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Up regulation of *ICAM-1* gene expression inhibits tumour growth and liver metastasis in colorectal carcinoma

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Abstract

We have previously reported that decreased intercellular adhesion molecule-1 (ICAM-1) expression in cancer cells is associated with liver metastasis of colorectal cancer. In this study, we have investigated the effect of *ICAM-1* gene transfection into the human colorectal cancer cell line LM-H3 on cell adhesiveness and cytotoxicity of peripheral blood mononuclear cells (PBMC) to cancer cells. Furthermore, we have investigated the effects of this gene transfer on subcutaneous tumour and liver metastases of LM-H3 in nude mice. More PBMC adhered to *ICAM-1* transfected LM-H3 cells, LM-H3/ICAM-1, than to non-transfected LM-H3 cells and control LM-H3/Vector. Lysis of LM-H3/ICAM-1 cells by PBMC was significantly increased compared with LM-H3/Vector. Liver metastases with LM-H3/ICAM-1 cells were fewer in number and smaller than metastases with LM-H3/Vector. Intra-tumoural injection of ICAM-1 adenoviral vector significantly inhibited the growth of subcutaneous LM-H3 tumour. In conclusion *ICAM-1* gene transfection using adenovirus vector might be an effective therapy for liver metastasis of colorectal carcinoma.

Keywords: ICAM-1; Colorectal carcinoma; Adenovirus vector; Liver metastasis; LFA-1; NK cells

1. Introduction

Liver metastasis is one prognostic factor in colorectal carcinoma. Chemotherapy against advanced colorectal carcinoma rarely achieves a satisfactory result, and metastasis is the main cause of death.

Patients with carcinoma have a functional disorder of immune effector cells, which results in immunological tumour tolerance and the promotion of carcinoma metastasis [1]. Tumour expression of the major histocompatibility complex (MHC) peptide and its co-stimulatory adhesion molecule are decreased [2,3]. Intercellular adhesion molecule-1 (ICAM-1), which belongs to the immunoglobulin superfamily, is not only an adhesion molecule but also a co-stimulatory molecule that provides signal to cytotoxic T lymphocytes (CTL) and natural killer (NK) cells [4,5]. ICAM-1 mediates cell adhesion through its receptor leukocyte-function-associated antigen-1 (LFA-1). Intercellular adhesion mediated by ICAM-1/LFA-1 plays a key role in the binding of CTL and NK cells to tumour cells, which stimulates immune activation [6]. Using immunohistological techniques, we have investigated the correlation between cancer cell expression of ICAM-1 and clinicopathological factors in patients with breast, gastric, and colon cancer [7–9]. The incidence of metastasis of these cancers was significantly lower in patients with ICAM-1-positive tumours than in ICAM-1-negative tumours, and the prognosis of patients with ICAM-1-negative tumours was significantly poorer. These results indicated that expression of ICAM-1 in cancer cells might play an important role in cancer metastasis.

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Immunogene therapy has been used for various types of cancers with effector cells, including macrophages, CTL, and NK cells. However, there have been few reports about immunogene therapy for liver metastasis of colon cancer. Our previous reports indicated that *ICAM-1* gene transfection may inhibit lymph node spread and peritoneal metastasis of gastric cancer [10,11]. In the present study, we have investigated the inhibitory effect of *ICAM-1* transfection on liver metastasis from colorectal carcinoma.

2. Materials and methods

2.1. Animals

Specific, pathogen-free, athymic BALB/c-*nu*/*nu* nude mice were purchased from Oriental Kobo (Tokyo, Japan). The mice were maintained under specific pathogen-free conditions and given sterile food and water *ad libitum*. Four-week-old female mice were used for the experiments. Studies were performed according to Osaka City University Medical School's standard guidelines for animal experiments.

2.2. Cell line

The highly metastatic colon carcinoma cell line, LM-H3, which was previously established in our laboratory was used in this study [12]. LM-H3 cells were cultured *in vitro* at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Bioproducts, Walkersville, MD), with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY), 100 mg/ml of streptomycin penicillin (ICN Biomedicals, Costa Mesa, CA), and 0.5 mM sodium pyruvate (Whittaker Bioproducts, Walkersville, MA).

2.3. ICAM-1 transfection

ICAM-1 transfection was performed as previously described [10]. The lipofectamine reagent is a 3:1 liposome formulation of polycationic lipid and neutral lipid. Briefly, the plasmid encoding the functional gene for *ICAM-1* cDNA (kindly provided by Dr. Timothy Springer, Center for Blood Research, Inc., Harvard Medical School, Boston, MA) was cotransfected with the gene for neomycin resistance using lipofection. The limiting dilution method isolated multiple G418-resistant clones and FACScan Calibur (Becton Dickinson, San Jose, CA) was used to confirm ICAM-1 expression in clones. A clone with high expression of ICAM-1 was designated LM-H3/ICAM-1. LM-H3 cells transfected with only the neomycin resistance-expression vector, designated as LM-H3/Vector, were used as negative control cell line.

