TGF-β1 down-regulates ICAM-1 expression and enhances liver metastasis of pancreatic cancer

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Abstract

Purpose: In order to study the regulation of adhesion-molecule expression by cytokines, we have investigated the effect of transforming growth factor-β1 (TGF-β1) on the expression of intercellular adhesion molecule-1 (ICAM-1) in human pancreatic cancer cell lines.

Material and methods: By using three pancreatic cancer cell lines, SW1990, CAPAN-2 and PANC-1, the effect of TGF-β1 on expression of ICAM-1, cancer cell immunogenicity and liver metastasis were investigated.

Results: Cell surface ICAM-1 expression by ELISA on three cell lines were all reduced significantly by following incubation with various concentrations of TGF-β1 and down-regulation of ICAM-1 expression was also observed at the mRNA level. Corresponding to the down expression of ICAM-1, the adhesion of peripheral blood mononuclear lymphocytes (PBMLs) to cancer cells and cancer cell cytotoxicity during co-culture with PBMLs were remarkably decreased by treatment with TGF-β1. Furthermore, enhanced liver metastatic potential by *in vivo* splenic injection was observed in CAPAN-2 cells pre-treated with TGF-β1.

Conclusions: Since decreased expression of ICAM-1 has been known to contribute to cancer cell escape from immunologic recognition and cytotoxicity by effector cells, the present results indicate that unknown function of TGF-β1 in the tumor progression and metastasis of pancreatic cancer.

Key words: pancreatic cancer, liver metastasis, ICAM-1, TGF-β1, immunosuppression.

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Introduction

The progression and metastasis of malignant tumor has been known to be mediated by various factors at microenvironment. Once tumor cells escape from the recognition by immunosurveillance system, it may acquire further metastatic properties. Cellular adhesion mediated by various membrane-associated adhesion molecules are essential for the proper function of immunologic processes. One of these adhesion molecules, intercellular adhesion molecule-1 (ICAM-1, CD54) is known to play an important role in the interaction between tumor cells and host cytotoxic effector cells [1].

ICAM-1 is a member of the immunoglobulin superfamily and a ligand for lymphocytes function-associated antigen-1 (LFA-1), which is expressed on T cells. Natural killer (NK) cells and lymphokine-activated killer (LAK) cells lead the recognition and subsequent lysis of appropriate target cells through ICAM-1/LFA-1 system, and cytotoxic T lymphocytes (CTLs) also require ICAM-1 expression as a co-stimulating accessory molecule in the activation of their immune response through T cell receptor [2,3]. The induction of ICAM-1 expression is regulated by several cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), interleukin-2 (IL-2) and interleukin-6 (IL-6) as well as lipopolysaccharide (LPS), and this up-regulation may be benefit for cancer cell killing [4]. However, down-regulation of ICAM-1 expression and regulation of sICAM-1 level still remain to be unknown.

Transforming growth factor-β1 (TGF-β1) inhibits potently tumor growth of normal and tumor epithelial cells *in vitro* and can exert multifunctional biologic effects in differentiation, inflammation, tissue repair and extracellular matrix remodelling besides cell proliferation [5]. TGF-β1 also stimulates cancer cell migration, production of proteolytic enzyme degradating extracellular matrix and suppression of immune response which play important roles in the tumor progression and metastasis [6]. Furthermore, stimulatory and inhibitory effects on cell adhesion by regulating the expression of cell surface adhesion molecules might be involved in the key potential function of TGF-β1.

In this study, we studied the relationship between metastatic potential and ICAM-1 expression in pancreatic cancer cells, and also investigated the modulating effect of TGF-β1 in ICAM-1 expression, cancer cell immunogenicity and liver metastasis.

Material and methods

Cell Lines and Culture

Human pancreatic cancer cell lines, SW1990, CAPAN-2 and PANC-1 were used in this study and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 2 mM glutamine at 37°C in 5% CO_2 humidified atmosphere.

