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Characterization of Mouse Monoclonal Antibody B1.4 Reactive with Human Invasive Bladder Cancer and Some Other Malignant Tumors but Not with Normal Urinary Epithelium

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Immunohistochemical analysis by indirect immunoperoxidase staining demonstrated that monoclonal antibody (mAb) B1.4 derived from a mouse immunized with a bladder cancer cell line EJ-1 was reactive with a high proportion of high-grade and invasive bladder tumors, but not with the majority of low-grade and superficial bladder tumors, or normal urinary epithelium. Among 71 primary bladder tumors classified by pathological grading, positive stainings were observed in 1 of 34 tumors (3%) of grade 1, 8 of 20 tumors (40%) of grade 2 and 14 of 17 tumors (82%) of grade 3. When the tumors were classified by pathological staging, positive stainings were observed in only 8 of 54 (15%) superficial tumors of stages Ta and T1, but in 15 of 17 (88%) invasive tumors of stages T2 and T3. mAb B1.4 showed restricted positive stainings with normal tissues including renal glomerulus, vascular endothelium, squamous epithelium of esophagus, glandular epithelium of prostate, and epithelium of pancreatic acinar gland and minute duct, while positive stainings were observed in a range of tumor tissues other than bladder tumor. Mixed hemadsorption assays with a panel of cell cultures showed also that the antigen recognized by mAb B1.4 was expressed on a range of tumor cell lines. These findings suggest that the antigen recognized by mAb B1.4 may appear after malignant transformation, and be an indicator of malignant potential of bladder cancer.

Key words: Monoclonal antibody — Bladder tumor — Invasive tumor

Several monoclonal antibodies (mAbs) to bladder cancer antigens have been established to investigate molecular changes during development, differentiation and malignant transformation.[1-8] Antigens detected by these mAbs can be divided into three categories: category 1 antigens[1-5] are expressed on almost all bladder tumors, but not on normal urinary epithelium; category 2 antigens[6] are present mainly on low-grade bladder tumors, but on few high-grade tumors; category 3 antigens[7,8] are found in a high proportion of high-grade and high-stage bladder tumors, but only a small proportion of superficial and low-grade tumors. Phenotyping of heterogenous bladder tumors by use of a panel of mAbs helps to predict biological activities including invasion and metastasis.[8]

In this study, in an attempt to obtain mAbs reactive with bladder tumors but not with normal urinary epithelium, we established mAb B1.4 by immunizing a mouse with a bladder cancer cell line EJ-1, and characterized its specificity. The expression of the antigen reactive with mAb B1.4 was restricted to a high proportion of high-grade and invasive bladder tumors, and was not detected in the majority of low-grade and superficial bladder tumors, or normal urinary epithelium.

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MATERIALS AND METHODS

Tissue culture Cultured cell lines of human cancer were obtained from the Laboratory of Human Tumor Immunology, Memorial Sloan-Kettering Cancer Center, NY. Bladder cancer cell lines EJ-1 and T24 were obtained from Japanese Cancer Research Resources Bank (JCRB), Tokyo. These cell lines were maintained in RPMI-1640 medium with 10% fetal calf serum.

Serological analysis The anti-mouse immunoglobulin (Ig) mixed hemadsorption (MHA) assay was performed as previously described.[9] To prepare the indicator cells, rabbit anti-mouse Ig (Dako Corp., Santa Barbara, CA) was conjugated to type O human RBC using 0.01% chromium chloride. Serological assays were performed on target cells previously cultured in Terasaki microplates. Antibodies were incubated with the target cells at room temperature for 1 h. The target cells were then washed. Indicator cells were added and incubated for 1 h, and hemadsorption results were scored.

Human tissues Fresh normal and malignant tissues were obtained from surgical or autopsy specimens in the Center for Adult Diseases, Osaka. The tissues were embedded in OCT compound (Miles Scientific, Naperville, IL) and promptly frozen on dry ice. The frozen tissues were stored at −80°C.

Preparation of mouse mAbs BALB/c mice were immunized intraperitoneally four times at 3-week intervals with 5×10^6 EJ-1 cells. Four days after the final immunization, the spleen cells were fused with NS-1 mouse myeloma cells. The supernatant of each clone was screened for anti EJ-1 antibodies by MHA assay using EJ-1 as target cells. Clones detected in this way were subcloned three times by limiting dilution. The immunoglobulin classes of culture supernatants were determined with an Amersham immunoglobulin isotyping kit (Amersham Int., England). The mAbs were purified using the MAPS-II kit (Bio-Rad, CA).