2.4. Recombinant adenovirus production

The replication-deficient adenovirus vectors used in this study were E1-, and E3- deleted vectors based on the adenovirus serotype 5 (Ad5) genome. Adenovirus vector with *ICAM-1* driven by CMV promoter (Ad-ICAM-1) and adenovirus vector with lacZ *Escherichia coli* (*E. coli*) driven by cytomegalovirus (CMV) promotor (Ad-lacZ) were constructed using Adeno X Expression Kit (Clonthech, Palo Alto, CA). Human *ICAM-1* gene and *E. coli lacZ* gene were inserted into the shuttle vector. Next, it was transferred to the adenoviral genome by means of *in vitro* ligation. The inserted *ICAM-1* gene was checked by direct sequence. Finally, the newly tailored recombinant adenoviral plasmid was packaged into infectious adenoviral particles by transfecting human embryonic kidney 293 (HEK293) cells. Recombinant adenoviruses were expanded in the HEK293 cells, after which the resultant viral solutions were stored at −80 °C. As a control, recombinant β-gal adenovirus (Ad-lacZ) infected cells were used. The virus titer was determined by 50% tissue culture infectious dose method (TCDI 50) [13–15].

2.5. In vitro adenovirus infection of cells

LM-H3 cells were plated in 6-well plates and grown until almost confluent. The cells were infected with 200 multiplicity of infection (MOI) of Ad-ICAM-1and incubated 24, 48, 72, and 96 h. After incubation, ICAM-1 expression was confirmed by FACScan Calibur.

2.6. Flow cytometry analysis

Cell lines LM-H3/Vector and LM-H3/ICAM-1 were incubated until 70% confluent. The cells were washed with phosphate-buffered saline (PBS) containing 0.01% sodium azide and 0.1% bovine serum albumin. An anti-ICAM-1 antibody with fluorescein isothiocyanate (FITC) (Immunotech, Marseille, France), at dilution of 40 μg/200 μl, was added to each sample and incubated for 30 min. An aliquot of 1×10^4 cells was subsequently analysed.

2.7. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers using mono-poly resolving medium (Dainippon Pharmaceutical, Tokyo, Japan) according to the manufacturer's instructions. Briefly, 6 ml of fresh anti-coagulated blood was laid gently onto 3 ml of the Resolving Medium in a 15 ml tube. The tube was centrifuged at 300*g* at 15 °C for 30 min. After drawing off the plasma, the upper fraction containing mononuclear cells was transferred to a clean tube and washed with PBS. The tube was centrifuged at 250*g* for 10 min,

and the pellet was resuspended to a concentration of 5×10^6/ml in DMEM with 10% FBS.

2.8. Adhesion assay

Evaluation of PMBC adhesion to cancer cells was performed as previously reported [10,16,17]. Briefly, cancer cells were cultured on a 96-well plate until confluent. PBMC (2×10^5 cells) were added to each well in a final volume of 200 μl. PBMC and cancer cells were co-incubated for 60 min at 37 °C. After incubation, these wells were washed gently to remove unattached PBMC. Cellular adhesion was quantified by 3-(4,5-dimethyl-2-thiazol)-2H tetrazolium bromide (MTT; Wako Pure Chemical Industries, Tokyo, Japan) calorimetric assay [18]. The assay was designed to measure the formazan product of MTT, using a MTP-120 microplate reader (Corona Electric, Ibaragi, Japan) to measure optical density (OD) at 550 nm. The percentage of adhering PBMC (% adhesion) of all PBMC was calculated as follows: % adhesion $= (A - B)/T \times 100$, where $A =$ OD of the experimental wells, $B =$ OD of cancer cell wells, and $T =$ OD of the total PBMC added to each well.

2.9. Cytotoxicity assay

We investigated non-MHC restricted cytotoxicity by PBMC. Cancer cells were incubated in 96-round bottom plates with PBMC at an effector to target ratio of 10. After 18 h incubation, 50 μl of supernatant was collected, and the concentration of lactate dehydrogenase in the cell lysate was measured using the Cyto-Tox 96 Assay Kit (Promega, Madison, WI). Absorbance data OD at 490 nm were obtained using the MTP-120 microplate reader (Corona Electric, Ibaragi, Japan). The percentage of specific lysis was calculated as follows: % cytotoxicity $= (A - B - C)/(D - E) \times 100$, where $A =$ experimental lysis, $B =$ effector spontaneous lysis, $C =$ target spontaneous lysis, $D =$ target maximum lysis, and $E =$ target spontaneous lysis.

2.10. Effects of anti-ICAM-1 neutralizing antibody on the adhesiveness and cytotoxicity of PBMC

LM-H3/ICAM-1 and LM-H3/Vector cells were treated with neutralising antibody against ICAM-1 for 1 h. Final solutions contained monoclonal anti-human ICAM-1 antibody (R&D Systems, Minneapolis, MN) at 10 mg/ml concentration. The adhesion and cytotoxicity assays were then performed as described above.

