphorylation by PKC is important for the functional activation of LFA-1-dependent adhesion.[6-8] However, it is not clear whether the expression of LFA-1 molecules is regulated by PKC. In this paper, we examined the effects of a PKC inhibitor, NA-382,[9] on the expression of LFA-1 molecules and mRNAs in AH66F cells, compared with those in thymocytes.

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MATERIALS AND METHODS

Cells Rat ascites hepatoma AH66F cells and AH130 cells were provided by the Department of Experimental Chemotherapeutics, Cancer Research Institute, Kanazawa University, Ishikawa. Cells were passaged weekly through female Donryu rats (6 weeks old, Nippon SLC, Hamamatsu) and harvested from tumor-bearing animals 6 to 10 days after transplantation. Thymocytes were prepared from the thymus of normal rats. M-cells were also prepared by trypsinizing normal rat mesentery, as reported previously.[4]

Cell viability was determined by the trypan-blue dye exclusion method.

Agents A PKC inhibitor, *N*-ethoxycarbonyl-7-oxostaurosporine (NA-382),[9] was kindly provided by the Pharmaceutical Research Center, Meiji Seika Kaisha Ltd., Yokohama. A PKA inhibitor, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89),[10] and the anti-rat LFA-1 β-chain (mouse IgG1; WT.3) monoclonal antibody (mAb) were purchased from Seikagaku Kogyo Co., Tokyo. NA-382 and H-89 were dissolved in dimethyl sulfoxide and used after 1000-fold dilution with the culture medium.

Adhesion assay The viable cells (4×10^4 AH66F cells or 1×10^6 thymocytes per well) were seeded on an M-cell monolayer and incubated for 1 h in the presence or absence of mAb in Dulbecco's modified Eagle's medium

at 37°C in a CO_2 incubator. After incubation, the plate was stirred for 30 s on a micro-mixer (Taiyo Kagaku Co., Ltd., Tokyo). The medium and washings of each well were combined in a microtube, and the number of non-adherent cells was counted under a microscope.

The dissociation of M-cell layers in this assay system was not observed.

Flow cytometric analysis Flow cytometry was done as previously described.[4] The viable cells, after treatment with or without a protein kinase inhibitor, were incubated on ice in a volume of 250 μl with WT.3 (20 μg/ml) for 45 min and stained with Texas red (TR)-conjugated anti-mouse 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).

Reverse transcription-polymerase chain reaction (RT-PCR) We determined the nucleotide sequences of partial cDNAs for rat LFA-1 α and β subunits from AH66F cells, corresponding to the mouse LFA-1 subunits (bases 344–1071 in α subunit and 237–844 in β subunit; refs. 11 and 12), using an RT-PCR procedure. The putative rat α subunit cDNA obtained was highly homologous to those of mouse (89.4% identity) and human (77.6%), and the putative rat β subunit cDNA also had high degree of homology to mouse β subunit (90.0%) and human β subunit (81.9%).

Then, primers for rat LFA-1 α and β subunits were designed on the basis of the obtained cDNA clones. mRNAs were prepared from cells treated with or without a protein kinase inhibitor using a QuickPrep micro mRNA purification kit (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions. RT reactions were carried out in 40 m*M* KCl, 50 m*M* Tris-HCl (pH 8.3), 6 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 0.1 mg/ml bovine serum albumin (BSA), 1 m*M* each of dATP, dCTP, dGTP, and dTTP, 10 units of RNase inhibitor (Promega, Madison, WI), 100 pmol of random hexamer, mRNA and 200 units of the Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Berlin, Germany) in a final volume of 50 μl at 37°C for 60 min. PCR reactions were carried out in a final volume of 25 μl containing 5 μl of RT reaction mixture, 50 m*M* KCl, 20 m*M* Tris-HCl (pH 8.3), 2.5 m*M* $MgCl_2$, 0.1 mg/ml BSA, 0.2 m*M* each of dATP, dCTP, dGTP, and dTTP, 10 μM each of the mixed oligonucleotide primer, and 0.5 unit of Taq DNA polymerase (Gibco-BRL). Each cycle consisted of 45 s at 94°C, 1 min at 55°C, and 1 min at 72°C. The sequences of the rat LFA-1 α subunit primers were 5′-GGG CAA TGT CGA CCT AGT GTT TCT G-3′ and 5′-GGC AAA TAT GTG GAG TTT CTC CTG C-3′, and the β subunit primers used were 5′-TCA AGG GTT GCC CAG CCG ATG ATA T-3′ and 5′-TCA GTT GCT TGC CGA CCT CTG TCT G-3′. The sequences of the β-actin primers were 5′-TTC TAC AAT GAG CTG CGT GTG GC-3′ and 5′-CTC (A/G)TA GCT CTT CTC CAG GGA GGA-3′, as shown in our previous paper.[13] The primers of rat CD45 were placed in exon 2 (P2) and in exon 9 (P9) of the CD45 gene, as presented by Hansson *et al.*[14] The P2 sense primer sequence was 5′-AAG TCT TTG TCA CAG GGC AA-3′ and the P9 antisense primer was 5′-TAA CGC ACA GTA ACG TTC CC-3′.

