

A Protein Kinase C Inhibitor NA-382 Prolongs the Life Span of AH66F-Bearing Rats as Well as Inhibiting Leukocyte Function-Associated Antigen-1 (LFA-1)-Dependent Adhesion of the Cells

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Rat ascites hepatoma AH66F is a high malignant tumor line, and AH66F-bearing rats died about 10 d after tumor inoculation. When treated with a protein kinase C (PKC) inhibitor, NA-382, the life span of AH66F-bearing rats was significantly prolonged, while a potent protein kinase A inhibitor, H-89, was not effective. In the adhesion assay, the adhesive ability to the mesentery-derived mesothelial cells (M-cells) of AH66F cells from rats injected with 10 mg/kg of NA-382 was significantly decreased, while the adhesion rate of the cells from the vehicle control group and from the H-89 (10 mg/kg)-treated group was about 50%. The adhesion of AH66F cells from the vehicle control group was curtailed to one half by leukocyte function-associated antigen-1 (LFA-1) β -chain monoclonal antibody (WT.3), but that from the NA-382 group was not further influenced by WT.3. In flow cytometric analysis using WT.3, the expression of LFA-1 β -chain on AH66F cells from the NA-382-treated group was also partially decreased, while that from the H-89-treated group was not changed. It was confirmed *in vitro* that after treatment with these protein kinase inhibitors for 48 h the expression of LFA-1 β -chain in the cells was decreased by NA-382, but not by H-89. These results suggested that the PKC inhibitor prolongs the life span of AH66F-bearing rats through inhibition of LFA-1-dependent adhesion of the cells.

Key words rat ascites hepatoma; AH66F; adhesion; LFA-1; protein kinase C; NA-382

Compelling clinical evidence indicates that tumor cells are malignant mainly because of their ability to spread throughout the body and give rise to metastatic foci.¹⁾ Tumor cells have a number of adhesive abnormalities which contribute significantly to their invasive ability.^{2–4)}

Rat ascites hepatoma (AH) cell lines have been induced with dimethylaminoazobenzene and established as transplantable tumors.⁵⁾ When AH cells were inoculated intraperitoneally into rats, the host animals died within a particular period. We have reported that a high malignant cell line AH66F is a unique hepatoma expressing leukocyte function-associated antigen-1 (LFA-1)⁶⁾ and has a higher metastatic ability to lung and liver in chick embryo than low malignant AH130 and AH66 cell lines.⁷⁾ The adhesive ability and the metastatic ability of AH66F cells treated with a protein kinase C (PKC) inhibitor, NA-382,⁸⁾ was significantly decreased.^{6,7)}

In this study, we showed that NA-382 prolongs the life span of AH66F-bearing rats and suggested that its effect relates to the inhibition of adhesive ability of AH66F cells to mesentery-derived mesothelial cells (M-cells) through a decrease in expression of an LFA-1 molecule on the cell surface.

MATERIALS AND METHODS

Materials N-Ethoxycarbonyl-7-oxostauroporine (NA-382) was generously provided by the Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., Yokohama. N-[2-(p-Bromocinnamyl-amino)ethyl]-5-isoquinoline-sulfonamide (H-89) was purchased from Seikagaku Kogyo Co., Tokyo. Anti-LFA-1 β -chain monoclonal antibody (WT.3) was purchased from Seikagaku Kogyo Co., and Texas red (TR)-conjugated anti-mouse IgG (H+L) was from Caltag Laboratories, San Francisco, CA.

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NA-382, a PKC inhibitor,⁸⁾ and H-89, a protein kinase A (PKA) inhibitor,⁹⁾ were suspended in 0.5% carboxymethyl cellulose (CMC) in physiological saline for *in vivo* use. For the *in vitro* adhesion assay, these agents were dissolved in dimethyl sulfoxide and used after 200-fold dilution with culture medium.