Immunohistochemical staining Assays were performed by an indirect immunoperoxidase method. Cryostat sections were fixed with 2% paraformaldehyde or acetone for 10 min at room temperature. After blocking of endogenous peroxidase activity, the sections were incubated with mAb for 60 min. Rabbit anti-mouse Ig conjugated with peroxidase was added for 60 min as a second antibody. Ascites from mice bearing NS-1 myeloma cells or normal mouse sera were routinely included as negative controls.

Criteria of stage and grade of bladder cancer Stages and grades were determined according to the general rules for clinical and pathological studies on bladder cancer of the Japanese Urological Association.[10] Staging was done according to the UICC criteria. Grading was based on both cellular and structural atypia: grade 1 (G1), well differentiated; grade 2 (G2), moderately differentiated; grade 3 (G3), poorly differentiated.

RESULTS

Analysis of the specificity of mAb B1.4 by indirect immunoperoxidase staining of cryopreserved tissues mAb B1.4 was obtained by the fusion of spleen cells from a mouse immunized with bladder cancer cell line EJ-1 with NS-1 mouse myeloma cells. The immunoglobulin class of mAb B1.4 was IgG1 as determined by the Amersham immunoglobulin isotyping kit. The specificity of mAb B1.4 was investigated in cryopreserved normal human tissues by the indirect immunoperoxidase method. mAb B1.4 was not reactive in formalin-fixed, paraffin-embedded tissues. As shown in Table I, none of the 22 normal urinary epithelia obtained from patients with benign prostatic hypertrophy was stained by mAb B1.4 (Fig. 1A). Among the other 18 types of normal tissues, positive stainings were restricted to renal glomerulus, vascular endothelial cells, squamous epithelial cells of the esophagus, luminal sites including cell membrane and cytoplasm of glandular epithelial cells of prostate, and epithelium of pancreatic acinar gland and minute duct (Fig. 1 D–G). mAb B1.4 did not stain renal glomerulus or vascular endothelial cells of canine, rabbit and rat (data not shown).

Cryopreserved bladder tumors from 71 patients were examined for reactivity with mAb B1.4. As shown in Table II, mAb B1.4 reacted with 23 of the 71 tumors (32%). We examined whether the reactivities of mAb B1.4 with bladder tumors were related to pathological grading and staging. In terms of pathological grading, positive stainings were observed in 1 of 34 tumors (3%) of grade 1, 8 of 20 tumors (40%) of grade 2, and 14 of 17 tumors (82%) of grade 3. In terms of pathological staging, positive stainings were observed in only 8 of 54 (15%) superficial tumors of stages Ta and T1, but 15 of 17 (88%) invasive tumors of stages T2 and T3. Thus, the frequency of positive reactions with mAb B1.4 correlated well with pathological grading and staging of bladder tumors. Typical stainings of bladder tumors by mAb B1.4 are shown in Fig. 1B and C. Positive reaction with mAb B1.4 was observed in the cell membrane and the cytoplasm, but not in the nucleus of bladder cancer cells.

Table I. Reactivity of mAb B1.4 with Normal Human Tissues by Immunoperoxidase Staining

Tissues	Positive stainings[a]
Urothelium	0/22
Kidney	
glomerulus[b]	10/10
tubule	0/10
Prostate[c]	10/10
Testis	0/3
Cerebrum	0/1
Cerebellum	0/1
Thyroid	0/2
Esophagus[d]	2/2
Heart	0/1
Lung	0/2
Liver	0/2
Stomach	0/2
Pancreas[e]	2/2
Spleen	0/2
Small intestine	0/2
Colon	0/5
Adrenal	0/1
Uterus	0/1
Lymph node	0/2

a) Number of specimens with positive staining/number of specimens tested.
b) Intraglomerular cells including endothelial cells, mesangial cells and podocytes were stained. Bowman's capsule was not stained.
c) Luminal sites including cell membrane and cytoplasm of glandular epithelial cells of prostate were stained.
d) Squamous epithelial cells of esophagus were stained.
e) Epithelial cells of pancreatic acinar gland and minute duct were stained, but epithelial cells of excretory duct and the islet of Langerhans were not.

