ABSTRACT

Gazi MH and Ito M have previously shown that novel monoclonal antibody (mAb) LAD-4 inhibited the binding of RL⁺¹ lymphoma cells to fibronectin (FN) in vitro and partially those migration to the liver in vivo. In this study, I further examined the ability of LAD-4 to inhibit the RL⁺¹ liver metastasis and to have a cytopathic effect.

Histological examination revealed s.c. inoculation of BALB/c mice with RL⁺¹ followed i.p. injection of LAD-4 inhibited liver metastasis. Furthermore, flow cytometry (FCM) measurement of number of RL⁺¹ cells into liver confirmed LAD-4 obviously decreased them. LAD-4 improved survival of mice inoculated intravenously with RL⁺¹, but was not curative. LAD-4 decreased RL⁺¹ proliferation in vitro. Adsorbed LAD-4 on the micro-plates downregulated their proliferation under serum-free conditions, but FN did not. Treatment of RL⁺¹ with LAD-4 for 3h increased Annexin V-positive cells and caspase-3 activation. Moreover, FCM analysis using terminal deoxynucleotidyl transferase (TdT) revealed that 24h treatment of RL⁺¹ with LAD-4 induced apoptotic DNA fragmentation. Inhibition of lymphoma cell adhesion to hepatocytic FN have been postulated the mechanism of LAD-4-mediated inhibition of RL⁺¹ liver metastasis. However, my results suggest that LAD-4 transduces an apoptotic signal through crosslinking of its receptor molecules and this may be another mechanism whereby LAD-4 inhibits RL⁺¹ liver metastasis.

Key words: fibronectin receptor, LAD-4, apoptosis, Annexin V, DNA fragmentation
function associated antigen-1 (LFA-1), which belongs to the $\alpha_2$-integrin subfamily, is involved in liver metastasis by certain lymphoma cells. As there is no basement membrane under the sinusoidal endothelium and hepatocytes express FN on their surfaces, if lymphoma cells express FN receptors, the FN/FN receptor interaction may also be involved in the liver metastasis.

Recently, Gazi MH and Ito M developed a mAb, LAD-4, that inhibits the binding of RL-1 lymphoma cells to FN in vitro and partially inhibits RL-1 cell migration to the liver. The molecular weight of the antigen recognized by LAD-4 is approximately 55 kDa, which differs from those of known any FN receptors. Therefore, this antigenic molecule appears to be a new FN receptor. As the ability of a tumor cell to migrate to a target organ does not correlate with its ability to metastasize in that organ, I examined whether LAD-4 can inhibit liver metastasis by RL-1 cells. Accumulating evidence indicates that the integrin family of cell adhesion receptors can transduce biochemical signals to modulate cell growth and differentiation. Therefore, in this study, I investigated whether LAD-4 had a cytopathic effect on RL-1 cells and the mechanisms whereby LAD-4 inhibits liver metastasis by RL-1 cells. I found that LAD-4 induced apoptosis of RL-1 cells in vitro. Other possible mechanisms responsible for the inhibition of liver metastasis by LAD-4 are discussed.

MATERIALS AND METHODS

The following investigations were performed under the protocol approved by the Animal Research Laboratory of Yamagata University School of Medicine.

Animals and Cell Lines.

BALB/c mice and BALB/c nude mice, 6-8 weeks of age, were purchased from Japan SLC Inc. (Hamamatsu, Japan) and kept in the facilities at Animal Research Laboratory of Yamagata University School of Medicine. T cell lymphoma cell line derived from BALB/c mice, RL-1, was obtained from National Cancer Institute (Bethesda, MD) and maintained in our laboratory with culture medium: RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Whittaker Bioproducts, Walkersville, MD), 50 $\mu$g/ml of gentamycin as reported previously. Ascites cell line of RL-1, the single lot stored in liquid nitrogen in 1980 was used for in vivo experiments described below.

Antibodies.

Rat mAb specific for CD44 (IM7) and phycoerythrin (PE)-conjugated streptavidin were purchased from BD PharMingen (San Diego, CA). Peroxidase-conjugated mouse IgG specific for rabbit IgG was from Jackson ImmunoResearch Laboratory (West Grove, PA). Rat hybridoma cell line producing anti-LFA-1 (M17/4) was obtained from American Type Cell Collection (Rockville, MD). LAD-4 and anti-LFA-1 mAb were purified from ascites on a protein G-Sepharose column (Amersham Pharmacia, UK) as reported previously.

In Vivo Experiments.