Cell Rat ascites hepatoma AH66F cells were maintained serially by intraperitoneal passage in female Donryu rats (Nippon SLC, Hamamatsu). Cells in the logarithmic growing phase were harvested from the abdominal cavity of the rats and used.

Treatment of AH66F-Bearing Rats Ten rats in a group were intraperitoneally inoculated with AH66F cells (10⁶) on day 0 and intraperitoneally administered a protein kinase inhibitor once a day from days 3 to 7. Blood samples for the hematocrit value were collected from the retroorbital sinus 7 d after tumor cell inoculation.

In Vitro Adhesion Assay Mesentery-derived mesothelial cells (M-cells), which were isolated as previously reported,⁶⁾ were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. AH66F cells (4 × 10⁴ per well), harvested from the tumor-bearing rats treated with or without a protein kinase inhibitor, were seeded on an M-cell monolayer. The cell mixture was incubated in the presence or absence of WT.3 for 1 h at 37 °C in a CO₂ incubator, then the plate was stirred for 30 s on a micro-mixer, washed twice, and the resulting nonadherent cell number was counted under a microscope.

Flow Cytometry AH66F cells were incubated on ice in a volume of 250 μ l with WT.3 (20 μ g/ml) for 45 min and stained with TR-conjugated anti-mouse IgG. The cells were washed twice, and the fluorescence intensity was measured using an EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL).

RESULTS

Effects of Protein Kinase Inhibitors on the Life Span of AH66F-Bearing Rats Figure 1 shows the survival rate of AH66F-bearing rats injected with protein kinase inhibitors. The vehicle control (0.5% CMC) group died within 14d after tumor inoculation. Similar survival behavior was observed in the group injected with a PKA inhibitor, H-89 (10 mg/kg), but a PKC inhibitor, NA-382, prolonged the life span in a dose-dependent manner (Fig. 1), without influencing the body weight.

The hematocrit values, as an indicator of anemia, of each group 7d after tumor inoculation (final day of injection) are shown in Table 1. While the values in the vehicle control group dropped, those in the group injected with NA-382, but not H-89, were maintained at a high level.

Adhesion to M-Cells of AH66F Cells from Rats Injected with Protein Kinase Inhibitors AH66F cells were harvested 8d after tumor inoculation (24h after the final injection of 10 mg/kg of NA-382 or H-89). While the adhesion rates of the cells from the 0.5% CMC group and the H-89-treated group were about 50%, that from the NA-382-treated group was significantly low ($34 \pm 2\%$) (Fig. 2).

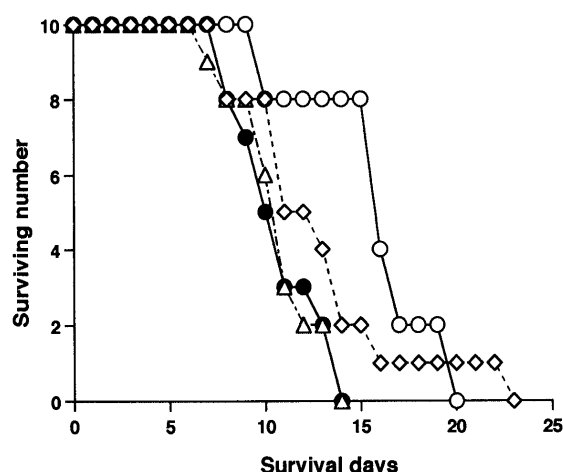


Fig. 1. Survival Rates of AH66F-Bearing Rats Injected with Protein Kinase Inhibitors

Ten rats in a group were intraperitoneally inoculated with AH66F cells (10^6) on day 0 and intraperitoneally injected with 0.5% CMC (—●—), 10 mg/kg (—○—) or 50 mg/kg NA-382 (—◇—), or 10 mg/kg H-89 (—△—) once a day from day 3 to 7.