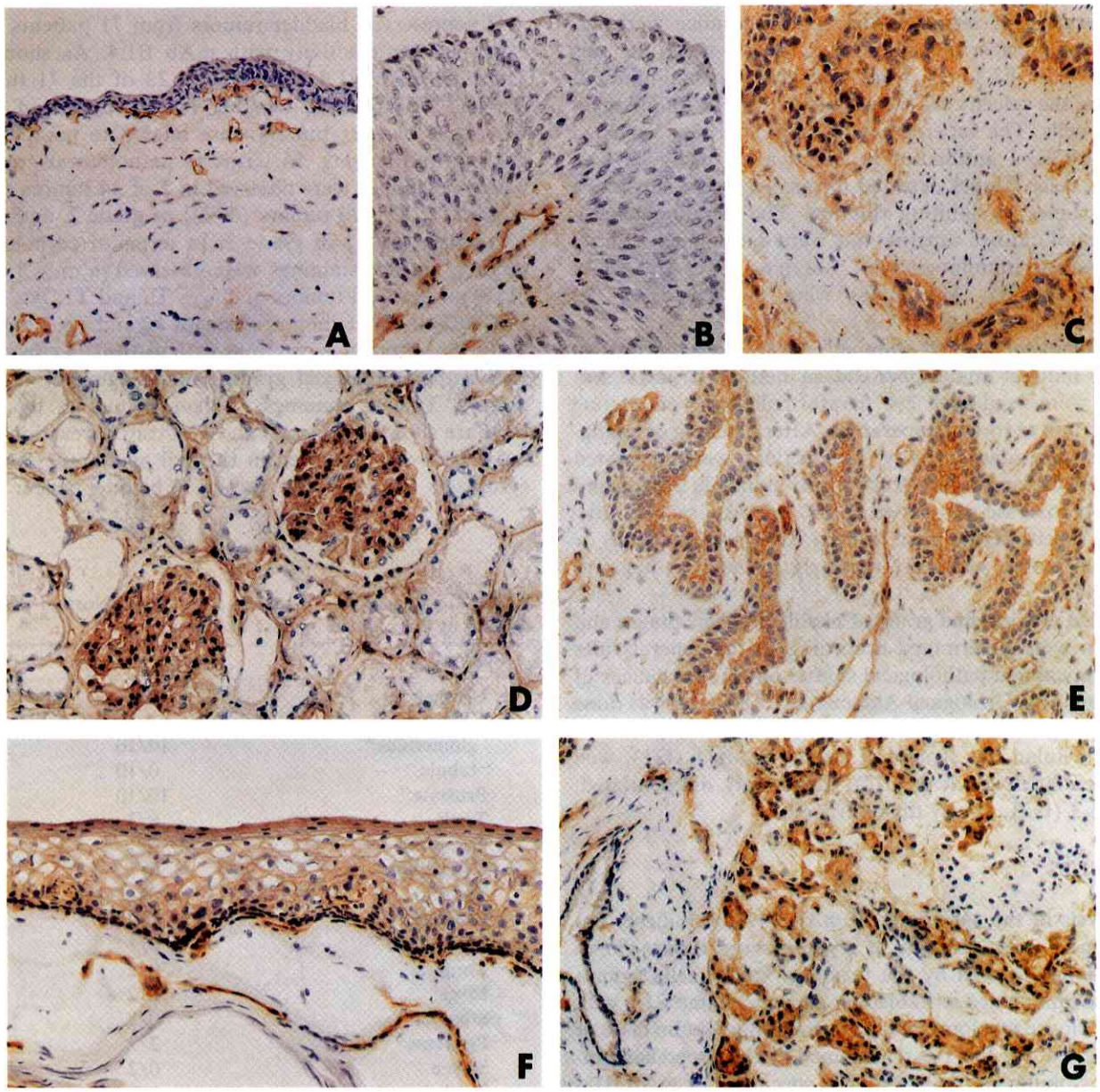

Fig. 1. Immunohistochemical staining with mAb B1.4 (×200). Normal urinary epithelium (A), and superficial bladder tumor (G1, Ta) (B) were not stained by mAb B1.4, but vascular endothelial cells in tissues were stained. Invasive bladder tumor (G3, T3a) (C) was positively stained. Among other normal organs, kidney (D), prostate (E), esophagus (F), and pancreas (G) showed positive staining. Intraglomerular cells including endothelial cells, mesangial cells and podocytes, and vascular endothelial cells in tissues were stained, but renal tubules were not. Squamous epithelial cells of esophagus, luminal sites including cell membrane and cytoplasm of glandular epithelial cells of prostate, and epithelial cells of pancreatic acinar gland and minute duct were stained. The islet of Langerhans and epithelial cells of excretory duct of pancreas were not stained.

