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Expression of Intercellular Adhesion Molecule-1 on Transitional Cell Cancer

Possible Significance in Immunity against Tumor Cells

Yoshihiko Tomita,* Hisami Watanabe,† Hisashi Kobayashi,‡ Tsutomu Nishiyama,* Shoji Tsuji,‡ Kohzoh Imai,§ Toru Abo,† Michio Fujiwara,‖ and Shotaro Sato*

From the Departments of Urology, and Immunology,† Niigata University School of Medicine, Niigata; Department of Neurology,‡ Niigata University Brain Research Institute, Niigata; Department of Internal Medicine (Section 1),§ Sapporo Medical College, Sapporo; Animal Center for Biomedical Research,‖ Faculty of Medicine, University of Tokyo, Tokyo, Japan*

Immunohistochemical examination demonstrated expression of intercellular adhesion molecule-1 (ICAM-1) on 17 of 44 transitional cell cancers (TCCs) but not on normal transitional cells. ICAM-1 was frequently expressed in higher stage tumors, especially in those with abundant immune cells scattered within tumor. Analysis of infiltrating immune cells showed that they were composed mainly of T lymphocytes and a smaller number of macrophages bearing the lymphocyte function-associated antigen-1 (LFA-1). Expression of ICAM-1 on transitional cell cancer cell lines was augmented by in vitro treatment with interferon-γ, tumor necrosis factor-α, and interleukin-1β. Furthermore, Northern blot analysis revealed higher quantities of a 3.3-kb RNA in T24 cells exposed to interferon-γ or tumor necrosis factor-α. These results suggest that the expression of ICAM-1 on transitional cell cancers might be modified by cytokines produced by infiltrating immune cells, which might facilitate immune responses against cancer cells. (Am J Pathol 1993, 143:191–198)

Cell-to-cell contact is required in some phases in immune reaction, including lysis of target cells by cytotoxic T lymphocyte (CTL).[1] Major histocompatibility complex molecule and peptide fragment, derived from specific antigen, constitute ligand to T cell receptor,[2] and play as restriction elements for immune response.[1,3] CTL can lyse target cells when they bind to target cells through its cell receptor and syngenic major histocompatibility complex class I molecule on target cell. Furthermore, for initiation of a specific immune response, antigen-independent interactions between adhesion molecules are also required.[4] Lymphocyte function-associated antigen-1 (LFA-1), one of these adhesion molecules, mediates the intercellular adhesion occurring in cytolytic conjugate formation by CTL and target cells.[5,6] Moreover, LFA-1 is involved in natural killer cell-mediated cytotoxicity and antibody-dependent cytotoxicity mediated by granulocytes or peripheral blood mononuclear cells.[7,8] Intercellular adhesion molecule-1 (ICAM-1), one of the ligands of LFA-1,[9,10] is also known to play an important role in target cell lysis by immune cells.[11] Recently, it was reported that ICAM-1 expression on melanoma cells determines its susceptibility against CTL[12] and natural killer cells.[13] Immunohistochemical studies have demonstrated that ICAM-1 is expressed not only on hematopoietic cells but also nonhematopoietic cells such as endothelial cells and dermal fibroblasts.[14] ICAM-1 expression has also been detected on malignant cells such as those of lymphomas,[15,16,17] melanomas,[18] and certain kinds of carcinomas.[19,20] Natali et al reported that ICAM-1 was not detected on normal urothelium nor on six transitional cell carcinomas (TCCs) of urinary bladder.[21] However, there seemed to be no report of TCC on ureter and renal pelvis.

In the present study, using immunoperoxidase staining, we obtained novel data demonstrating frequent expression of ICAM-1 on TCCs with immune

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Address reprint requests to Dr. Yoshihiko Tomita, Department of Tumor Biology, Karolinska Institute, Box 60 400, 104 01 Stockholm, Sweden.

cell infiltration, and surface expression of ICAM-1 and ICAM-1-specific messenger (m)RNA could be significantly increased by incubating transitional cancer cells with interferon (IFN)-γ and tumor necrosis factor (TNF)-α.