Table 1. Effects of Protein Kinase Inhibitors on the Hematocrit Value of AH66F-Bearing Rats

Treatment	Dose (mg/kg)	Hematocrit value (Mean \pm S.D., %)
Healthy control		45.0 \pm 3.3
0.5% CMC		31.1 \pm 4.9*
NA-382	10	36.8 \pm 1.9* [#]
	50	42.1 \pm 11.3 [#]
H-89	10	32.2 \pm 6.6*

Ten rats in a group were intraperitoneally inoculated with AH66F cells (10^6) on day 0 and intraperitoneally administered NA-382 or H-89 once a day from day 3 to 7. The hematocrit value of blood collected from the retroorbital sinus on the final day of administration was determined. * Significantly different from the healthy control at $p < 0.001$. [#] Significantly different from the 0.5% CMC group at $p < 0.05$.

Our previous paper showed that part of the adhesion of AH66F cells to M-cells results from an interaction between LFA-1 on AH66F cells and intercellular adhesion molecule-1 (ICAM-1) on M-cells, because the adhesion of AH66F cells to M-cells is reduced by half in the presence of monoclonal antibodies for LFA-1 α -chain, β -chain and/or ICAM-1.⁶⁾ We therefore examined here the adhesive ability of AH66F cells from rats treated with protein kinase inhibitors and the effects of WT.3 on the cell adhesion. The adhesion rates of the cells from the vehicle and H-89 groups were inhibited by addition of 10 μ g/ml of WT.3, but the decreased adhesion of the cells from the NA-382 group was not further inhibited by the antibody (Fig. 2).

Expression of LFA-1 β -Chain on AH66F Cells from Rats Treated with Protein Kinase Inhibitors Figure 3 shows a typical cytogram of sequential flow cytometric analysis of LFA-1 β -chain on AH66F cells from groups treated with or without protein kinase inhibitors. LFA-1 β -chain on the cells from rats treated with 10 mg/kg of NA-382 was shifted to a low level of expression, but treatment with

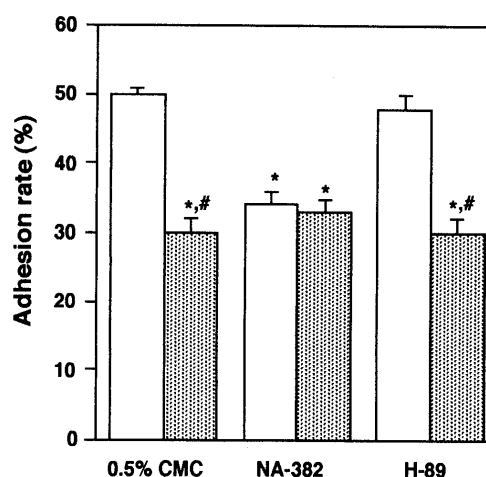


Fig. 2. Effects of Anti-LFA-1 β -Chain Monoclonal Antibody (WT.3) on Adhesion of AH66F Cells from the Tumor-Bearing Rats Treated with 10 mg/kg of NA-382 or H-89

The adhesion assay was done in the absence (□) or presence of WT.3 (▨). Data are the mean \pm S.E. * Significantly different from the 0.5% CMC group at $p < 0.001$; [#] Significantly different from the adhesion rate in the absence of WT.1 at $p < 0.001$.

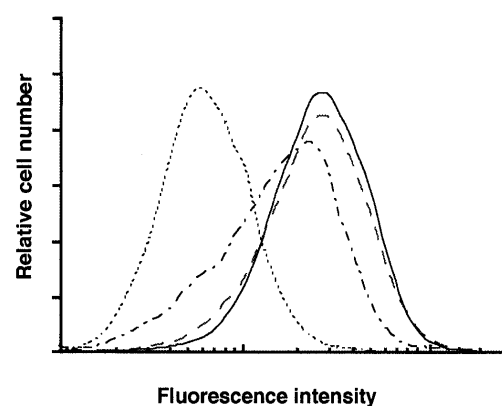


Fig. 3. The Expression of LFA-1 β -Chain of AH66F Cells from the Tumor-Bearing Rats Treated with Protein Kinase Inhibitors

Cells were harvested from the tumor-bearing rats treated with 0.5% CMC (—), 10 mg/kg NA-382 (---) or 10 mg/kg H-89 (···) 8d after tumor inoculation. Background reactivity (— · —) of cells was stained with TR-conjugated second antibody only.