We examined 64 tumor tissues from 10 other organs. Positive stainings were observed in all of the 17 prostatic tumors, including low and high grades, 1 laryngeal tumor, 9 of 33 renal tumors, and 1 of 3 gastric tumors, but not in the other tumors, as shown in Table III. Thus, based on the expression of B1.4 antigen, tumor tissues can be classified into two phenotypic patterns; those that originated from normal cells stained by mAb B1.4 (e.g.,

Table II. Relationship of the Reactivities of mAb B1.4 with Pathological Grades and Stages of Bladder Tumors

Stage	Positive staining[a] (%)			
	G1	G2	G3	Total
Ta	1/22	4/11	1/2	6/35
T1	0/12	0/4	2/3	2/19
				8/54 (15)
T2		2/2	3/3	5/5
T3a		2/3	6/7	8/10
T3b			2/2	2/2
				15/17 (88)
Total	1/34 (3)	8/20 (40)	14/17 (82)	23/71 (32)

a) Number of specimens with positive staining/number of specimens tested.

Table III. Reactivity of mAb B1.4 with Other Human Tumors by Immunoperoxidase Staining

Tumor	Histology	Positive staining[a]
Prostate	Adenocarcinoma	17/17
Kidney	Adenocarcinoma	9/33
Testis	Seminoma	0/3
Uterus	Squamous cell carcinoma	0/1
Stomach	Adenocarcinoma	1/3
Colon	Adenocarcinoma	0/2
Rectum	Adenocarcinoma	0/1
Liver	Hepatocellular carcinoma	0/2
Breast	Medullary adenocarcinoma	0/1
Larynx	Squamous cell carcinoma	1/1

a) Number of specimens with positive staining/number of specimens tested.

Table IV. Reactivities of mAb B1.4 with Human Cancer Cell Lines Evaluated by Mixed Hemadsorption Assay

Cells	Reactivity[a] ($\times 10^{-3}$)
Bladder (transitional cell carcinoma)	
EJ-1	256
T24	16
RT-4	<1
647-V	32
639-V	<1
253-J	128
Renal (adenocarcinoma)	
SK-RC-6	256
SK-RC-9	128
SK-RC-26	16
SK-RC-28	256
SK-RC-29	256
SK-RC-47	256
SK-RC-49	256
SK-RC-56	128
Colon (adenocarcinoma)	
HT-29	128
HG-15	64
SK-CO-10	64
Lung (anaplastic carcinoma)	
SK-LC-1	<1
Breast (ductal adenocarcinoma)	
MCF-7	<1
Astrocytoma	
SK-AO-2	<1
SK-MG-3	64
SK-MG-12	16
Neuroblastoma	
NSNb	<1
Melanoma	
MEWO	128
SK-MEL-23	<1
SK-MEL-28	128
SK-MEL-31	16
SK-MEL-173	16

a) Dilution titers of ascites of mAb B1.4 reactive with more than 90% of each target.

prostate, larynx), and those that originated from normal cells not stained by mAb B1.4 (e.g., kidney, stomach).

Analysis of the reactivity of mAb B1.4 with cultured cell lines by rabbit anti-mouse Ig MHA assay The reactivities of mAb B1.4 with various tumor cell lines were examined further by serological assay. Four of the 6 bladder cell lines and almost all the cell lines from kidney, colon, brain and skin also reacted with mAb B1.4, as shown in Table IV. These results were consistent with the results of immunohistochemical stainings of tumor tissues.

Chemical characterization of antigens recognized by mAb B1.4 We could not determine the molecule of B1.4 antigen by immunoprecipitation after labeling EJ-1 with ^{125}I by the lactoperoxidase and iodogen methods. mAb B1.4 was IgG1, but even after adding rabbit anti-mouse IgG as a second antibody, a specific band was not precipitated. We could not identify a specific band by Western blotting. To enrich molecules reactive with mAb B1.4, EJ-1 cells were pretreated with mAb B1.4 and rabbit anti-mouse IgG, then antigen-antibody complex lysate was applied to a protein A Sepharose column. Eluted lysate was subjected to Western blotting, but no specific band was found. Thus, we could not find a specific band by various kinds of immunochemical analyses. Treatment of cryostat sections fixed with 2% paraformaldehyde with 0.1% protease, heating at 100°C or 0.02 *M* sodium periodate decreased the reactivity of mAb B1.4. The molecule reactive with mAb B1.4 may be a glycoprotein.

