ANALYSIS OF ANTIGENICITY OF MUC-1 MUCIN AS A TUMOR ANTIGEN AND POSSIBILITY OF THIS MOLECULE FOR THE USE OF COMBINED ANTI-CANCER IMMUNOTHERAPY WITH GENE TRANSFER

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Shuhei Inoue
Second Department of Surgery
Shiga University of Medical Science

PREFACE

CONTRIBUTORS

Head Investigator

Shuhei Inoue, Associated Professor, Second Department of Surgery, Shiga University of Medical Science

Investigators

Yoshio Okada, Principal, Shiga University of Medical Science

Hirofumi Kato, Associated Professor, Second Department of Surgery, Shiga University of Medical Science

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RESEARCH RESULTS

Loss of the Expression or Modulation of the Carbohydrate Structures of MUC-1 Mucin Is A Major Escape Mechanism of Cancer Cells from Being Recognized by Peripheral Tumor-Specific T Lymphocytes and Natural Killer Cells in Patients with Breast Cancer.

ABSTRACT

In the present study, we analyzed the antigenicity of MUC-1 mucin expressed on breast cancer cell lines. Of\7 cell lines, 6 expressing the mucin were readily lyzed by PBMCs obtained from normal donors in a 51Cr-release assay. On the other hand, no cell lysis was observed against a MUC-1 deficient cell. The cell lysis observed against the targets with high levels of MUC-1 expression was not inhibited by adding cold K562, and was mediated by TCRαβ/CD4 positive T lymphocytes. The recognition of this molecule was blocked by anti-MUC-1 core antibodies, or an anti-carbohydrate antibody, CC49. The cell lysis against the targets with the low expression was partially inhibited by cold K562. Also the antigenicity of autologous cancer cells from patients with breast cancer was studied. In 7 of 26 patients, the strong lytic activity was seen. The patients possessing the activity showed higher reactivity to anti-MUC-1 core antibodies and CC49 of cancer cells in their tumor tissues than the patients without the activity, by an immunohistochemical study. However, as many as 19 patients showed negligible lytic activity against their targets. Of these patients without the activity, 4 showed lack or a scarcity of MUC-1 expression on the cancer cells, and 7 demonstrated high levels of the expression but lack of CC49-reactivity. A MUC-1 defficient cancer cell line obtained from a patient without the lytic activity was transfected with MUC-1 cDNA, and it was tested for its antigenicity. A strong activity was observed against the autologous transfectant. These data suggested that MUC-1 mucin has a crucial role in inducing a specific anti-tumor immunity as a tumor antigen and that the immune effector cells, T lymphocytes and NK cells can be easily obtained in freshly isolated PBMCs. Also it was suggested, in contrast, that the unresponsiveness to cancer cells observed in many of the patients was mainly due to loss of MUC-1 expression or modulation of carbohydrate structures of the molecule. Since immunotherapy designed to target natural MUC-1 mucin on cancer cells was expected to have the limitation of its effect, for the reason, it would be necessary to improve a means to generate or enhance its antigenicity to activate the immunity.

Keywords: MUC-1 mucin, Breast cancer, Anti-tumor immunity, T lymophocyte, NK cell, Modulation of the antigenicity, Escape mechanism of cancer cells

INTRODUCTION

There have been reported many tumor antigens so far, such as tyrosinase, MAGE and GAGE families, MART-1, HER-2/new, and so on, many of which were expressed in melanoma cells (1-6). The detailed investigations have clarified their nucleotide and amino acid sequences, physiological characteristics, tissue distributions and antigenicities. Each has a favor of MHC haplotypes to be presented on and acquires the ability to activate T lymphocytes followed by target cell lysis when it is presented on the appropriate MHC molecule. On the other hand, MUC-1 mucin has been demonstrated to be recognized by T lymphocytes in a MHC unrestricted manner (7). The mechanism of the recongnition by T lymphocytes, and cell surface molecules and lymphokines involved in the recognition still remain unclear. It is likely, however, that this mucin is recognized by CTLs not according to the already-known interaction of antigenic peptides presented on MHC molecules and TCR. The unique structure of the molecule should be taken into account to explain the unusual manner. A lot of tandem repeats, approximately twenty to a hundred, which are encoded in the core protein and contain conserved twenty amino acid sequences (8-12), may acquire the adequate affinity to antigen binding regions of TCR. It results in the activation of CTL, despite the antigenic peptide presented not in the context of MHC molecules. On the other hand, the exposure of the epitopes in the tandem repeat domain was greatly affected by carbohydrate structures bound on the core protein (13). The mucin is also known to be expressed abundantly on cancer cells such as breast, pancreatic and ovarian cancer (14-16). Recent studies demonstrated that the mucin was abberantly expressed on various cancer cells, i.e., in lung cancer, gastric cancer and colon cancers, etc. (17-19). Because of the abundant expression, MUC-1 mucin has been widely used as a tumor marker, CA15-3, to predict the cancer progression or to evaluate the effect of an anti-cancer treatment to perform. It is expressed on normal epithelial cells of the ducts as well. However, glycosylations of this molecule on normal cells was reported to be different from those on cancer cells (20). Long and complexly branched carbohydrates on normal cells ordinarily mask MUC-1 core proteins and surface molecules on the cells, suggesting that antigenic determinants