Flow Cytometric Analysis

Flow cytometric analysis was performed according to the method previously described [7]. All cells were prepared as cell suspension, and incubated with anti-human ICAM-1 antibody (84H10, Immunotech, France). Cells were washed twice and incubated with fluorescein isothio-cyanate-conjugated goat anti-mouse immunoglobulin (Tago, Buringame, CA, USA), and were analysed using EPICS ELITE ESP flow-cytometer (Coulter Co., USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

ICAM-1 expression on cancer cell surface was determined by ELISA system according to the method previously described [8]. Cells were cultured in 96-well microplates over a 48 h period till reaching to confluent and treated by recombinant human TGF-β1 (Austral Biologicals, CA, USA) at various concentrations (0.8, 4.0, 20.0 ng/ml) and 2.0, 10.0 and 50.0 ng/ml of recombinant human TNF-α (Genzyme, MA, USA) at 37°C for 24 h. After cells were washed with PBS, they were fixed with 0.25% glutaraldehyde and incubated with anti-ICAM-1 antibody after blocking with 100 mM glycine, 1% bovine serum albumin (BSA) in PBS. The quantitative expression of ICAM-1 was determined with horseradish peroxidase-conjugated second antibodies.

Northern Blot Analysis

Cells were spread on to 10 cm plastic dish and incubated over night, then treated with 20 ng of TGF-β1 or 50 ng of TNF-α for 24 h. Total RNA was extracted by Acid Guanidium-Phenol-Chloroform method and Northern blot hybridization was performed as previously described [9]. Briefly, twenty-five mg of total RNA was electrophoresed in 1% denaturing agarose gel and blotted on to nylon membrane. The blot was hybridized to ICAM-1 oligonucleotide probe (#ON398, Oncogene Science Inc., Cambridge, MA) labeled with 32P using endlabeling method (Megalabel 5'-endlabeling kit, Takara, Shiga, Japan). Excess probes were then washed out. Autoradiography was subsequently performed, and the intensity of the band displaying specific ICAM-1 expression was measured by BAS 2000 system (Fuji Film, Tokyo, Japan).

Lymphocytes adhesion assay to cancer cells

Human peripheral blood mononuclear lymphocytes (PBMLs) were prepared from healthy volunteers by Ficoll-Hypaque density gradient centrifugation. Mono-Poly Resolving Medium was added to plastic tubes and fresh anti-coagulated blood were added onto the medium. After centrifugation at 1500 rpm for 30 min and elimination of supernatant, PBMLs were collected and resuspended in DMEM with 10% FCS. Cancer cells (2.5×10^5/well) were incubated in DMEM with 10% FCS alone or 10% FCS and TGF-β1 (20 ng/ml) or anti-ICAM-1 neutralizing antibody (2 μg/ml) for 48 h in 96-well microplates. After washing plates with PBS, PBMLs (2.5×10^6/well) suspended in serum-free medium were allowed to attach to cancer cells for 1 h at 37°C. The binding of PBMLs was quantified by colorimeric assay with MTT [10] using a MTP-120 Microplate reader at 550 nm. The percentage of total PBMLs that adhered to cancer cells was calculated as % adhesion = (OD of experimental wells – OD of cancer cell wells)/OD of total PBML wells×100.

Cancer cell cytotoxicity assay

The cell cytotoxicity assay was performed using a Cytotox 96 Non Radioactive Cytotoxicity Assay Kit (Promega Co., Madison, WI), that measured lactate dehydrogenase (LDH). After cancer cells were pretreated with or without TGF-β1 (20 ng/ml) or anti-ICAM-1 neutralizing antibody (2 μg/ml) for 48 h prior to assay, approximately 1×10^4/well cancer cells were placed in 96-well plate, and 1×10^5/well PBMLs were added and incubated at 37°C for 18 h. Absorbance related to released LDH level in the medium was measured with Microplate reader at 492 nm. The percentage of total cancer cells that underwent lysis was calculated as % cytotoxicty=(experimental – PBML spontaneous – cancer spontaneous)/(cancer maximum – cancer spontaneous)×100.