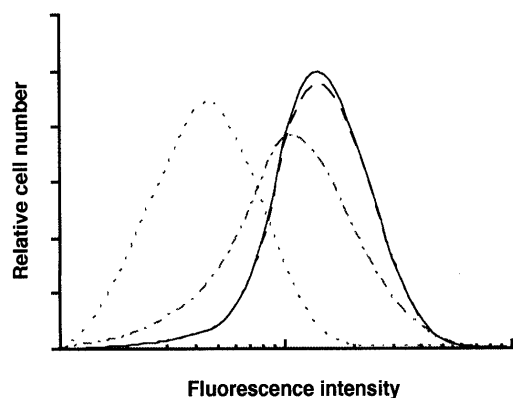


Fig. 4. The Expression of LFA-1 β -Chain on AH66F Cells Treated with Protein Kinase Inhibitors *in Vitro*.

Cells (10^6 /ml) were cultured without (—) or with $0.5 \mu\text{M}$ NA-382 (---) or $6 \mu\text{M}$ H-89 (···) at 37°C for 48 h, and flow cytometry was done. Background reactivity (---) of cells was stained with TR-conjugated second antibody only.

10 mg/kg of H-89 did not influence the expression of LFA-1.

***In Vitro* Effects of Protein Kinase Inhibitors on the Expression of LFA-1 β -Chain on AH66F Cells** Since we previously indicated that the adhesion ability of viable AH66F cells to M-cells was significantly inhibited by NA-382, but not by H-89, after culture with the 50% growth inhibitory concentration of the agents for 48 h,⁷⁾ the expression of LFA-1 β -chain on AH66F cells was analyzed under the same experimental conditions. As shown in Fig. 4, the expression level of the adhesion molecule was clearly decreased after treatment with $0.5 \mu\text{M}$ NA-382 but was hardly changed by $6 \mu\text{M}$ H-89.

DISCUSSION

We previously reported that highly malignant AH66F cells express LFA-1 on the cell surface and adhere to M-cells through an LFA-1/ICAM-1 interaction,⁶⁾ and that the adhesion was inhibited by a PKC inhibitor, NA-382, among several protein kinase inhibitors.⁷⁾ In this study, we compared the effects of NA-382 on AH66F-bearing rats with those of a PKA inhibitor, H-89, and its mechanism. NA-382 clearly prolonged the life span of AH66F-bearing rats and inhibited decrease in the hematocrit value as an indicator of bleeding in the peritoneal cavity after tumor inoculation, but this was not true of H-89 (Fig. 1, Table 1). The adhesive ability to M-cells of AH66F cells harvested from rats injected with NA-382 (10 mg/kg) was significantly lower than that from the vehicle control group (Fig. 2), as true in the *in vitro* treatment with the agent ($0.5 \mu\text{M}$) for 48 h.⁷⁾ The lower adhesion rate of AH66F cells from the NA-382 group was not further influenced by WT.3, while the adhesion rate of the cells from the H-89 group was similar to the level of the vehicle control and dropped to half in the presence of WT.3. These results suggest that NA-382 intraperitoneally injected inhibits the LFA-1-mediated adhesion of AH66F cells to the mesentery in the peritoneal cavity.