DISCUSSION

We demonstrated immunohistochemically that mAb B1.4 reacted with bladder tissues in a high proportion of muscle invasive tumors, but in only a few superficial tumors. It did not react with normal urinary epithelium. The frequency of positive staining with mAb B1.4 correlated with pathological grading and staging. Thus, B1.4 antigen would seem to appear after highly malignant transformation of bladder tissues. B1.4 antigen belongs to the category 3 antigens. In immunohistochemical staining of normal tissues by mAbs reactive with the category 3 antigens, mAb T43 stained renal tubules, mAb T138 stained endothelial cells, and mAb T23 stained skin, fibroblasts, smooth muscle and cartilage. These three mAbs also showed positive reactions to a range of panel cell lines by serological analysis, and to some other tumors by immunohistochemical analysis.[11] mAb 3G2-C6 stained some other tumors including those of testis and kidney.[7] Therefore mAb B1.4 seems to react with an antigen different from other category 3 antigens. mAb P7A5-4 established by Ben-Aissa *et al.* showed similar reactivities to those of mAb B1.4.[4] In immunohistochemical analysis, mAb P7A5-4 reacted with 7 of 8 bladder tumors, though the grades and stages of these tumors were not cited. Positive stainings were observed in endothelial cells in most tissues and glandular epithelial cells of prostate among normal tissues they examined. Further analysis of the specificity of mAb P7A5-4 by immunohistochemical staining of normal tissues and a large number of bladder tumors would help to discriminate B1.4 antigen from P7A5-4 antigen, and further immunochemical analysis of the antigen reactive with mAb B1.4 should be worthwhile.

mAb B1.4 reacted with some renal or gastric cancers, but renal tubules and gastric mucosa were not stained by mAb B1.4. Preliminary data also suggested that mAb B1.4 reacted with high-stage and high-grade tumors of the kidney, but not with low-grade and low-stage tumors (N. Meguro *et al.*, in preparation). The appearance of B1.4 antigen in tumors could be a reflection of the greater proliferative nature of tumor cells, particularly those with more aggressive characteristics. The fact that B1.4 antigen is expressed in a range of cell lines is consistent with this hypothesis. Another possibility is that B1.4 antigen is expressed as a result of malignancy-related alterations of gene regulation, leading to activation of normally silent genes.

Phenotyping with mAbs has been useful in defining lineage and differentiation markers of human tumors, though little is known about the relationship of antigen phenotype to the biological characteristics of tumors, such as invasiveness and metastasis. Fradet *et al.* demonstrated that T138 antigen was expressed on invasive or metastatic bladder tumors, and endothelial cells,[11] and further examined whether T138 antigen expression and ploidy in bladder tumor cells were correlated with cancer progression and prognosis.[12] The expression of T138 antigen as a single variable was a better indicator of cancer progression than ploidy, and preliminary evidence suggested the association of T138 antigen expression with poor prognosis of bladder tumors. In superficial bladder tumors, mAb B1.4 reacted with 1 of 34 (3%) in grade 1, 4 of 15 (27%) in grade 2, and 3 of 5 (60%) in grade 3. In this small-scale analysis, the prognosis of B1.4 antigen-positive patients among these superficial bladder tumors was significantly worse than that of B1.4 antigen-negative patients (data not shown). B1.4 antigen may be an indicator of malignant potential of bladder cancer, and B1.4 antigen-positive patients with superficial bladder tumors should be followed carefully after bladder conservative operation. The finding that B1.4 antigen and T138 antigen were expressed on invasive tumors and were also markers of endothelial cells raises the possibility that these antigens might be involved in the ability of cancer cells to invade blood or lymphatic vessels, a prerequisite for cancer metastasis. Fradet *et al.*[12] found that T138 antigen was expressed on the majority of metastatic carcinomas of various origins (unpublished results). The mechanisms of invasion and metastasis are very complicated, and many molecules related to cell-cell interaction may be involved in each step during the establishment of metastasis. The expression of endothelial antigens during tumor progression deserves further study.

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