In vivo liver metastatic assay

Cancer cells were incubated in 10% FCS-DMEM with or without 20 ng/ml TGF-β1 for 48 h. The cells were suspended to a final concentration of 5×10^5/0.1 ml PBS and injected into the spleen of 6-week-old female Balb/c nude mice under ether anesthesia. After injection, the spleen was extracted and mice were sacrificed after 5 weeks to measure the number of metastatic tumor.

Statistical analysis

Results were expressed as the mean ±SD. Student's t-test was used for statistical analysis and P values less than 0.05 were considered to indicate statistical significance.

Results

ICAM-1 Expression on Pancreatic Cancer Cells

The percentage of ICAM-1 positively expressing cells in SW1990, CAPAN-2 and PANC-1 were 27.1, 73.2 and 94.1, respectively by analysis with flow cytometry. While the expression of HLA class I antigen on these three cell lines was also analysed with monoclonal antibody B9.12.1 against this antigen (Immunotech), there was no difference between these cell lines with high percentage of expression (99.7, 100 and 99.6, respectively) (*Fig. 1*).

Cell surface ICAM-1 expression by ELISA

Subsequently, the expression of ICAM-1 were determined quantitatively by ELISA, and SW1990 cells demonstrated signifi-

Figure 1. **HLA class I antigen and ICAM-1 expression on pancreatic cancer cell lines by flow cytometric analysis**

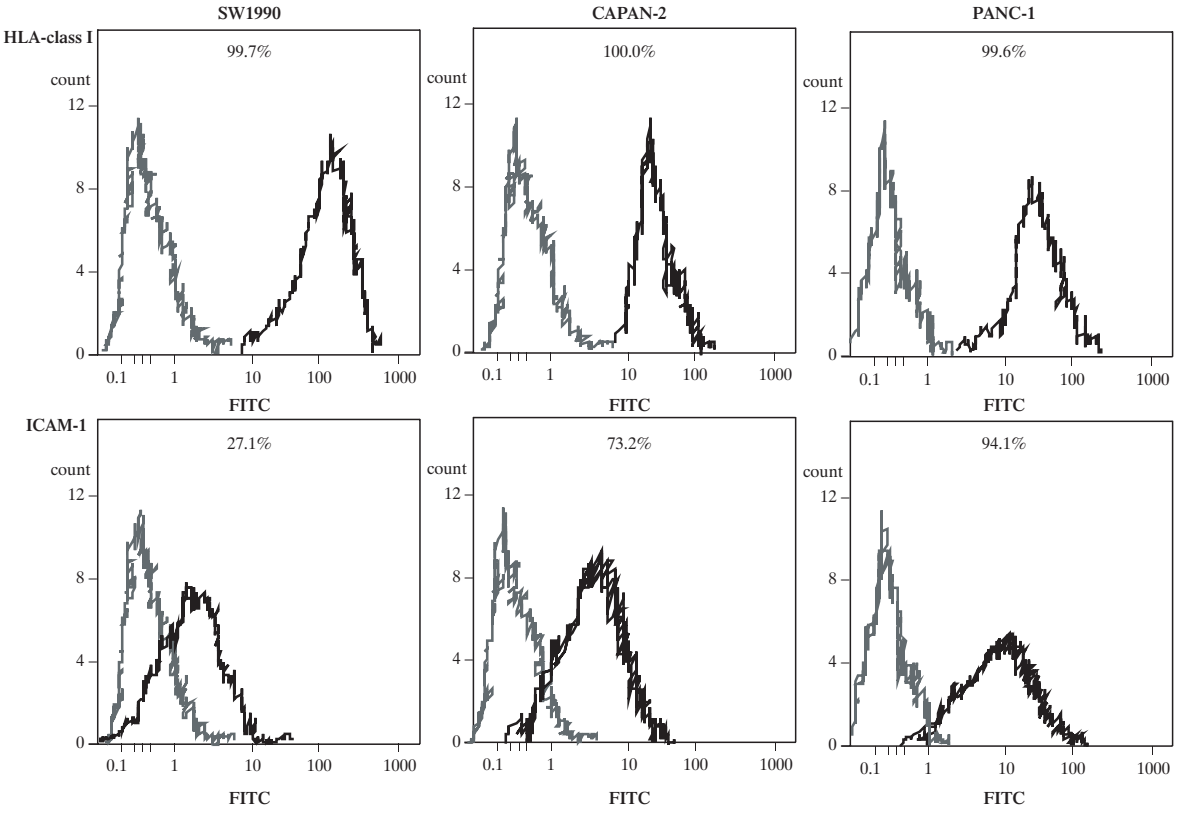
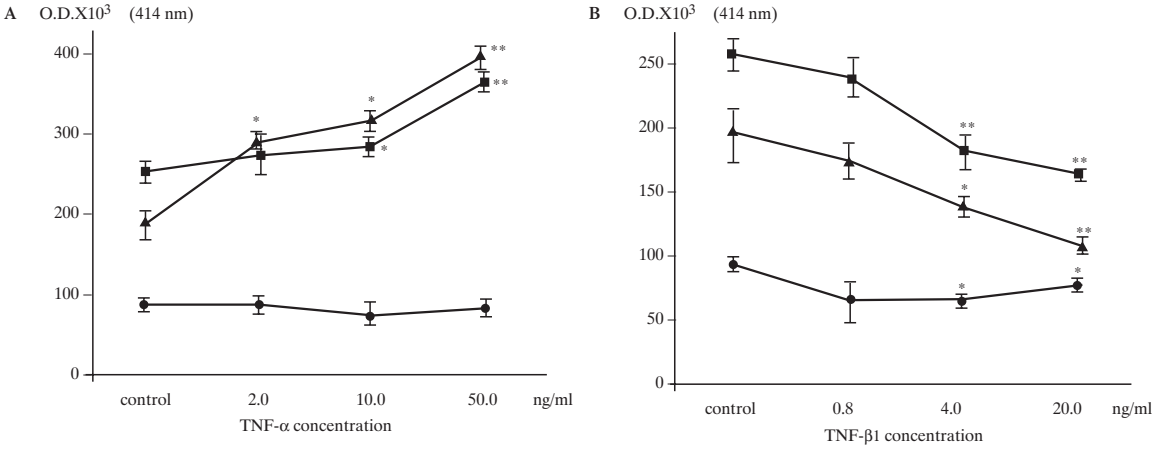