Mice were injected with $2 \times 10^5$ RL-1 cells either s.c. in the dorsal flank or i.v. into the tail vein, and then received i.p. 200 $\mu$g of LAD-4, anti-LFA-1 mAb, or normal rat IgG. The efficacy of mAb administration was evaluated by the prolongation of mean survival time of tumor bearing hosts, which were observed for
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23 to 27 days.

**Histological Examination.**

Mice (5 mice per group) were sacrificed at 3 weeks postinjection and examined for the presence of lymphoid tumors at necropsy. Liver, kidneys, spleen, and lungs were removed and fixed in 10% formalin and embedded in paraffin, and 3 μm sections (4 per liver) were stained with hematoxilin and eosin. The liver samples were subjected to microwave treatment and stained immunohistochemically using peroxidase-conjugated anti proliferating cell nuclear antigen (PCNA) (PC10, DAKO, Copenhagen, Denmark) antibody as described elsewhere. The images of each section (100 fields per liver) were captured by a digital still camera (FUJIFILM, Tokyo, Japan). The number of lymphoma cells, estimated by PCNA positivity and nuclear size, was measured by a computer-associated image analysis system using NIHimage software. The experiment was repeated at least three times.

**Flow Cytometric Analysis.**

Cell suspensions were prepared from liver using a glass homogenizer and then overlayed on a 60% solution of Percoll (Amersham Pharmacia) to remove hepatocytes and red blood cells. After centrifugation at 400 g for 20 min, cells of the interface were collected and washed with culture medium. The cells were incubated with biotynilated LAD-4 that was made according to the manufacturer's instructions (Pierce Chemical Co., Rockfold, IL). After 60 min on ice, cells were washed with PBS and subsequently incubated with PE-conjugated streptavidin for 30 min on ice. After washing with PBS, the cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Prior to the analysis, the gate was set using the mixture of ascites-line RL–1 cells and spleen cells according to forward and side scatter. The experiment was repeated once with similar results.

**In Vitro Experiments.**

The culture cell line of RL–1 was used for following in vitro experiments.

**Measurement of Cell Growth.**

Growth of RL–1 cells was determined by use of a colorimetric assay using a WST-1 tetrazolium salt reagent (TaKaRa Biomedicals, Tokyo, Japan). 1-2 × 10^5 cells were cultured in 100 μl of culture medium with various doses of normal rat IgG, LAD-4, or anti-LFA-1 mAb. After 24h or 48h at 37°C, 10 μl of WST-1 reagent was added to each well of a 96-well tissue culture plate (Coster, Cambridge, MA) and allowed react for 1.5 h at 37°C. Substrate cleavage was monitored at 450 nm by use of a Model 450 microplate reader (Bio-Rad Laboratories, Hercules, CA) and analyzed using a Microplate Manager software (Bio-Rad). In some experiments, 2 × 10^5 cells were cultured in a well of microtest plate coated with human fibronectin (Sigma), human laminin (Sigma), or LAD-4 in a serum free medium: RPMI-1640 containing 2 % bovine serum albumin (Sigma). Prior to this assay, cells were stimulated with PMA and ionomycin for 10 min at 37°C as described previously. After 24h of incubation, growth of cells was determined as described above. The experiment was done in triplicate and repeated at least three times.

**Annexin V staining**

Phosphatidylserine expression on early apoptotic cells was measured by the binding of Annexin V using Annexin V-FITC kit (Beckman Coulter). 5 × 10^6 of RL–1 cells were cultured in 4 ml culture medium in the presence of LAD-4, anti-CD44 mAb, or normal rat IgG (final concentration: 20 μg/ml) for 3h.
at 37 ºC. After incubation, the cells were collected and stained with FITC-labeled Annexin V and propidium iodide (PI) under the manufacturer’s instructions. The cells were analyzed with a FACSCalibur flow cytometer. The experiment was repeated once with similar results.

**Measurement of Caspase-3 Activation.**

Caspase-3 like protease activity in cells was analyzed by use of a CPP32/Caspase-3 colorimetric protease assay kit (Chemicon International Inc., Temecula, CA). 5 × 10^6 RL ´ 1 cells were cultured in 4 ml culture medium with LAD-4, control antibody, or cyclophosphamide (Sigma) for 3h or 24h. After washing with PBS, cell lysate was prepared as described by Nicholson et al. Protein concentrations were determined using a protein microassay (Bio-Rad, Tokyo, Japan). The cell lysate (25 μg protein) was mixed with 400 μM of the caspase-3 specific substrate (DEVD-para-Nitroanilide) in the absence or presence of caspase-3 inhibitor (Ac-DEVD-CHO) and incubated at 37 ºC for 2h. Subsequently, substrate cleavage was monitored at 405 nm. To determine specific caspase-3 activity, the value of the absorbance signal of the inhibitor sample was subtracted from that of the noninhibited sample. The experiment was done in duplicate and repeated three times.