Materials and Methods

Tissue Specimens

Tumor specimens were obtained from 44 patients (30 males and 14 females) who had undergone total cystectomy for bladder cancer and nephroureterectomy for renal pelvic or ureteral cancer. These patients had not received chemotherapeutic or immunomodulatory agents preoperatively, and they had been treated with antibiotics if there was evidence of urinary tract infections. The mean age at the time of operation was 67.1 years, ranging from 52 to 80 years. Ten specimens of normal urinary bladder epithelium were collected at transurethral resection for benign prostatic hyperplasia. Five specimens of renal pelvis and two of ureter were collected at radical nephrectomy for renal cell cancer (RCC). Tissue samples were embedded in optimum cold temperature compound (Miles Laboratories, Naperville, IL) and were quickly frozen in isopentane, precooled in dry ice acetone. These blocks were stored at −80 C until 5-μ serial sections were cut using cryostat. Histological examination was performed on hematoxylin and eosin-stained tissue sections. Tumors were graded and staged according to the criteria of the World Health Organization.[22]

Reagents

Monoclonal antibodies (MAbs) used in this study are as follows: 84H10 against ICAM-1 (CD54, IgG1) (Immunotech, S. A., Marseille, Cedex 9, France), MHM24 (Dakopatts a/s, Glostrup, Denmark), and TS1/22 (HB202, obtained from American Tissue Culture Collection) against LFA-1 α-chain (CD11a, IgG2a). Anti-Leu1 against pan T cells (CD5, IgG1), anti-Leu2a against killer/suppressor T cells (CD8, IgG1) anti-Leu3a against helper/inducer T cells (CD4, IgG1), anti-Leu12 against B cells (CD19, IgG1), and anti-LeuM3 against macrophages (CD14, IgG2b) were used for evaluating phenotypes of tumor infiltrating mononuclear cells. These MAbs for phenotyping were purchased from Becton-Dickinson (Mountain View, CA). Optimal dilution for each antibody was determined by staining, on three specimens of lymph nodes obtained at nephrectomy for RCC without tumor metastasis.

Immunoperoxidase Staining

Immunoperoxidase staining was performed using the streptavidin-biotin bridge technique as described elsewhere.[23] Briefly, serial sections were air-dried and fixed in cold acetone. After rehydration with phosphate buffered saline (PBS), sections were incubated in PBS containing 20% normal sheep serum (Antibodies Inc., Davis, CA) for 30 minutes, and endogenous biotin was blocked by using an Endogenous Biotin Blocking Kit (Vector Laboratories, Burlingame, CA). They were then incubated with mouse MAbs for 60 minutes, followed by incubation with biotinylated sheep anti-mouse immunoglobulin (Amersham International, Amersham, Bucks, UK) in PBS containing 20% human type AB serum (Biological Speciality Co., Lansdale, PA). Subsequently, they were incubated with streptavidin peroxidase (Amersham) for 45 minutes. Each step was followed by washing in PBS with three changes of buffer. Finally, the sections were immersed in 0.05 mol/L Tris-HCl buffer containing 0.05% diaminobenzidine and 0.01% H_2O_2 for 3 to 5 minutes to visualize the reaction products. Some specimens were counterstained in Mayer's hematoxylin and mounted after dehydrating in graded ethanol and xylene. As negative controls for ICAM-1 staining, serial sections of tumor tissue were stained with the same subclass of monoclonal antibodies against a variety of phenotypes as described previously. For intrinsic positive controls of ICAM-1, staining patterns of endothelial cells were checked.

When reacted with anti-ICAM-1 MAbs, the tumor tissue showed various staining patterns. The degree of positive staining of tumor cells, which were distinguishable from nontumor cells, was expressed as approximate percentage of positive cells. Evaluation of infiltrating immune cells was performed in a semi-quantitative manner: +++: marked; ++: moderate; +: light; ±: occasional; −: none.