Figure 2. **Modulation of ICAM-1 expression by TNF-α (A) and TGF-β1 (B) on pancreatic cancer cells. The ICAM-1 expression on cell surface of SW1990 (●), CAPAN-2 (▲) and PANC-1 (■) were determined by ELISA system. (*P<0.05, **P<0.01)**



cantly lower expression of ICAM-1 compared with CAPAN-2 and PANC-1 consistently with the results of flow cytometry (*Fig. 2*). The ICAM-1 expression in CAPAN-2 and PANC-1 were significantly enhanced to 1.5-5 fold of untreated control by the pretreatment with 2.0-50.0 ng/ml of TNF-α in dose dependent fashion (*Fig. 2A*). On the other hand, the high expression of ICAM-1 on CAPAN-2 and PANC-1 were decreased dose-dependently and the expression on cells pretreated with 20.0 ng/ml of TGF-β1 were significantly suppressed to 54.8 and 62.7% of untreated CAPAN-2 and PANC-1 respectively. The expression on SW1990 was also decreased significantly in the fewest range by the pretreatment with 4.0 and 20.0 ng/ml of TGF-β1 (*Fig. 2B*).

Modulation of ICAM-1 mRNA level on Northern Blot Analysis

All three cell lines were stimulated by the treatment with TNF-α in their ICAM-1 mRNA level. After TNF-α treatment, the level of ICAM-1 mRNA was increased than untreated control. A larger transcript, considered as immature ICAM-1 mRNA, was also demonstrated after TNF-α treatment. On the other hand, slightly decreased but no significant change in ICAM-1 mRNA expression was found after TGF-β1 treatment compared with untreated control (*Fig. 3*).