**Western Blotting.**

Immunodetection of proteins separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) was performed with enhanced chemiluminescence Western blotting reagents (New England Nuclear, Boston) as described previously. 5 × 10^6 cells were cultured with LAD-4 (20 μg/ml) at 37 ºC for various time (0.5h to 3h). After washing with PBS, cells were extracted in a lysis buffer: 50 mM Pipes NaOH pH 6.5 with 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 10 μM APMSF (Wako Pure Chemical Industries, Osaka, Japan). Samples were adjusted to equal concentration and volume. Each sample (40 μg) was separated by SDS-PAGE on 5-20% gels and electroblotted onto PVDF membrane. Rabbit polyclonal antibodies to caspase-3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and cleaved caspase-3 (Cell Signaling Technology, Beverly, MA) were used as primary antibodies. The experiment was repeated once with similar results.

**Detection of apoptotic DNA fragmentation.**

2 × 10^6 cells were cultured in 4 ml of culture medium containing LAD-4 or control antibodies for 3h or 24h. The cells were treated with TdT and stained with FITC-labeled dUTP under manufacturer’s instructions (Beckman Coulter). The stained cells were then analyzed using a FACSCalibur flow cytometer (Becton Dickinson). The experiment was repeated three times with similar results.

**Statistical Analysis.**

Differences between the experimental results were evaluated by means of one factor ANOVA with Scheffe’s F test. All values were expressed as mean ± SD, and statistical significance was set at P<0.05.

Kinetics of survival were evaluated by Breslow-Gehan-Wilcoxon test.

**RESULTS**

As Gazi MH and Ito M have demonstrated that LAD-4 inhibited migration of RL ´ 1 cells into the liver, I performed a series of experiments to determine whether LAD-4 could inhibit liver metastasis and prolong survival of mice inoculated with lymphoma...
cells in vivo.

Effect of LAD-4 Administration on Liver Metastasis.

BALB/c mice were injected s.c. with $2 \times 10^5$ RL-1 cells, followed 30 min later by i.p. injection of 200 μg normal rat IgG, anti-LFA-1 mAb (M17/4) or LAD-4. On day 21, the mice were killed and their livers were removed for histological examination as follows. Sections (4 per liver) were immunostained with the anti-PCNA antibody (Fig.1A) and, the labeled cells were counted using computer-associated image analysis. LAD-4 as well as anti-LFA-1 mAb significantly inhibited liver metastasis by RL-1 cells (Fig. 1B). Due to the heterogeneity and scattered distribution of metastasis within the liver, histological examination of several sections may not reflect whole liver metastases. So, to overcome this weakness, I tried to quantify whole liver metastasis by FACS analysis. Single-cell suspensions were prepared from each liver, immunostained with biotinylated LAD-4, and analyzed by FACS, which gated live cells according to their forward and side scatter. Scatter analysis revealed that RL-1 cells were distributed in gate R1, whereas infiltrating lymphocytes in gate R3, and cell

![Image](image_url)

Fig. 1. Histological analysis of the liver metastasis by RL-1 cells. BALB/c mice (5 mice per group) were inoculated s.c. with $2 \times 10^5$ RL-1 cells and injected i.p. either with 200 μg of normal rat IgG, LAD-4, or anti-LFA-1 mAb (M17/4). On day 21 after tumor inoculation, mice were sacrificed and histologically evaluated for liver metastasis. A, Typical photomicrographs of liver sections stained immunohistochemically with anti-PCNA mAb. B, The number of PCNA positive cells analyzed by a computer-associated image analysis system. *p<0.01 by one factor ANOVA with Scheffe’s F test. Data represent mean ± SD obtained with 5 mice. Representative results from three similar experiments were shown.

![Image](image_url)

Fig. 2. Quantification of whole liver metastasis. Mice received injections of RL-1 cells and each antibody as in Fig. 1. A, Forward and side scattergrams of RL-1 cells, spleen cells, and cell suspensions prepared from the liver of tumor bearing mice (lower panels). RL-1 cells distributed in gate R1 and spleen cells in gate R3. This gate setting was used for analyzing cell suspensions from the tumor-bearing liver. B, Summary of the flow cytometric analysis. Data represent mean ± SD obtained with 5 mice. *p<0.01 by one factor ANOVA with Scheffe’s F test. Representative results from two similar experiments were shown.
debris in R2 (Fig.2A). The FACS data showed that LAD-4 significantly inhibited whole liver metastasis to the same extent as anti-LFA-1 mAb (Fig.2B).