Treatment with Cytokines and Flow Cytometric Analysis

TCC cell lines were cultured in 3055 (Coaster, CA) 25-cm^2 tissue culture flasks in complete medium (Roswell Park Memorial Institute medium 1640 containing 10% fetal calf serum). These cell lines were treated initially with a fixed dose (2,500 U/ml for recombinant (r)IFN-α and 500 U/ml for the others) of

four different cytokines (rIFN-α, rIFN-γ, natural (n)-TNF-α, and rIL-1β) known to have modulating effects on the expression of ICAM-1 on melanoma cells[24] and RCC cells.[20] The concentrations of 2,500 U/ml and 500 U/ml were determined to compare our results with other studies using cytokines. Furthermore, dose- and time-response experiments were performed for enhancement of ICAM-1 expression on T24 by IFN-γ. For flow cytometric analysis, cells were stained by the indirect immunofluorescence method as described before.[20] Briefly, tumor cell suspensions were prepared by treatment with 0.125% trypsin and 0.02% ethylenediaminetetraacetic acid. Tumor cells (1×10^6) were reacted for 30 minutes at 4 C with anti-ICAM-1 MAb at a final dilution of 1:10 in PBS supplemented with 2% fetal calf serum and 0.02% sodium azide. After washing twice by centrifugation, cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse Ig (Tago Inc., Burlingame, CA) for 30 minutes at 4 C at a final dilution of 1:30. Subsequently, the cells were washed three times and suspended at a concentration of 1×10^6 cells/ml and analyzed by flow cytometry (FACScan™, Becton-Dickinson). All data are represented in mean fluorescence intensity units that were calculated as described by Guarini et al.[25]

RNA Extraction and Northern Blot Analysis of ICAM-1

A 520-bp ICAM-1 complementary DNA probe was used.[26] A β-actin complementary DNA probe,[27] which was kindly provided by Dr. H. Hamada (Tokyo University, Faculty of Med., Tokyo, Japan), was used as a control probe. T24 cells were cultured in 3375 (Costar, CA) 75-cm^2 tissue culture flasks in Roswell Park Memorial Institute medium 1640 containing 10% fetal calf serum with or without exposure to IFN-α or TNF-α for 48 hours. These cells were harvested by cell scraper, washed twice with PBS, and were extracted using a guanidinium isothiocyanate procedure to yield a total RNA preparation.[28] Twenty μg of total RNA were size-fractionated on 1% agarose-formaldehyde gel and transferred to a nylon membrane (Gene Screen, NEN). Hybridization was performed at 65 C in 7% polyethylene glycol and 10% sodium dodecyl sulfate[29] using multi-primed ^{32}P-labeled probe Amersham, Multiprime DNA labeling system, RPN 1600). Filters were washed finally in 0.2 × standard saline citrate (1 × standard saline citrate = 150 mmol/L NaCl, 15 mmol/L sodium citrate) for 30 minutes at 65 C, and autoradiographed using an intensifying screen at −80 C.

Results

ICAM-1 Expression and Infiltration of Immune Cells in TCC

When normal urothelium was stained with anti-ICAM-1 MAb, in all transitional cells examined in this study were negative. On the other hand, ICAM-1 was detected on 27 out of 44 cases of TCC and more than 30% of the tumor cells were positive in eight of 44 cases (Table 1). ICAM-1 was expressed more frequently in invasive TCC compared with superficial TCC, although significant correlation was not noticed (Figure 1, A and B). Invasive tumors in which there were a larger number of scattered immune cells expressed ICAM-1 more frequently. The degree of ICAM-1 expression was significantly higher than that without immune cell infiltration (Table 2). The degree of infiltration was evaluated in a semi-quantitative manner as described in Materials and Methods. Immunohistochemical staining using a panel of MAbs for characterization of tumor infiltrating mononuclear cells showed that infiltrating cells were composed of T cells and a smaller number of macrophages, both bearing LFA-1 (Table 1, Figure 2). In several tumors, B cells formed follicles at the submucosal layer or peritumoral area. However, characters of infiltrating immune cells such as composition of T cells, B cells, and macrophages were not different between ICAM-1-positive and negative TCCs (data not shown).