capable of activating immune response are physically seggregated from the immune system. Therefore, to determine what structure of the mucin has the antigenicity recognizable by the host immune system, what population of effector cells are responsible for the recognition, and whether it is possible to manupulate the antigenicity and the effector function, for the purpose of immunotherapy against the cancer.

We investigated the relationship between expression levels and patterns of MUC-1 mucin in cancer cells and its antigenicity as a tumor antigen, using breast cancer cell lines showing different expressions of the mucin and breast cancer cells obtained from cancer patients. From the MUC-1 phenotype of the cancer cells, major causes for resistance of the cancer cells to tumor cell lysis by PBMCs were concluded. Three dimentional structures of carbohydrate side chains as well as tandem repeat domains containing the antigenic determinants as reported by Jerome et al. (21) on the molecule were important in eliciting anti-tumor immune response in hosts. In freshly isolated PBMC, two effector populations, CD3/CD4 positive T lymphocytes and NK cells, were involved in the MUC-1 specific cytotoxicity. However, our conlusions were not supportive to targeting natural MUC-1 molecule as a tumor antigen for anti-cancer immunotherapy.

MATERIALS AND METHODS

Monoclonal Antibodies. DF3, 115D8, B72.3 and CC49 were provided from Toray-Fuji Bionics Inc. (Tokyo, Japan). SM3 was purchased from Cymbus Bioscience Ltd. (Southampton, UK), and TKH-6 was from Otsuka Pharmaceutical Co. (Tokushima, Japan). Antigenic determinants with which these antibodies react of MUC-1 mucin are summarized in table 1. SNH3, which is reactive with sialyl-Lex, was kindly given from Dr. Sen-itiroh Hakomori, Biomembrane Institute (Seattle, WA). /\Anti-human CD4 and CD8 monoclonal antibodies were purchased from Seikagaku corp. (Tokyo, Japan), and anti-human TCRab monoclonal antibody was from Cosmo Bio. Co., Ltd. (Tokyo, Japan).

Table 1 Monoclonal antibodies or a lectin used and their antigenic determinants on MUC-1 mucin

Antibodies used	Antigenic structures detected with antibodies
Anti-MUC-1 Core	
DF3	TRPAPGS
115D8	? Core Peptide
SM3	PDTRP
Anti-Carbohydrate	
TKH-6	GalNAc-O-Ser/Thr (Tn)
B72.3	NeuAcα2,6GalNAc-O-Ser/Thr (sialyl-Tn)
PNA	Galβ1,3GalNAc-O-Ser/Thr (TF)
CC49	Galβ1,3(NeuAcα2,6)GalNAc-O-Ser/Thr

Reagents. Dispase was purchased from Godo Shusei Co. Ltd. (Tokyo, Japan), Ficoll-Conray Lymphosepal from Immuno-Biological Laboratories (Gunma, Japan) and Na251CrO4 from DuPont NEN (Boston, MA). PNA reactive with TF, mucin-associated carbohydrate structures was purchased from Seikagaku corp.(Tokyo, Japan). Sialydase and Geneticin (G418) were from GIBCO BRL (Tokyo, Japan).

Cell lines. ZR75-1, T47D, BT-20, SK-BR3, MDA-MB231 and K562 were purchased from American Type Culture Collection (Rockvill, MD). MCF-7 was kindly given from Dr. Shunzo Kobayashi, Second Department of Surgery, Nagoya City University (Nagoya, Japan). NZK-K1 was established from a main tumor of a 45-year-old female breast cancer patient, by grafting the tumor specimens subcutaneously in female Scid mice which were purchased from Charles River Inc. (Hino, Japan).