**Effect of LAD-4 Administration on Survival.**

In the first experiment, mice were injected s.c. with RL 1 cells and i.p. with antibodies as described above. Survival did not differ significantly among the three groups (Fig.3A). In the second experiment, the effect of LAD-4 administration on the survival of mice inoculated i.v. with lymphoma cells was examined. Mice that received LAD-4 survived significantly longer than control mice (p<0.01), as shown in Fig. 3B. Furthermore, LAD-4 administration significantly reduced the liver weight of mice inoculated i.v., but not s.c., with lymphoma cells, (inoculated i.v.: control IgG vs LAD-4 = 3.7 ± 0.4 g vs 2.7 ± 0.3 g, p<0.01; inoculated s.c.: 1.5 ± 0.2 g vs 1.49 ± 0.2 g, N.S.). Postmortem examination revealed that mice inoculated s.c. with lymphoma cells died as a result direct invasion of the retroperitoneum involving the intestine, whereas those inoculated i.v. died as a result of diffuse metastasis in the lungs, liver, and kidneys (data not shown). Therefore, I concluded that reduction of hepatic metastasis by LAD-4 administration led to prolongation of survival in mice inoculated i.v., but not s.c., with lymphoma cells.

**LAD-4 Inhibited Proliferation of RL 1 Cells in Vitro.**

In preliminary experiments, we found that LAD-4 in a soluble form, but not in a fixed form adsorbed on the microtest plate, triggered homotypic aggregation of RL 1 cells. As this effect of LAD-4 is similar to that of an anti-β1-integrin mAb and the integrin family of cell adhesion receptors can transduce signals that modulate cell growth and differentiation, I examined whether LAD-4 inhibited proliferation of RL 1 cells and induced apoptosis in vitro. RL 1 cells were cultured in the presence of normal rat IgG, anti-LFA-1 mAb, or LAD-4 and the numbers of viable cells were determined by a colorimetric assay. LAD-4 significantly inhibited RL 1 cell proliferation in a dose-dependent manner, whereas the anti-LFA-1 mAb (M17/4) did not (Fig.4A). FN adsorbed on the microtest plate did not downregulate RL 1 cell proliferation under serum-free conditions, whereas LAD-4 ad-
sorbed on the microtest plate did. (Fig.4B)

**LAD-4 Enhanced Annexin V Positivity of the RL-1 Cell Population.**

To determine whether LAD-4 could influence apoptotic cell death, RL-1 cells were cultured in the presence of LAD-4 or control antibodies and the percentages of cells undergoing apoptosis were determined by Annexin V binding. The proportions of Annexin V-positive/PI negative cells increased from the control value of 4% to 24% of total RL-1 cell population after treatment with LAD-4 for 3h, whereas no such increase occurred after treatment with the anti-LAF-1 mAb or anti-CD44 mAb (Fig.5A). The number of Annexin V-positive cells increased in a time-dependent manner (Fig.5B), suggesting that LAD-4-mediated growth inhibition was related to apoptosis of RL-1 cells.

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**Fig. 4.**

Downregulation of in vitro proliferation of RL-1 cells by LAD-4. A, cells were cultured in the presence of the indicated doses of normal rat IgG (□), LAD-4 (○), or anti-LFA-1 mAb (■) at 37°C for 24h or 48h. B, Cells were cultured in a well of tissue culture plate coated with LAD-4 (○), laminin (□) or fibronectin (□) at 37°C for 24h. Dotted lines indicate the initial number of cells in each assay. The numbers of viable cells were calculated with absorbance values of standard (known cell numbers). The results are the mean ± SD of three separate experiments with triplicate determinations in each experiment. *p<0.01 by one factor ANOVA with Scheffe’s F test.

**Fig. 5.**

Increase in Annexin V-positivity of RL-1 cells by LAD-4. A, Annexin V and propidium iodide (PI) staining of RL-1 cells treated with the indicated mAbs (20 μg/ml) for 3h. The percentage of Annexin V+/PI-cells (early apoptotic cells) is indicated in the lower right quadrant. B, The percentage of Annexin V+ cells after 3h, 24h or 48h treatment with normal rat IgG (□), LAD-4 (○), or anti-LFA-1 mAb (■). Representative data from three similar experiments are shown.
LAD-4 Induced Activation of Caspase-3 in RL-1 Cells.