Expression of ICAM-1 on TCC Cell Lines and the Effect of Treatment with Cytokines

It was also of interest whether or not TCC cell lines express ICAM-1. Three TCC cell lines were examined for their response to various types of cytokines that are potent inducers of ICAM-1 expression.[24] Two (T24 and Scaber) of three cell lines moderately expressed ICAM-1 spontaneously, and less than 5% of cells of the other line (HT1376) did so (Table 3, Figure 3). IFN-γ, TNF-α, and IL-1β augmented the expression of ICAM-1 on three TCC cell lines examined (Table 3, Figure 3). Subsequently, dose- and time-response experiments for enhancement of ICAM-1 expression on T24 cells by IFN-γ were carried out. IFN-γ augmented the expression of ICAM-1 on T24 in a dose- and time-dependent

Table 1. *Clinical and Histopathological Features, ICAM-1 Expression, and Immune Cell Infiltration in TCC*

No.	Age	Sex	Site	Grade	T-stage	ICAM-1	LFA-1+ cells within tumor	Degree of immune cell infiltration
1	54	F	BT*	3	2	40	yes	++†
2	62	M	BT	2	2	0	no	+
3	52	M	BT	3	4	70	yes	+++
4	59	M	BT	3	1	0	no	+
5	58	M	BT	2	1	0	no	++
6	72	F	BT	3	1	0	no	++
7	72	M	BT	3	4	>95	yes	++
8	72	M	BT	3	2	10	no	+
9	65	M	BT	3	3	5	no	++
10	73	M	BT	3	3	>95	yes	+++
11	64	F	BT	3	3	0	no	+
12	62	M	BT	3	2	5	yes	+
13	66	M	BT	3	2	0	no	++
14	73	M	BT	3	3	5	no	+++
15	62	F	BT	3	2	0	no	+
16	69	F	BT	2	3	10	no	+++
17	67	F	BT	3	3	5	no	++
18	79	M	BT	3	4	30	no	±
19	76	F	BT	3	1	0	no	+
20	72	F	BT	3	1	0	no	+++
21	63	M	BT	3	1	50	yes	++
22	75	M	BT	3	4	40	yes	++
23	72	M	BT	3	1	10	yes	++
24	67	F	BT	2	2	0	no	+
25	72	M	BT	2	1	20	no	++
26	75	M	UT	1	1	0	no	+
27	80	M	UT	2	1	0	no	++
28	60	M	UT	1	1	5	no	++
29	71	M	UT	3	3	15	no	+++
30	63	F	UT	2	3	5	yes	+
31	72	F	UT	3	3	0	no	+
32	67	F	UT	2	2	0	no	++
33	63	M	UT	2	3	20	no	+++
34	72	M	UT	3	2	70	yes	+++
35	59	M	UT	2	2	0	no	++
36	57	M	PT	2	1	10	no	+++
37	67	F	PT	2	3	20	no	+++
38	60	M	PT	2	1	5	no	+
39	74	F	PT	2	2	5	no	+
40	72	M	PT	2	1	30	no	++
41	53	M	PT	2	1	0	no	+
42	73	M	PT	3	3	5	no	++
43	61	M	PT	2	3	10	no	++
44	77	M	PT	2	1	0	no	+

* Site of TCC; BT: urinary bladder; UT: ureter; PT: renal pelvis.
† Degree of immune cell infiltration was evaluated in a semi-quantitative manner as described in Materials and Methods.

manner (Figure 4, A and B). Concentrations as low as 50 U/ml IFN-γ enhanced the expression of ICAM-1, and the maximal effect was shown at concentrations ranging between 1,000 and 2,000 U/ml IFN-γ (Figure 4A). Time-response experiments showed that a marked increase in the expression of ICAM-1 was already detectable after 10 hours of incubation, that a maximal increase was observed after 36 hours of incubation, and that a plateau was reached between 36 and 72 hours (Figure 4B).

Enhancement of ICAM-1 mRNA in T24 Cells Treated with IFN-γ or TNF-α

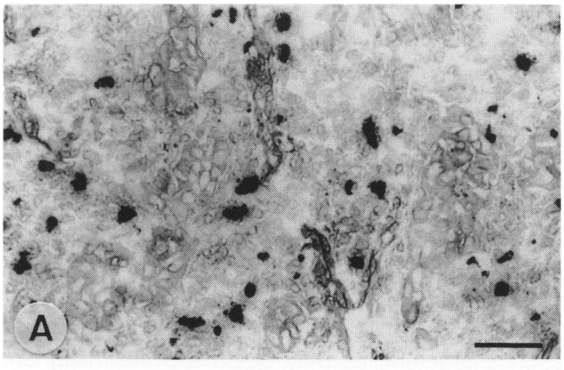
Total RNAs extract were isolated from T24 cells with or without exposure to IFN-γ or TNF-α for 48 hours and analyzed by Northern blotting. Results of autoradiography are shown in Figure 5. A mRNA band (2.2 kb) of ICAM-1 was visible on untreated T24 cells (lane 1), and levels of ICAM-1 mRNA in T24 cells became higher than untreated cells by the treatment with IFN-γ (lane 2) or TNF-α (lane 3).