2.11. In vitro and in vitro proliferation assay

After 1×10^5 cells of LM-H3/Vector and LM-H3/ICAM-1 were incubated for 24, 48, and 72 h, the number of cells were counted. LM-H3/Vector (1×10^7) or LM-H3/ICAM-1 cells in 200 μl of DMEM were inoculated subcutaneously into the lateral abdominal wall of nude mice. Tumour areas were then measured with a caliper every 2 days.

2.12. Formation of liver metastasis

BALB/c nude mice were used. To detect liver metastasis. Under anesthesia, the mice abdomen was opened and in 0.1 ml PBS, 5×10^5 LM-H3/Vector or LM-H3/ICAM-1 cells were injected into the lower pole of the spleen. Splenectomy was conducted 2–3 min after injection. If tumour formation in the spleen was allowed to occur, the process of liver metastasis would have been more complex. At 4 weeks, mice were killed, and the number of metastatic nodules on the liver surface were counted.

2.13. In vivo gene transfer by adenovirus

LM-H3 cells (1×10^7) were injected subcutaneously into the lateral abdominal wall of 10 BALB/c nude mice, and the mice were divided into two groups. At 5 days after injection of LM-H3 cells, 1.0×10^8 plaque-forming units (PFU) of Ad-ICAM-1 was injected into each subcutaneous tumour every 3 days. Similarly, Ad-LacZ was injected into each subcutaneous tumour as a control. Tumour areas were measured with a caliper every 3 days. For histopathological examination, tissue paraffin blocks were cut into 4 μm thick sections and were stained with hematoxylin and eosin (H&E) or anti-ICAM-1 antibody (Chemicon International, Temacula, CA) by the avidin–biotin peroxidase complex method. To detect expression of the lacZ gene, tissues were stained with 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) as previously described [19].

2.14. Statistical analysis

Data are expressed as mean ± SD and taken from at least four independent determinations. Significant differences were analysed using the unpaired Student's t test. A value $P < 0.05$ was considered statistically significant.

3. Results

3.1. ICAM-1 transfection by lipofection

ICAM-1 was strongly expressed on LM-H3/ICAM-1 cells. The percentage of positive cells was 3.6% of LM-H3/Vector cells and 71.7% of LM-H3/ICAM-1 cells (Fig. 1).

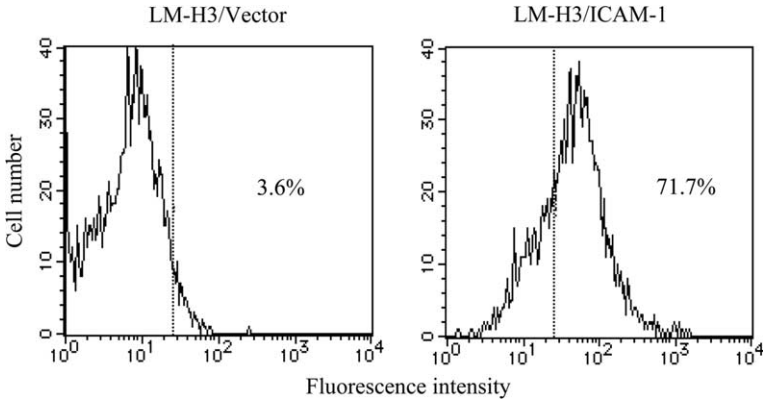


Fig. 1. ICAM-1 expression on LM-H3 cells after *ICAM-1* transfection by lipofection.

3.2. ICAM-1 transfection by adenovirus

As shown in Fig. 2, ICAM-1 expression on the cell surface was confirmed at 24, 48, 72, and 96 h after infection with Ad-ICAM-1. The basal expression of ICAM-1 on the surface of parent LM-H3 cells was 2.4% in the control. The percentages of positive cells were 10.3% at 24 h, 25.9% at 48 h, 67.9% at 72 h, and 20.3% at 96 h. LM-H3 cells transfected by Ad-ICAM-1 at 72 h demonstrated maximal expression of ICAM-1.

3.3. Effect of ICAM-1 transfection on PBMC adhesion

PBMC adhering to LM-H3/ICAM-1 was significantly higher than for LM-H3/Vector cells ($P < 0.01$). Forty-one percent of PBMC adhered to LM-H3/ICAM-1 cells, whereas only 7% of PBMC adhered to LM-H3/Vector cells (Fig. 3). The adhe- siveness was significantly ($P < 0.01$) decreased by co-administration of anti-ICAM-1 antibody to LM-H3/ICAM-1 cells, while LM-H3/Vector cells were not affected by anti-ICAM-1.