Figure 3. **Northern blot analysis of ICAM-1 mRNA level in pancreatic cancer cells after treatment with TGF-β1 (20 ng/ml) or TNF-α (50.0 ng/ml). Lane1:SW1990 no treatment, 2: SW1990 TGF-β1 treatment, 3: SW1990 TNF-α treatment, 4: CAPAN-2 no treatment, 5: CAPAN-2 TGF-β1, 6: CAPAN-2 TNF-α, 7: PANC-1 no treatment, 8: PANC-1 TGF-β1, 9: PANC-1 TNF-α. ICAM-1 mRNA was detected as 3.3 k.b. transcript (arrow). A larger transcript was detected after TNF-α treatment in each cell lines (arrow head)**

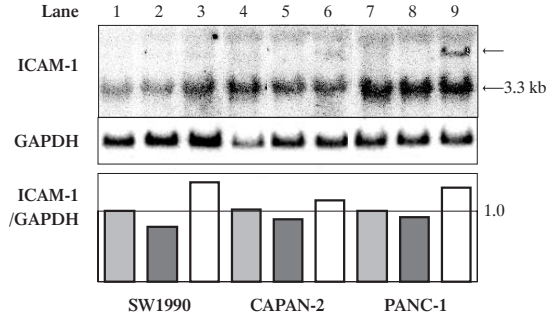
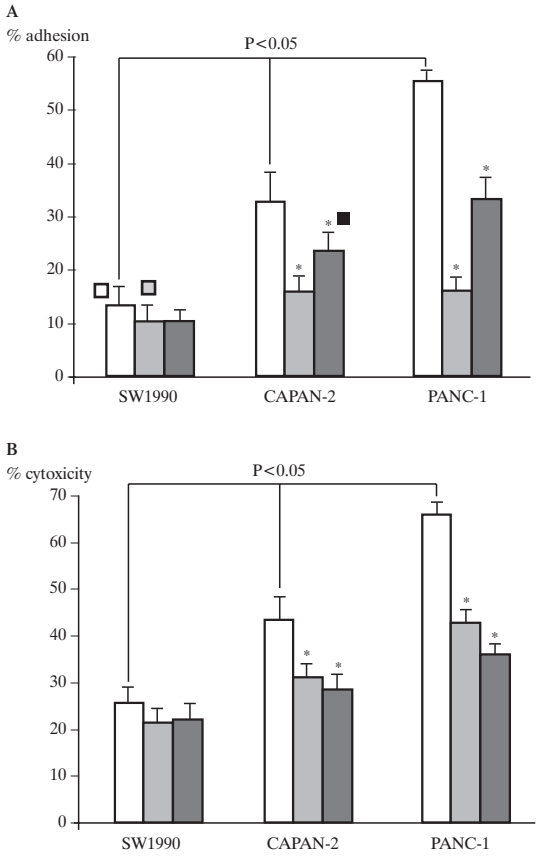


Figure 4. **Effect of anti-ICAM-1 neutralizing antibody and TGF-β1 on adhesion of lymphocytes to cancer cells (A) and cancer cell cytotoxicity by lymphocytes (B). Cancer cells were pretreated without (□) or with anti-ICAM-1 neutralizing antibody (2 μg/ml) (▨) or TGF-β1 (20 ng/ml) (■) for 48 h prior to assay. (*P<0.05)**



Effect of TGF-β1 on adhesion and cytotoxicity by PBMLs

The adhesion of PBMLs to SW1990 and SW1990 cytotoxicity by PBMLs were significantly lower than CAPAN-2 or PANC-1, corresponding to the lowest ICAM-1 expression of SW1990. While the high adhesion of PBMLs to CAPAN-2 or PANC-1 and these cancer cell high cytotoxicity by PBMLs were significantly inhibited by the addition of anti-ICAM-1 neutralizing antibody (2 μg/ml), these high adhesion and cytotoxicity were also significantly inhibited by the treatment with TGF-β1 (20 ng/ml), which is consistent with the results of decreased ICAM-1 expression by TGF-β1 (*Fig. 4*). In SW1990 cells, the adhesion and cytotoxicity were slightly inhibited by anti-ICAM-1 neutralizing antibody and TGF-β1, but significant difference was not observed.