Discussion

Cell adhesion molecules are thought to be necessary in cellular interactions for development of effective immune responses[4] in addition to antigen-receptor and major histocompatibility complex molecules. LFA-1 and ICAM-1 are representative for such adhesion molecules. The LFA-1 molecule is expressed on hematopoietic cells, and ICAM-1, one

of the ligands of LFA-1, is present on both nonhematopoietic and hematopoietic cells.[14] LFA-1 is involved in effector-target interaction, and therefore anti-LFA-1 MAb inhibits CTL-mediated lysis.[5,6] The presence of ICAM-1 on target cells facilitated cell-mediated lysis in some systems involving tumor cells.[11–13] Furthermore, tumor cells with ICAM-1 and class II molecules might function as antigen-presenting cells, because mouse L cells transfected with the ICAM-1 and HLA-DR genes can activate class II-restricted, allospecific T cells.[30] In this context, ICAM-1 was examined in several kinds of tumor cells, and its de novo expression was detected on lymphomas,[15–17] melanomas,[18] and some kinds of carcinomas.[19] Our previous study showed that ICAM-1 was frequently expressed on RCC.[20] Burkitt's lymphoma cell lines, which have lower susceptibility to Epstein-Barr virus-specific CTL, express a lower amount of ICAM-1, whereas cell lines with higher susceptibility frequently express ICAM-1.[15,16] ICAM-1 expression by non-Hodgkin lymphoma is reported to depend on the maturation stage of lymphocytes, and higher expression has been detected on the follicular type than on the diffuse type, which has a poorer prognosis.[17] A relationship between differentiation stage and ICAM-1 expression has been reported in lymphoid leukemia.[31] In RCC, expression of ICAM-1 in the granular cell type, which is reported to have a worse prognosis,[32] was significantly lower than the clear cell type.[20] These findings suggest that ICAM-1 on tumor cells might contribute to immune surveillance by the host.

ICAM-1 on tumor cells was found to be inducible or augmentable by cytokines,[20,24,33,34] suggesting that cytokines produced by infiltrating mononuclear cells might influence the expression of ICAM-1 on tumor cells. A significant correlation between the degree of mononuclear cell infiltration and the expression of ICAM-1 by stromal cells in breast can-



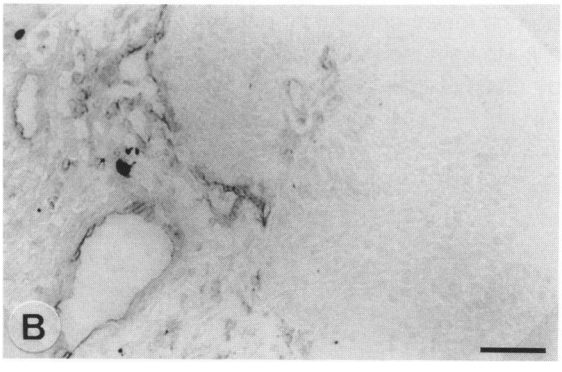


Figure 1. *Immunoperoxidase staining of (TCC) against ICAM-1. ICAM-1 was expressed frequently in invasive TCCs with marked immune cell infiltration* **(A)** *but was not detected in superficial TCCs that showed papillary growth* **(B)**.

Table 2. *Correlation between Expression of ICAM-1 in TCC and Immune Cell Infiltration within Tumor*

Immune cell infiltration within tumor	ICAM-1 expression in TCC	
	Less than 20% of tumor cells	More than 20% of tumor cells
+++	29*	5
++, +, ±	3	7

* Number of cases. Expression of ICAM-1 in TCC is significantly correlated with immune cell infiltration within tumor (χ square = 11.911; $P < 0.01$).