3.4. Effect of ICAM-1 transfection on cytotoxicity

Fig. 4 shows the cytotoxicity of PBMC against cancer cells. LM-H3/ICAM-1 cells showed a significantly higher rate (71.7%) of cell lysis than LM-H3/Vector cells (4.6%) at 18 h after co-incubation with PBMC ($P < 0.01$). The cell lysis of LM-H3/ICAM-1 cells was

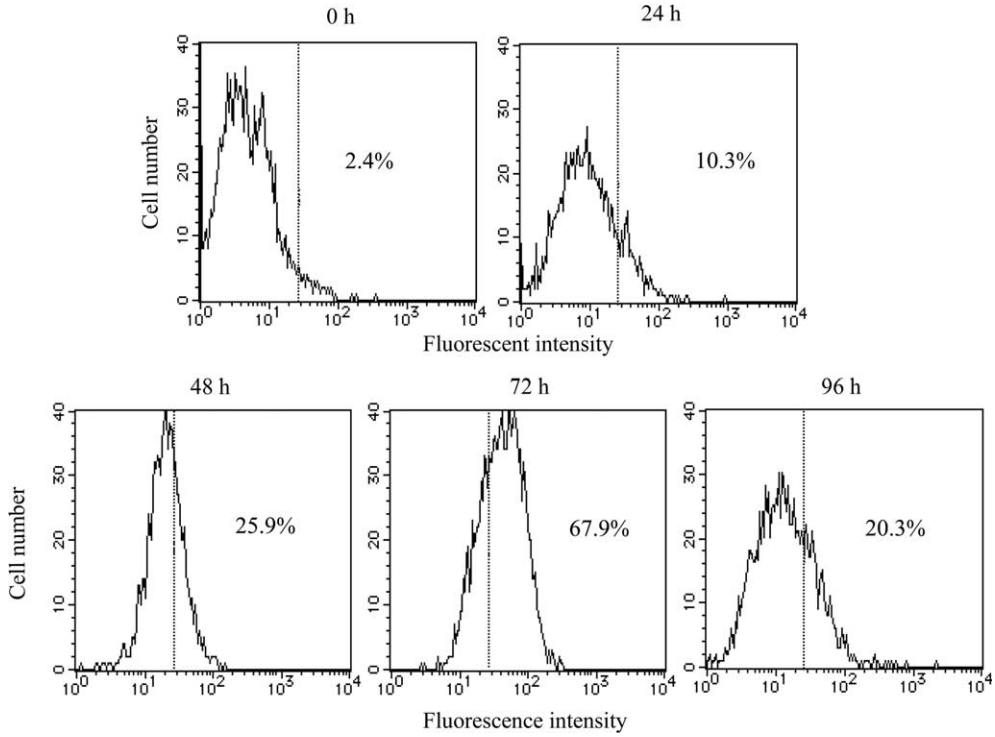


Fig. 2. ICAM-1 expression on LM-H3 cells following Ad-ICAM-1 transfection.

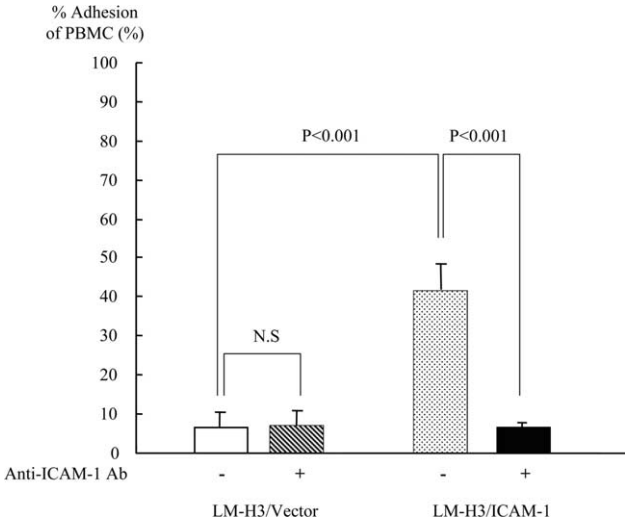


Fig. 3. Effect of transfection of *ICAM-1* on adhesion of PBMC to cancer cells.

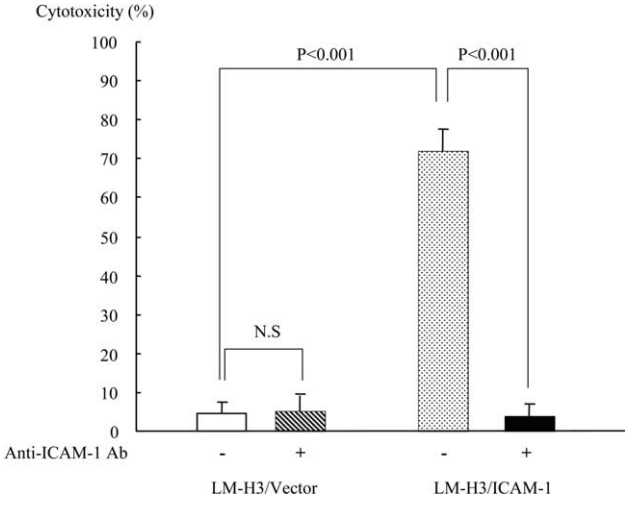


Fig. 4. Effect of *ICAM-1* transfection on cytotoxicity of PBMC to cancer cells.

significantly ($P < 0.01$) decreased (3.8%) by neutralising antibody against ICAM-1.