Effect of TGF-β1 on *in vivo* liver metastatic assay

SW1990 which has low expression of ICAM-1 demonstrated high metastatic ability in 9 of 10 mice (90%) under untreated condition, but CAPAN-2 and PANC-1 which show relatively high ICAM-1 expression, demonstrated low incidence of liver metastasis, 18.2% and 0% respectively under untreated condition. After pretreatment with TGF-β1, the metastatic potential of CAPAN-2 was significantly enhanced from 18.2% to 84.6% (11 of 13 mice) and TGF-β1 increased not only the incidence but also the number of metastatic nodules in CAPAN-2. TGF-β1 enhanced the metastasis of SW1990 from 90 to 100%, but PANC-1 cells did not induce any liver metastasis even by the treatment with TGF-β1 (*Fig. 5*).

Discussion

ICAM-1 is expressed on various type of cells, including white blood cells, fibroblasts, endothelial and some epithelial cells. Although the expression of ICAM-1 is found on various cancer cell surface, low ICAM-1 expressing cancer cells demonstrate resistance to lysis by effector cells and correlate to their malignant and metastatic potential compared with highly ICAM-1 expressing cells [7]. Soluble ICAM-1 molecules (sICAM-1) have been known to be released by ICAM-1 expressing cells and elevated serum levels of sICAM-1 have been identified in various inflammation, infection and also malignant diseases. The elevated release of sICAM-1 by cancer cells have been reported to block the recognition of cancer cells by T lymphocytes and let them escape from host immune defense [11]. These immunosuppression may be suggested to induce cancer progression and metastasis.

As we investigated ICAM-1 expressions on three pancreatic cancer cells by flow cytometry and ELISA assay in the present study, SW1990 cell which has lowest ICAM-1 expression revealed highest liver metastatic ability in nude mice splenic injection model [12]. On the other hand, CAPAN-2 and PANC-1 cell which have high ICAM-1 expression demonstrated weak or no liver metastatic potential. These present results suggested that the decreased ICAM-1 expression on pancreatic cancer cells correlated with their metastatic potential, which were consistent with other previous reports [7].

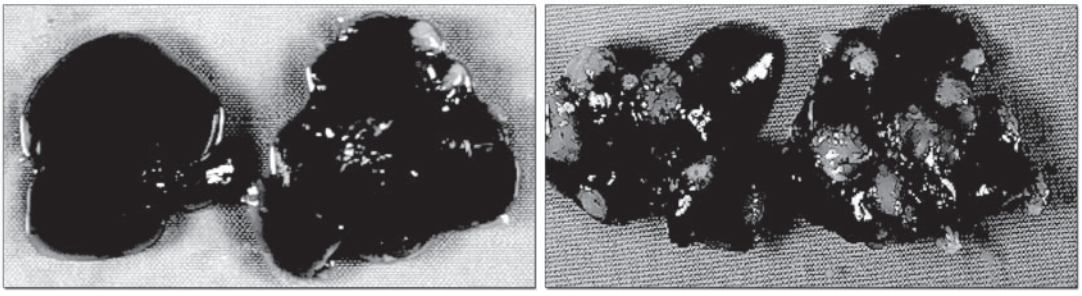
The ICAM-1 expression on cancer cells have been reported to be induced by various cytokines, and TNF-α has also known to be a strong inducer of its expression and involved in the metastatic process at host microenvironment on several steps

***Figure 5*. Effect of TGF-β1 on *in vivo* liver metastasis. Upper table is the ratio of liver metastasis bearing nude mice after splenic injection of pancreatic cancer cells treated with or without TGF-β1. Lower figure is macroscopic findings of the resected liver in nude mice injected of CAPAN-2 cells**