Statistics Statistical analyses were done by using Student's *t* test.

Table I. Effects of Protein Kinase Inhibitors and Anti-leukocyte Function-associated Antigen (LFA)-1 Monoclonal Antibody (mAb) on Adhesion of the Cells to Mesothelial Cells (M-cells)

	AH66F cells		Thymocytes	
	−WT.3	+WT.3	−WT.3	+WT.3
Untreated	46.1±2.1	25.4±2.1[b, d]	32.3±2.4	9.3±1.2[b, d]
NA-382 (0.5 μM)				
2 h	44.5±4.1	26.8±2.6[b, d]		
24 h	36.4±3.6[a]	23.4±2.0[b, c]		
48 h	25.1±3.1[b]	26.2±3.0[b]	31.8±3.4	10.0±0.6[b, d]
H-89 (6 μM)				
48 h	44.2±1.8	25.2±2.8[b, d]	32.4±4.1	10.9±1.0[b, d]

After treatment of AH66F cells and thymocytes with 0.5 μM NA-382 or 6 μM H-89 for the indicated periods, the adhesion ability of the cells to the M-cell layer was measured in the absence (−WT.3) or presence (+WT.3) of 10 μg/ml anti-LFA-1 mAb (WT.3). The adhesion rate (%) was calculated as described in "Materials and Methods." Data are the means±SE of at least three experiments.

a, b) Significantly different from the adhesion rate in the untreated control at $P<0.05$ and 0.01, respectively.

c, d) Significantly different from the adhesion rate in the absence of WT.3 at $P<0.05$ and 0.01, respectively.

RESULTS

Effects of protein kinase inhibitors on adhesion to M-cells Table I shows the adhesion ability of AH66F cells and thymocytes to the M-cell layer after treatment with protein kinase inhibitors. When AH66F cells were treated with a 50%-growth-inhibitory concentration of each protein kinase inhibitor, their adhesion ability was significantly suppressed by NA-382 (0.5 μM) treatment for more than 24 h, but was hardly affected by H-89 (6 μM) treatment, even for 48 h. It was confirmed that after treatment with and washing out of these compounds, the cells retained proliferating ability. The adhesion rate of AH66F cells (0 h) to M-cells was clearly decreased from ca. 46% to 25% in the presence of mAb to LFA-1 β, WT.3 (10 μg/ml). The adhesion rate of AH66F cells in the presence of WT.3 was not changed by treatment with NA-382, and the adhesion of the cells treated with 0.5

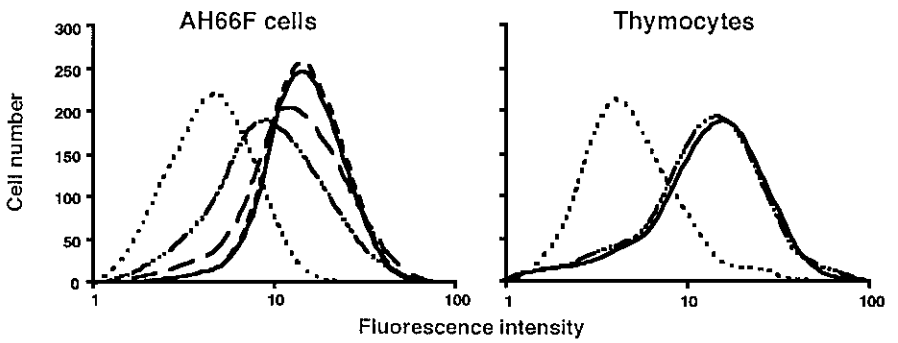

Fig. 1. Effects of NA-382 on the expression of LFA-1β on the surface of AH66F cells and thymocytes. Cells were untreated for 48 h (——) or treated with the protein kinase C inhibitor (0.5 μM) for 2 h (– – –), 24 h (— —) or 48 h (—·····—), and cells positive to WT.3 were analyzed. Background reactivity (·····) of the cells was that obtained with TR-conjugated second antibody alone. NA-382, *N*-ethoxycarbonyl-7-oxostaurosporine; LFA, leukocyte function-associated antigen; TR, Texas red.