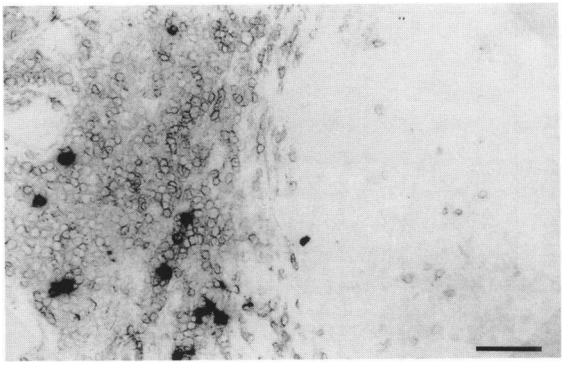


Figure 2. *Immunoperoxidase staining with anti-LFA-1 MAb on immune cells infiltrating within tumor.*

Table 3. *Effect of Cytokines on ICAM-1 Expression on TCC Cell Lines*

Cell lines	Cytokines added				
	(–)	rIFN-α	rIFN-γ	nTNF-α	rIL-1β
T24	1002*	3325	11742	6891	1873
HT1376	168	104	903	1064	282
Scaber	860	ND†	10214	5280	1326

Lined TCC cells were treated with a fixed dose (2,500 U/ml for rIFN-α, and 500 U/ml for the others) of four different cytokines (rIFN-α, rIFN-γ, nTNF-α, and rIL-1β) for 48 hours and analyzed by FACScan.

* Mean fluorescence intensity unit calculated as described in Materials and Methods.

† ND: not done.

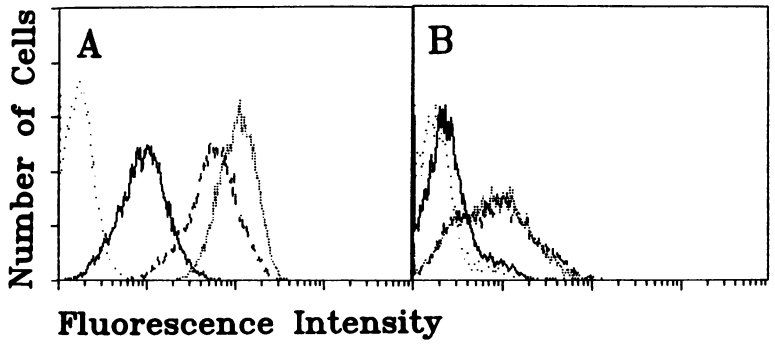


Figure 3. *Flow cytometric analysis of ICAM-1 expression on T24* (A) *and HT1376* (B) *cells. Both cell lines were incubated with IFN-γ (500 U/ml; tight dotted line) or TNF-α (500 U/ml; broken line) for 48 hours. Dispersed dotted line and solid line represent negative control and untreated cells, respectively.*