Effect of TGF-β1 on *in vivio* liver metastatic ability;
the ratio of liver metastasis bearing nude mice

	no treatment		TGF-β1 treatment
SW1990	90.0% (9/10)		100% (10/10)
CAPAN-2	18.2% (2/11)	P<0.05	84.6% (11/13)
PANC-1	0% (0/5)		0% (0/5)

5 weeks after splenic injection



Control of CAPAN-2 TGF-β1 treatment of CAPAN-2

[4,7]. Increased ICAM-1 expression on cell surface may lead a convenient circumstance for the cancer cell recognition by host effector cells. While we investigated the modulating effect of multifunctional TGF-β1 in ICAM-1 expression on pancreatic cancer cell, TGF-β1 significantly induced decreasing ICAM-1 expression on all cell lines. According to the immune system, this behavior of TGF-β1 might be a disadvantage in the recognition of cancer cells by cytotoxic effector cells and the supplement of TGF-β1 may possibly increase the metastatic ability.

Our present study also demonstrated that TGF-β1 treatment slightly decreased ICAM-1 mRNA expression level in all cell lines, but its mRNA modulation was not significantly inhibited compared to the decreasing effect in ICAM-1 protein expression. This results suggested that little influence was supposed to be obtained by TGF-β1 on transcriptional regulation of ICAM-1 expression, and modulation in ICAM-1 expression by TGF-β1 might be limited to post-transcriptional level, such as the manner or the location of ICAM-1 protein existence.

While TGF-β1 was found to be increased in tissue remodeling after acute pancreatitis, pancreatic cancer sometimes accompany inflammatory change by obstructing exocrine pancreatic duct which may be involved in increased production of TGF-β1 [13]. TGF-β1 generally acts to inhibit cell proliferation through binding to its receptor on cell surface, and the loss of these growth suppression induced by deficient receptor function has been known to be involved in carcinogenesis [14]. Furthermore, TGF-β1 produced in autocrine by tumor cells and in paracrine by host parenchymal stromal cells at microenvironment were reported to be implicated in host immunosuppression and also involved in tumor progression and metastasis by modulating tumor biological characteristics [5].

In addition to these effect, the present study suggested that TGF-β1 may inhibit the immune defence function against not only host effector cells but also cancer cells by modulating ICAM-1 expression. Besides the decreasing ICAM-1 expression on cancer cells by TGF-β1, the adhesion of PBMLs to cancer cells and consequent cancer cell cytotoxicity by PBMLs were apparently inhibited by TGF-β1 treatment of cancer cells as well as the additional treatment with anti-ICAM-1 neutralizing antibody, and these modulation of immunogenicity in cancer cell induced by TGF-β1 was found to be correlated to the enhancement of *in vivo* liver metastasis in CAPAN-2 cells. This function must be newly nominated in one of the multifunction of TGF-β1 and be implicated in the mechanism of highly metastatic behavior of pancreatic cancer. Highly metastatic SW1990 demonstrated slight increasing liver metastasis by pretreatment with TGF-β1, but no enhancement of metastasis was observed in PANC-1. PANC-1 basically reveals the lack of the requiring factors for the accomplishment of liver meatstasis such as expression of sialyl-Le^a or -Le^x , u-PA high activity [8,12] and others, therefore that is supposed to be the reason for no enhancing metastasis by TGF-β1 in PANC-1, despite of the decreasing ICAM-1 expression.

In conclusion, the present study suggested that decreased ICAM-1 expression induced by TGF-β1 contributes to cancer cell escape from immunological recognition and cytotoxicity by effector cells, and this biological TGF-β1 function may play an important role as one of unknown function in the tumor progression and metastatic mechanism of pancreatic cancer. Since TGF-β1 has been known to reveal other invasion or metastasis enhancing functions besides the current ICAM-1 decreasing effect, the inhibiting therapy of these TGF-β1 functions might be one of the new possible approach to inhibit or treat the metastasis of aggressive pancreatic cancer.

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