3.5. Tumour growth

The *in vitro* doubling times of LM-H3/Vector cells and LM-H3/ICAM-1 cells were 24 and 26 h, respectively, and no differences in the *in vitro* growth rate was found between LM-H3/Vector cells ($n = 6$) and LM-H3/ICAM-1 cells ($n = 6$). To investigate the effect of enhanced ICAM-1 expression on *in vivo* tumour growth, LM-H3/Vector and LM-H3/ICAM-1 cells were injected subcutaneously into nude mice. At any time, the tumour sizes following inoculation of LM-H3/ICAM-1 were significantly smaller than LM-H3/Vector cells (Fig. 5).

3.6. The inhibitory effect of ICAM-1 transfection on liver metastasis

As summarised in Table 1, the average number of metastatic nodules in the LM-H3/Vector group ($n = 6$) was 54 ± 23. In contrast, in the LM-H3/ICAM-1 group ($n = 6$), this number was only 0.167 ± 0.408 ($P < 0.01$). The average liver weight in the LM-H3/ICAM-1 group was 1815 ± 74 mg. In contrast, in the LM-H3/Vector group, this weight was 2022 ± 190 mg ($P < 0.05$; Fig. 6).

Table 1
The inhibitory effects of *ICAM-1* transfection on liver metastasis of colorectal cancer

Cell line	Number of metastatic nodules of liver (mean ± SD)		Weight of liver (mean ± SD) (mg)	
LM-H3/Vector	54 ± 23	$P < 0.01$	2022 ± 190	$P < 0.05$
LM-H3/ICAM-1	0.167 ± 0.408		1815 ± 74	

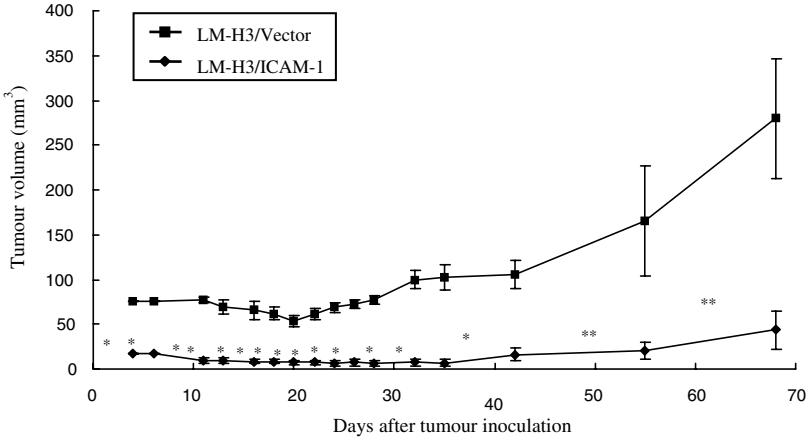
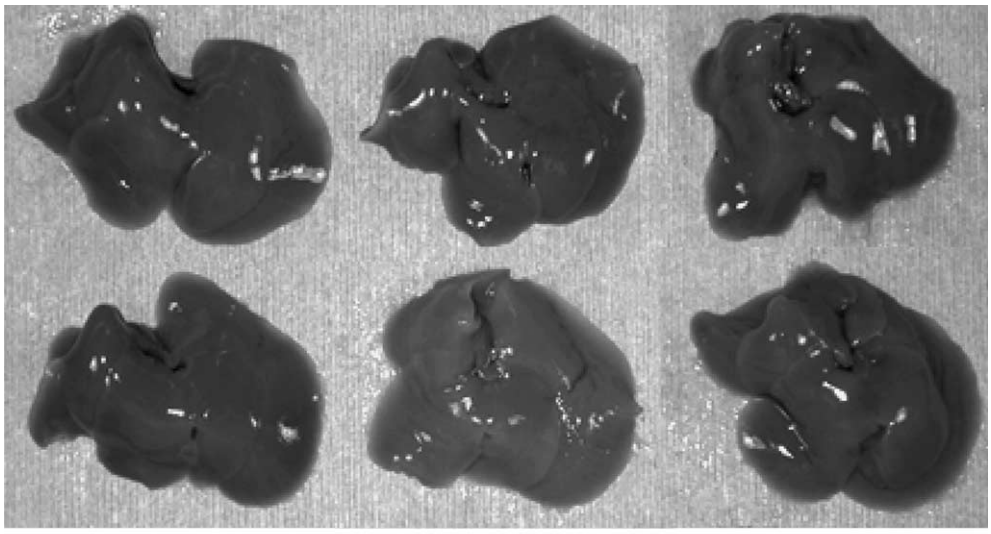
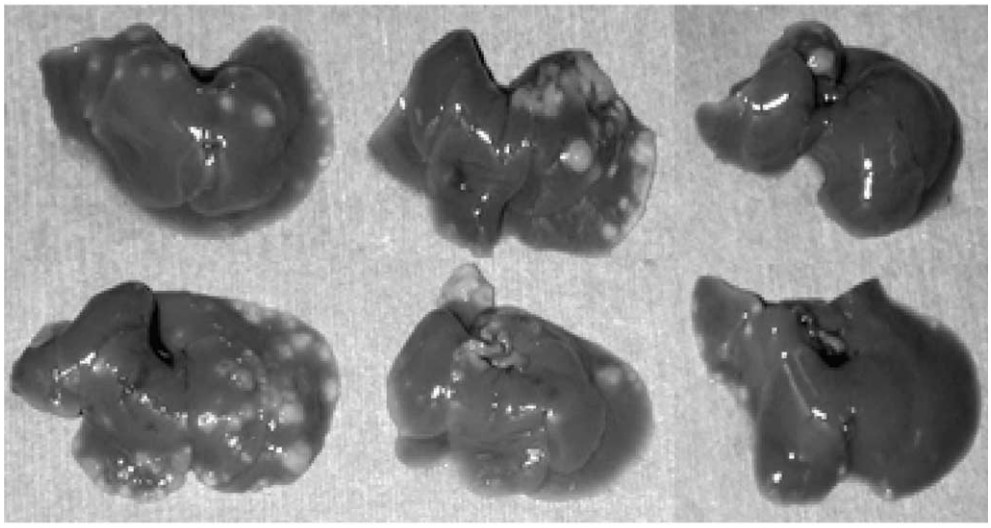