To determine whether the observed Annexin V binding increases other well-characterized markers of apoptosis, I performed a colorimetric assay to measure the caspase-3 activities of RL-1 cells after treatment with LAD-4 or the anti-LFA-1 mAb. Induction of caspase-3 activation by LAD-4 was observed after treatment for 3h and this effect was dependent on the dose of LAD-4 added to the culture (Fig.6A), whereas no effect was observed with the anti-LFA-1 mAb (M17/4) (Fig.6B). After treatment with LAD-4 for 24h, the caspase-3 activity was not significantly higher than the control value and was lower than that induced by cyclophosphamide (Fig.6B).

I performed western blotting to verify these results and demonstrated that caspase-3 activation was accompanied by cleavage of a 36-kDa procaspase-3 to an active 17-kDa fragment during LAD-4-induced apoptosis. The intensity of the 17-kDa cleaved caspase-3 band increased in a time-dependent manner, while that of the 36-kDa procaspase-3 band decreased slightly with time (Fig.7).
LAD-4 Increased Apoptotic DNA Fragmentation in RL-1 Cells.

I analyzed LAD-4-treated cells for apoptotic DNA fragmentation by the TUNEL assay. The percentage of TUNEL positive cells was approximately 6% to 36% after treating with 3h and 24h, respectively, whereas only marginal effect was observed with anti-LFA-1 mAb (Fig.8). These results demonstrated that LAD-4 gradually induced apoptosis of RL-1 cells.

DISCUSSION

My results show that LAD-4, which recognizes a new FN receptor, inhibits liver metastasis by RL-1 lymphoma cells in vivo. Several mechanisms governing the inhibition of liver metastasis have been proposed. Lymphoma cells inoculated either s.c. or i.v. are thought to disseminate through the bloodstream and accumulate in the preferred organs such as the liver, spleen, and kidneys. Receptor occupancy by LAD-4 has been reported to inhibit the adhesion of RL-1 cells to hepatocytic FN and to partially inhibit accumulation of these cells in the liver. This mechanism may be involved in partial inhibition of metastasis formation in the liver 2 to 3 weeks after tumor inoculation.

Surprisingly, our data showed clearly that LAD-4 induced apoptosis of RL-1 cells in vitro, i.e., expression of phosphatidylserine detected by Annexin V binding, caspase-3 activation, and apoptotic DNA fragmentation, and that LAD-4 downregulated RL-1 cell proliferation. As FN adsorbed on the microtest plates neither induced caspase-3 activation nor downregulated RL-1 cell proliferation, homotypic aggregation of RL-1 cells and/or cross-linking of the receptor molecules triggered by LAD-4 may transduce a death signal. The later mechanism may also contribute to inhibition of liver metastasis. Another possible mechanism of the anti-tumor effect of LAD-4 is antibody-mediated cellular cytotoxicity (ADCC), as reported with an anti-CD20 mAb.

However, I performed a 4h 51Cr-release assay on spleen cells and detected no ADCC (data not shown), suggesting that ADCC contributed little or nothing to the anti-metastatic effect of LAD-4.

Integrin receptors mediate not only cell adhesion but also signal transduction through focal adhesion kinase. It has been reported that integrin receptor occupancy by its ligand transduces signals leading to cell growth and,
furthermore, adhesion of small cell carcinomas of the lung to extracellular matrix protein confers resistance to chemotherapeutic agents as a result of 1-integrin-stimulated tyrosine kinase activation\(^{22}\). In agreement with these reports, binding of RL 1 cells to fixed FN did not downregulate, but actually upregulated RL 1 cell proliferation (Fig.4B). Although LAD-4 may have therapeutic potential by virtue of effect on survival of mice inoculated with lymphoma cells, it was not curative. This may be because LAD-4 administered i.p. was absorbed by circulating leukocytes bearing the antigenic molecule and consequently, the amount of LAD-4 that reached the lymphoma cells was not enough to induce apoptosis. Another explanation is that anti-apoptotic factors, such as survivin that inhibit caspase activity \(^{23,24}\) may have been present. Although the precise mechanisms responsible for LAD-4-induced apoptosis are unknown, the combination of LAD-4 with a survival factor inhibitor, such as anti-sense survivin oligonucleotides, may have a therapeutic effect against lymphomas.

In conclusion, LAD-4 partially but significantly inhibited liver metastasis by RL 1 lymphoma cells. One of the mechanisms responsible for this effect may be induction of lymphoma cell apoptosis, which may be initiated through cross-linking of the receptor molecules by LAD-4.

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