There is much evidence that stimulation of PKC and phosphorylation of LFA-1 β -chain are important to

LFA-1-dependent cell adhesion in lymphocytes.^{10–15)} The stimulation of T lymphocytes by 12-*O*-tetradecanoylphorbol-13-acetate and triggering of CD2 or T cell receptor (CD3) strongly activated LFA-1-dependent adhesion.^{10,13,15)} The triggering by T cell receptor cross-linking induces PKC-mediated rearrangement of the cytoskeleton, actin polymerization, and LFA-1 association with the cytoskeleton.^{11–14)} It has also been reported that stimulation of the integrins in melanoma cells modulated the expression of type IV collagenase and invasive ability of these cells.^{16,17)} Moreover, Mattila *et al.* reported that expression of ICAM-1, a counter ligand of LFA-1, in endothelial cells is regulated by a PKC pathway.¹⁸⁾ This study indicated that the expression of LFA-1 β -chain was lowered on AH66F cells by the PKC inhibitor NA-382 not only in *in vivo* but *in vitro* (Figs. 3 and 4). From these results and evidence, it seems that NA-382 modulates the adhesion-invasion pathway of AH66F cells in the host animal through inhibition of the PKC-dependent phosphorylation in AH66F cells and mesothelial cells and prolongs the life span of tumor-bearing rats.

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REFERENCES

- Poste G., Fidler I. J., *Nature* (London), **283**, 139–146 (1980).
- Ruoslahti E., *Annu. Rev. Biochem.*, **57**, 375–413 (1988).
- Chan B. M. C., Matsuura N., Takada Y., Zetter B. R., Hemler M. E., *Science*, **251**, 1600–1602 (1991).
- Dedhar S., Saulnier R., Nagle R., Overall C. M., *Clin. Exp. Metastasis*, **11**, 391–400 (1993).
- Yoshida T., *Ann. N. Y. Acad. Sci.*, **63**, 852–881 (1956).
- Nomura M., Yamamoto H., Sugiura N., Kuroda K., Kawaguchi H., Miyamoto K., *Jpn. J. Cancer Res. (Gann)*, **87**, 86–90 (1996).
- Yamamoto H., Endo Y., Nomura M., Miyamoto K., Sasaki T., *Anticancer Res.*, **16**, 413–418 (1996).
- Miyamoto K., Inoko K., Ikeda K., Wakusawa S., Kajita S., Hasegawa T., Koyama M., *J. Pharm. Pharmacol.*, **45**, 43–47 (1992).
- Chijiwa T., Mishima A., Hagiwara M., Sano M., Hayashi K., Inoue T., Naito K., Toshioka T., Hidaka H., *J. Biol. Chem.*, **265**, 5267–5272 (1990).
- van Kooyk Y., Kemenade P. vd Wiel-v., Weder P., Kujipers T. W., Figdor C. G., *Nature* (London), **333**, 811–813 (1989).
- Kelleher D., Murphy A., Cullen D., *Eur. J. Immunol.*, **20**, 2351–2354 (1990).
- Hibbs M. L., Xu H., Stacker S. A., Springer T. A., *Science*, **251**, 1611–1613 (1991).
- Pardi R., Inveradi L., Rugarli C., Bender J. R., *J. Cell Biol.*, **116**, 1211–1220 (1992).
- Haverstick D. M., Sakai H., Gray L. S., *Am. J. Physiol.*, **262**, C916–C926 (1992).
- Lollo B. A., Chan K. W. H., Hanson E. M., Moy V. T., Brian A. A., *J. Biol. Chem.*, **268**, 21693–21700 (1993).
- Seftor R. E. B., Seftor E. A., Gehlsen K. R., Stetler-Stevenson W. G., Brown P. D., Ruoslahti E., Hendrix M. J. C., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 1557–1561 (1992).
- Seftor R. E. B., Seftor E. A., Stetler-Stevenson W. G., Hendrix M. J. C., *Cancer Res.*, **53**, 3411–3415 (1993).
- Mattila P., Majuri M. L., Mattila P. S., Renkonen R., *Scand. J. Immunol.*, **36**, 159–165 (1992).