Fig. 5. Effect of *ICAM-1* transfection on xenografted tumour growth in nude mice. $*P < 0.05$, $**P < 0.001$.



LM-H3/ICAM-1



LM-H3/Vector

Fig. 6. The liver metastasis of nude mice. The photographs were taken 4 weeks after inoculation with cancer cells.

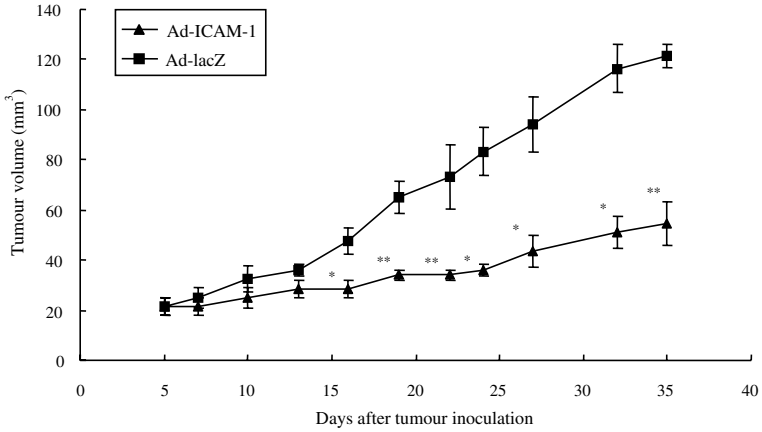


Fig. 7. Growth of subcutaneous tumour by injection of adenoviral vector. $*P < 0.05$, $**P < 0.01$.

3.7. Inhibitory effect of ICAM-1 transfection on subcutaneous tumour

We investigated the inhibitory effect of *ICAM-1* transfection on xenografted tumour in nude mice after tumour injection of adenoviral vector. Tumour sizes following treatment of Ad-ICAM-1 ($n = 5$) were significantly smaller than that of Ad-lacZ ($n = 5$) (Fig. 7). In order to investigate whether transfection was successful, X-Gal staining was performed. Beta-gal expression was recognised in cancer cells of subcutaneous tumour surrounding the adenoviral injection at 48 h after Ad-lacZ injection (Fig. 8). To investigate whether gene delivery into cancer cells was successful after transfection, immmunohistoical analysis with anti-ICAM-1 antibody was performed. LM-H3 cells injected by Ad-ICAM-1 were stained with anti-ICAM-1 antibody, stroma was also positive for transfection by Ad-ICM-1 (Fig. 9(a)). As a control LM-H3 cells injected by Ad-lacZ were also stained (Fig. 9(b)). Histological findings showed that many leukocytes were infiltrating into the LM-H3/ICAM-1 tumours, but only few leukocytes were observed in the lesions associated with LM-H3/Vector cells (Fig. 10).