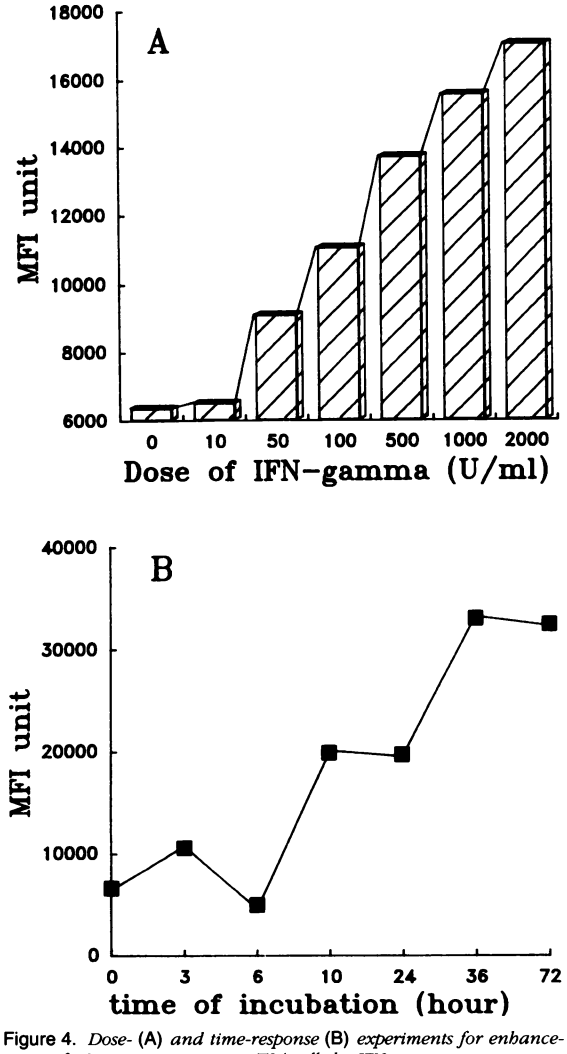


Figure 4. *Dose-* (A) *and time-response* (B) *experiments for enhancement of ICAM-1 expression on T24 cells by IFN-γ.*

cer,[19] by RCC,[20] and by malignant melanoma[35] supports this possibility. In the present study, frequent expression of ICAM-1 was detected more on invasive tumors than superficial tumors, although a significant correlation was not noticed. Immune cells did not scatter within superficial TCCs that showed papillary growth, and such TCCs also had little adjacent stroma in which immune cells can infiltrate. On the other hand, immune cells often scattered within invasive TCCs. Therefore, ICAM-1 might be frequently expressed on TCC in consequence of cytokines that can be produced by infiltrating immune cells within the tumor. Several invasive TCCs, however, did not express high amounts of ICAM-1, despite the infiltration of many immune cells (patients 12 and 23). Composition of immune cells of those TCCs were not different from that of TCCs with high positivity of ICAM-1, suggesting that ICAM-1 might not be inducible in all TCCs and that such characteristics may determine the malignant potential of TCC because a level of ICAM-1 expression on target cells might determine their susceptibility to immune cells. This idea may be supported by result of flow cytometric analysis of demonstrat-

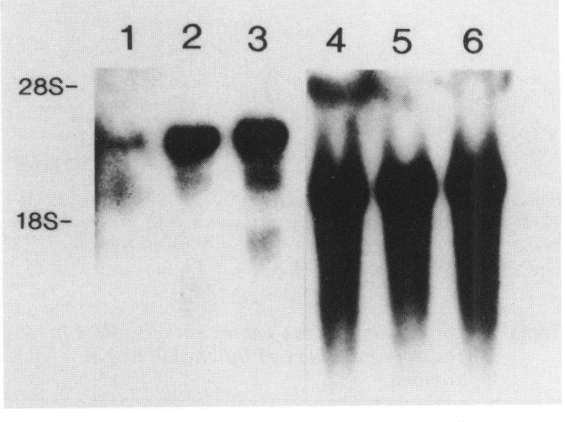


Figure 5. *Influence of pretreatment with IFN-γ and TNF-α on ICAM-1 mRNA expressions in human TCC cell lines (T24). T24 cells were cultured for 48 hours in the presence of 500 U/ml of IFN-γ* (lanes 2 *and* 5) *or TNF-α* (lanes 3 *and* 6), *or without cytokines* (lanes 1 *and* 4). *Twenty μg of total RNA extracted from T24 cells were run through 1% agarose-formaldehyde gel and transferred to nylon membrane. The filter was hybridized with the probe for ICAM-1* (lanes 1 *to* 3) *and β-actin* (lanes 4 *to* 6), *successively. Enhancement of mRNA was observed in T24 cells that were treated with IFN-γ or TNF-α.*

ing variable response against cytokines among three TCC cell lines.

Finally, we examined the expression of ICAM-1 on 44 cases of TCC, and TCC cell lines in response to various types of cytokine. ICAM-1 expression on TCC was more remarkable in cases showing a high degree of immune cell infiltration. ICAM-1 on TCC cell lines was also increased by treatment *in vitro* with IFN-γ, TNF-α, and IL-1β. ICAM-1-specific mRNA could be significantly increased by incubating T24 cells with IFN-γ and TNF-α. These results suggest that TCC tumor infiltrating mononuclear cells might influence ICAM-1 expression on tumor cells, and ICAM-1 expression on TCC might influence host immune reaction against TCC.

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