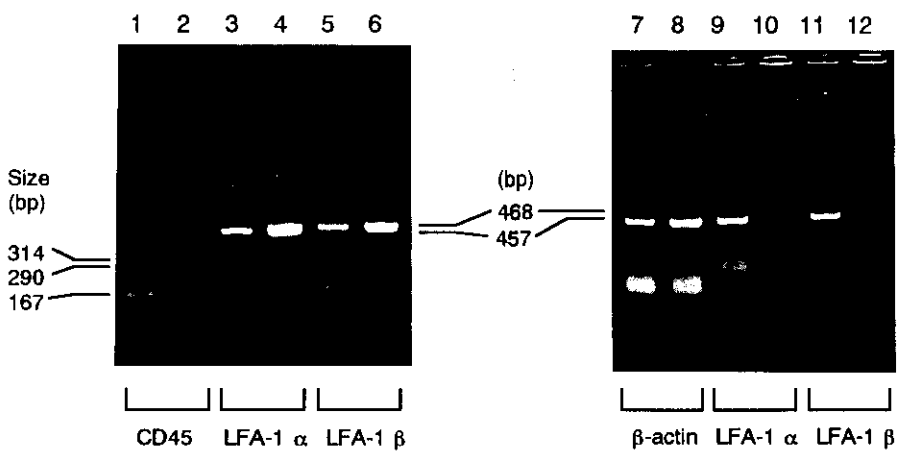

Fig. 2. Expression of LFA-1 α and β subunit mRNAs in rat thymocytes, AH66F cells and AH130 cells. Purified mRNA was reverse-transcribed and then amplified for 30 cycles of polymerase chain reaction (PCR), according to "Materials and Methods." Lanes 1, 3 and 5, mRNAs from rat thymocytes; lanes 2, 4, 6, 7, 9 and 11, mRNAs from AH66F cells; lanes 8, 10 and 12, mRNAs from AH130 cells. Lanes 1 and 2 show the expression of mRNA splicing variants of CD45, a leukocyte common antigen; the sizes of the RT-PCR products are 314 bp, 290 bp, and 167 bp. The expression of mRNAs of LFA-1 α subunit (457 bp) and β subunit (468 bp) is seen in lanes 3, 4, 9 and 10, and in lanes 5, 6, 11 and 12, respectively. Lanes 7 and 8 show the expression of mRNAs of β-actin (456 bp). LFA, leukocyte function-associated antigen.

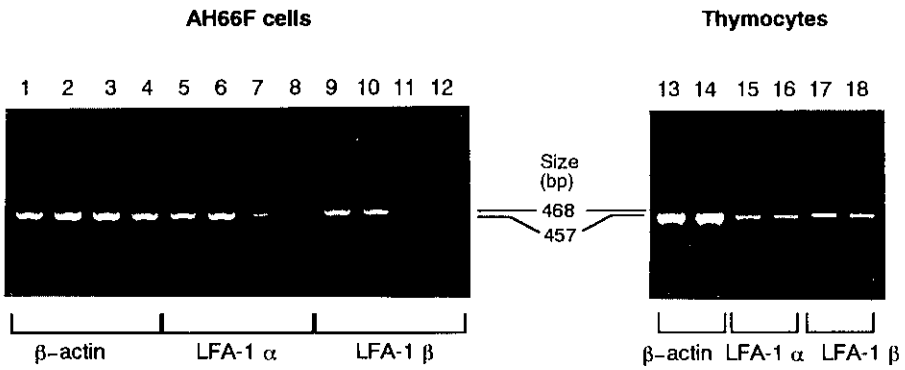

Fig. 3. Effects of NA-382 on the expression of LFA-1 α and β subunit mRNAs in AH66F cells and thymocytes. Cells were treated with 0.5 μM NA-382 for 0 h (lanes 1, 5, 9, 13, 15, 17), 2 h (lanes 2, 6, 10), 24 h (lanes 3, 7, 11) or 48 h (lanes 4, 8, 12, 14, 16, 18). cDNA was amplified for 20 cycles. NA-382, *N*-ethoxycarbonyl-7-oxostaurosporine; LFA, leukocyte function-associated antigen.

μM NA-382 for 48 h was similar to that observed in the presence of WT.3. On the other hand, the adhesion of thymocytes to M-cells was also suppressed by WT.3, but was not changed after treatment with either of the protein kinase inhibitors for 48 h.

Effects of protein kinase inhibitors on the expression of LFA-1β on AH66F cells and thymocytes When cells were treated with NA-382 (0.5 μM), the expression of LFA-1β subunit on AH66F cells was decreased in a time-dependent manner, but that on thymocytes was not influenced by the PKC inhibitor (Fig. 1). The expression of LFA-1 on these cells was not influenced by H-89 (data not shown).