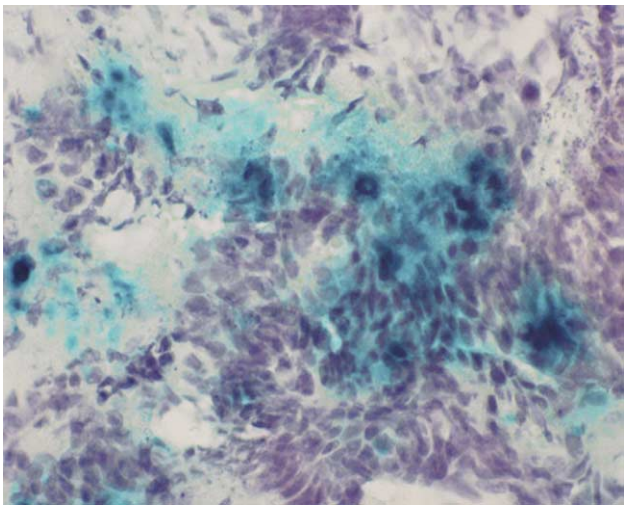


Fig. 8. Evaluation of adenoviral transfer into subcutaneous tumour by injection of adenovirus. X-Gal staining (200×).

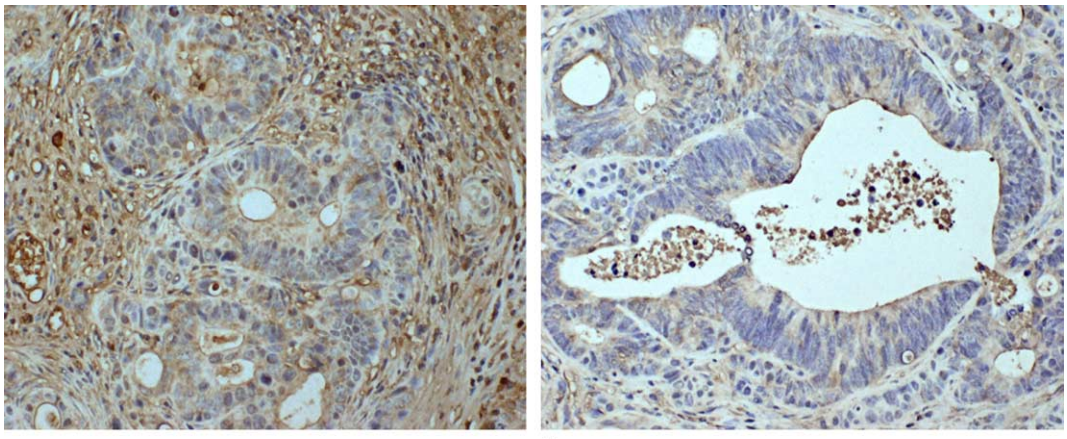


Fig. 9. Evaluation of *ICAM-1* gene transfer into cancer cells after injection of adenovirus. Immunohistochemical staining (200×).

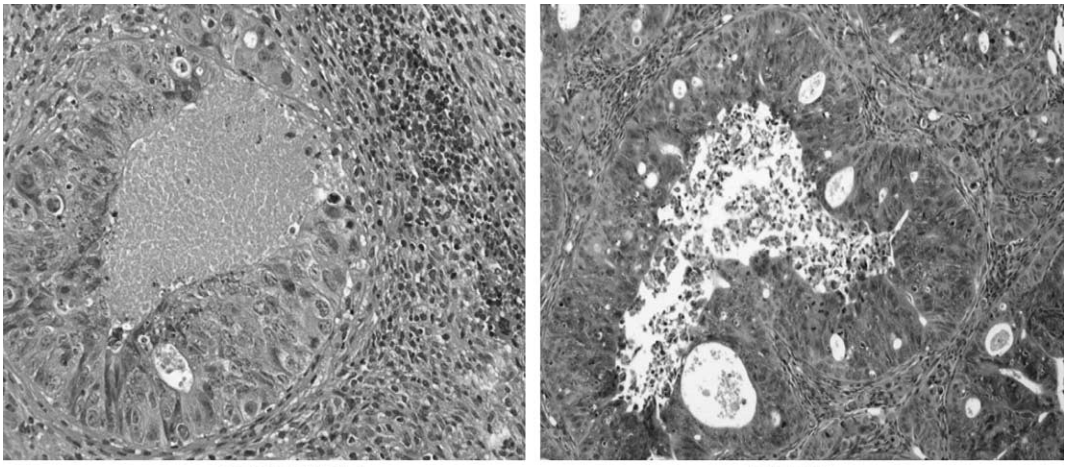


Fig. 10. Histochemical staining of LM-H3/ICAM-1 and LM-H3/Vector tumour to show infiltrating leukocytes. H&E staining (200×).

4. Discussion

We previously investigated resected specimens from 96 patients with colorectal carcinoma using immunohistochemical staining with a monoclonal antibody against ICAM-1. The incidence of liver metastasis of colorectal carcinoma was significantly lower in patients with ICAM-1-positive tumours than in those with ICAM-1-negative tumours. Five-year disease-free survival of patients with ICAM-1-negative tumours was 65%, significantly lower than that of ICAM-1-positive tumours (85%) [9]. These findings indicated that the loss or decrease of ICAM-1 expression on cancer cells was responsible for cancer cell escape from immune surveillance. Cancer cells without ICAM-1 expression can grow without recognition and avoid cell lysis by CTL and NK cells, and may survive to metastasis. Such impairment of immune surveillance may contribute to liver metastasis and poor clinical outcome. Therefore, we investigated whether *ICAM-1* gene transfection could be an effective therapy for liver metastasis of colorectal cancer.