Preparation of breast cancer cells. Breast cancer cells were freshly isolated from main breast tumors of the patients at the time of operations. Resected tumor samples were minced and incubated in RPMI supplemented with dispase at the concentration of 10,000 PU/ml at 37°C for 60 min to single cell suspensions. The suspensions were layered on the top of step gradients, from the bottom comprising 100% and 75% lymphosepal and centrifuged at 400 x g for 30min. Tumor cells were collected from the interface of the sample and 75% lymphosepal layer and resuspended in RPMI containing 2% FCS.

Preparation of Peripheral Blood Mononuclear Cells (PBMC). Heparinized periphral blood samples obtained from the patients were diluted in the same

volume of PBS, layered on lymphosepal and centrifuged at 400 x g at room temperature for 30 min. PBMCs were collected from the interface of the sample and lymphosepal layer and resuspended in RPMI containing 2% FCS.

Immunohistochemical Study. Tumor specimens obtained from the main breast tumors were either fixed in 10 % formalin and embedded in paraffin or frozen in OCT compound in liquid nitrogen. Paraffin-embedded secrtions were incubated with monoclonal antibodies diluted to their appropriate concentrations in PBS at room temperature for 2 hours following pre-incubation in 0.2% hydrogen peroxide-containning methanol to block non-specific binding sites on them. After three washes in PBS, they were incubated with peroxidase-conjugated anti-mouse IgG diluted at 1/200 in PBS at room temparature for 30 min. They were washed in PBS three times and incubated in 5 mM Tris-HCl containing diaminobentidine to visualize for 10 min. After washing in distilled water, nuclear compartment of cancer cells were stained in hematoxylin solution. They were dehydrated in 100% ethanol and mounted in 50% glycerin. Frozen sections were fixed in 50% chloroform solution and reacted to be stained according to an indirect peroxidase-staining technique as described above.

Flow Cytometric Analysis. Breast cancer cell lines were suspended in PBS and incubated with anti-MUC-1 monoclonal antibodies diluted on ice for 30 min. After three washes in cold PBS, the cells were incubated with FITC-conjugated anti-mouse IgG diluted at 1/1000 on ice for 30 min. Flow cytometric analyses were perfomed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) following three washes in cold PBS.

Cytotoxicity Assay. Breast cancer cell lines or cancer cells obtained from cancer patients were incubated with Na251CrO4 at 37°C for 60 min. 104 cells radio-labeled were transfered to a 96 well U-bottom culture plate containing 5 x 105 PBMCs prepared in 200ml RPMI supplemented with 10% FCS and 5 x 10-5M 2-Mercaptoethonol following three washes in medium. They were incubated at 37°C. 18 hour incubation time was employed to measure the cytotoxicity mediated by cytokines such as TNFa or IFNg as well as by cytotoxic granules, as previously reported (1, 22-23). To abrogate NK-mediated cytotoxicity, 2 x 105 K562 were added to each well containing effector and radio-labeled target cells. To inhibit the recognition of antigenic structures by effector cells, target cells were pre-incubated with anti-MUC-1 monoclonal antibodies at 37°C for 45 min in prior to co-culture with effector cells. To investigate what population of effector cells was responsible for the tumor cell lysis, PBMCs were treated with anti-TCR$\alpha\beta$, CD4 or CD8 antibodies at 37°C for 45 min followed by co-incubation with radio-labeled target cells. After 18 hours incubation of

target cells and PBMCs, the supernatant was collected from each well and counted for the release of 51Cr from the lyzed target cells, using LKB-Wallac Minigamma 1275 (Wallac Oy., Finland). The percentage of chromium release was determined by the formula:

$$\%\text{lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100$$

Tansfection of a Breast Cancer Cell Line with MUC-1 cDNA. MUC-1 cDNA expression vector, pDKOF.muc1 (30) was kindly given by Dr. O. J. Finn (University of Pittsburgh, Pittsburgh, PA). Transfection of a MUC-1 defficient cell line, NZK-K1 with the cDNA was performed, using the electroporation technique as described previously (24).