RT-PCR analysis Fig. 2 shows the results of RT-PCR analysis of LFA-1 mRNAs expressed in thymocytes, AH66F cells, and AH130 cells. Thymocytes expressed several CD45 mRNA splicing variants, as reported by Hansson *et al.*,[14] while AH66F cells did not express any CD45 fragments. RT-PCR products of LFA-1 α and β mRNAs from AH66F cells were 457 bp and 468 bp in size, respectively, as were those from thymocytes. These RT-PCR products were confirmed by digestion with several restriction enzymes (*Eco*T14 and *Hae* III for α-subunit and *Eco*T14, *Pst* and *Hin*c II for β-subunit) to produce the same size fragments in the cases of AH66F cells and thymocytes (data not shown). On the other hand, these products were not detected in AH130 cells, which have no LFA-1 on the cell surface.[4]

Next, the levels of mRNA for LFA-1 subunits in AH66F cells and thymocytes treated with or without 0.5 μM NA-382 were compared. The mRNA levels of both LFA-1 α and β subunits in AH66F cells were decreased after treatment with NA-382 for more than 24 h, without any change in the expression of β-actin, while the levels in thymocytes were not changed even after a 48 h treatment (Fig . 3).

DISCUSSION

AH66F cells display a high avidity for adhesion to M-cells through LFA-1/ICAM-1 interaction without any stimulus, and this adhesion is inhibited by PKC inhibitors, while the adhesion of AH130 cells, which do not express LFA-1 on the membrane, is not influenced by the protein kinase inhibitors.[5] This study indicated that a selective PKC inhibitor, NA-382, inhibited not only the adhesion of AH66F cells to M-cells, but also the expression of LFA-1β on the membrane (Table I and Fig. 1). Moreover, in this study, we determined partial cDNA sequences for LFA-1α and β subunits of AH66F cells, corresponding to the mouse LFA-1 subunits, using an RT-PCR procedure. The cDNAs obtained had high degrees of homology to mouse and human LFA-1 subunits. Then, the expression levels of mRNAs in AH66F cells and thymocytes from normal rats were compared. The molecular weight of the RT-PCR products for these LFA-1 subunits of AH66F cells was similar to that in the case of thymocytes (Fig. 2). However, after treatment with NA-382 for more than 24 h, the expression of mRNAs of both LFA-1 α and β subunits in AH66F cells was decreased as well as the protein expression, whereas in thymocytes this PKC inhibitor did not affect the expression levels of protein and mRNA of LFA-1 subunits (Figs. 1 and 3).

There are numerous reports which have suggested that stimulation of PKC and the phosphorylation of β subunit are important in functional regulation of LFA-1-dependent adhesion.[6–8, 15–17] In this study, the effects of NA-382

appeared after a long-term (more than 24 h) treatment, but not after a short-term (2 h) treatment of AH66F cells. Therefore, these results suggest that the inhibitory effect of the PKC inhibitor on AH66F cell adhesion is based on the inhibition of LFA-1 expression rather than on the inhibition of phosphorylation of the adhesion molecule(s). On the other hand, although the promoter region of LFA-1 α subunit (CD11a) gene was cloned by several investigators and has been characterized,[18–20] it has not been clear whether PKC contributes to the expression of LFA-1 in lymphatic cells, as was shown in this study (Fig. 3). However, Pedrinaci *et al.*[21] reported that phorbol ester induces the expression of LFA-1 in a monoblastoid cell line. Back *et al.*[22] have indicated that the PKC activator increased the LFA-1 mRNA levels in HL-60 cells and demonstrated that the increased expression was controlled by posttranscriptional mechanisms in cell differentiation. The expression of LFA-1 in lymphatic cells may be enhanced through PKC by the stimuli, but not under the unstimulated conditions, as indicated in this study. However, though the mechanism by which AH66F cells express LFA-1 molecules is still unclear, we can not rule out the possibility that the gene expression and/or transcriptional processes may be regulated through the PKC pathway even under unstimulated conditions.

In conclusion, we have demonstrated that the decrease in the LFA-1-mediated adhesion of AH66F cells by PKC inhibitor was based on a reduction of LFA-1 molecules on the cell surface by inhibition of the transcriptional process. Because the adhesion ability and LFA-1 mRNA levels in thymocytes from normal rats were not influenced by the PKC inhibitor, not only expression of LFA-1, but also its regulation in the malignant tumor cells is thought to be quite different from the usual mechanisms.

ACKNOWLEDGMENTS

We are grateful to Professor M. Seiki of the Cancer Research Institute, Kanazawa University for helpful discussions and support for the molecular biological studies. This work was partially supported by the Special Research Fund of Hokuriku University and by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan.

(Received November 5, 1996/Accepted January 6, 1997)

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