In *in vitro* experiments, we were able to transfect the *ICAM-1* gene at a high rate. LM-H3/ICAM-1 cells had a higher potential of adhesion to PBMC than LM-H3/Vector, and the cytotoxicity of PMBC against LM-H3/ICAM-1 cells was greater than that against LM-H3/Vector cells. The adhesiveness and cytotoxicity to LM-H3/ICAM-1 cells were significantly decreased by anti-ICAM-1 antibody, while those to LM-H3/Vector cells were not affected by anti-ICAM-1. Thus, increased expression of ICAM-1 on *ICAM-1* transfected cells enhanced the adhesion of cancer cells to PBMC and amplified the cytotoxicity of PBMC to cancer cells. Increased adhesion might allow PBMC more opportunity to lyse target cancer cells. These results suggested that transfection of *ICAM-1* into cancer cells could increase cell immmunogenicity. The ICAM-1/LFA-1 interaction has been reported to participate in non-MHC restricted cytotoxicity mediated by NK cells [20,21] and monocytes [22]. This interaction has also been reported to serve as a co-stimulus in the activation of CTL by adhesion and signal transduction [4]. The cytotoxicity to LM-H3/ICAM-1 cells could be amplified, not only by MHC-restricted immune cells, but also by non-MHC-restricted immune cells.

The subcutaneous tumour from LM-H3/ICAM-1 cells was significantly smaller compared with LM-H3/Vector cells, whereas no differences between LM-H3/ICAM-1 cells and LM-H3/Vector cells were found for *in vitro* growth rate.

Most of the mononuclear cells were murine NK cells due to the lack of T cells in the nude mouse. Human ICAM-1 has a 53% structural homology with the murine variant [23]. It has been reported that murine LFA-1 on NK cells adheres to human ICAM-1 on tumour cells and provides an anti-tumour effect [24], and ICAM-1 plays an important role in cytolysis by NK cells [10]. Cytotoxicity of murine NK cells to cancer cells was enhanced by *ICAM-1* transfection, and subcutaneous tumour growth was suppressed.

Liver metastasis of tumours arising from LM-H3/ICAM-1 cells was significantly suppressed compared with LM-H3/Vector cells. The number of liver metastatic nodules of LM-H3/ICAM-1 was significantly lower than that of LM-H3/Vector. We suggest that the cytotoxicity of murine NK cells to cancer cells was enhanced by *ICAM-1* transfection, and liver metastasis was suppressed in nude mice. Since other immune cells, Kupffer cells and pit cells, occur in the liver, it is possible that the suppressive effect of *ICAM-1* transfection may be stronger for liver metastasis than subcutaneous tumour.

The growth of subcutaneous tumour in nude mice was suppressed by direct injection of ICAM-1 adenoviral vector into the tumour. In our previous studies, *ICAM-1* gene transfection into cancer cells was useful for peritoneal and lymph node spread of gastric cancer [16,17]. There have been few reports about gene therapy for liver metastasis of colorectal carcinoma. In a clinical study, a phase I/II trial of hepatic artery infusion with wild type *p53* gene in liver metastasis of colorectal carcinoma has been attempted [25]. One recent strategy for carcinoma was to use cytokines which activate cytotoxic T cells and NK cells [26]. Caruso and colleagues [27] have reported that intratumoural injection of the interleukin-12 gene inhibited liver metastasis of murine colon cancer and prolonged median survival time. Shiratori and colleagues [28] has reported that intravenous injection of the interleukin-2 gene inhibited liver metastasis of murine colon cancer by activation of liver associated lymphocytes. In contrast, *ICAM-1* gene therapy enhanced the expression of ICAM-1 on cancer cells and prohibited them from escaping immune surveillance. It is expected that transfer of both ICAM-1 and cytokine genes together could be even more effective.

Direct injection under ultrasonography (US), computed tomography (CT), or magnetic resonance imaging (MRI) guidance, hepatic arterial infusion, portal infusion, and systemic venous infusion are methods of gene delivery to liver metastasis. It is ideal that a gene is transfected only into cancer cells and not normal cells. However, a gene currently cannot be selectively transfected and in view of transfection efficiency and side effects [29], direct injection may be better than other methods. In the present study, *ICAM-1* transfection to colon carcinoma cells inhibited liver metastasis, and intratumoural injection of Ad-ICAM-1 suppressed the growth of subcutaneous tumour. Endoscopic direct injection of Ad-ICAM-1 into primary colorectal carcinoma might suppress primary growth and prevent liver metastasis. Direct injection of Ad-ICAM-1 into liver metastasis of

colorectal carcinoma may represent an effective salvage therapy as well.

Conflict of interest statement

None declared.

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