RESULTS

Phenotypic Analysis of Breast Cancer Cell Lines by FACS. Seven breast cancer cell lines were stained with monoclonal antibodies directed to the MUC-1 core, the associated carbohydrate or MHC molecules as described in 'Materials and Methods'. They were tested for their phenotypes on the cell surface by FACS (Fig. 1). NZK-K1 showed lack of MUC-1 mucin and high levels of both class I and II expressions. MDA-MB231 and SK-BR3 demonstrated low expressions of the tandem repeat domain, detected with DF3 and 115D8, and no expression of the mucin-associated carbohyrates. Although high expressions of the tandem repeat, detected with DF3 and 115D8, was seen in ZR75-1, BT-20 and T47D, a strong reactivity to SM3 was shown in BT-20 and T47D, but not in ZR75-1. Of carbohydrate structures, the determinants detected with CC49 were strongly expressed in ZR75-1 and T47D, and weakly but apparently expressed in BT-20. Other oligosaccahride structures, detected with TKH-6 or B72.3, were not found in these cells. MCF-7 showed intermediate levels of tandem repeat and a scarcity of the cabohydrate expression.

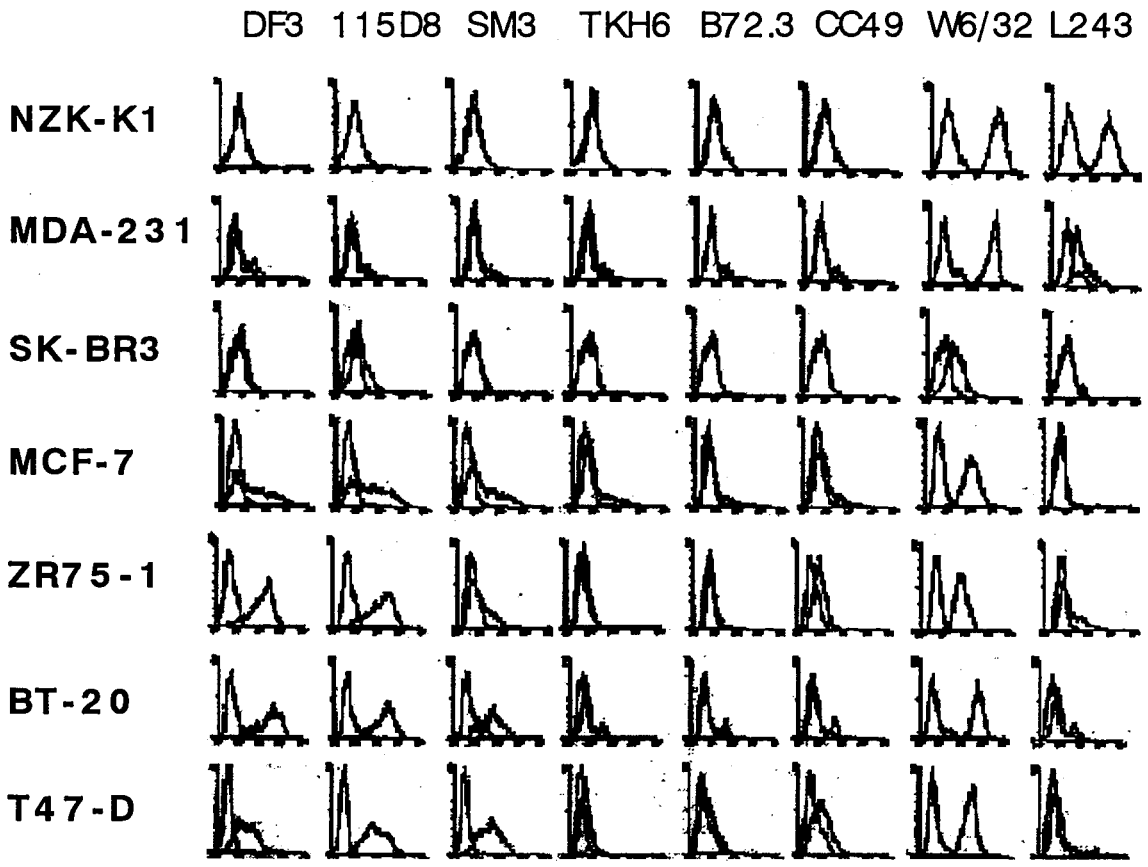


Figure 1. Flow cytometric analysis of MUC-1 expressions in breast cancer cell lines. Monoclonal antibodies directed to the MUC-1 core, the associated carbohydrate or MHC molecules listed in table 1 were used for the assay. Seven breast cancer cell lines were stained with the antibodies at the appropriate concentration on ice for 30 min. After three washes in PBS, they were incubated with FITC-conjugated anti-mouse IgG antibody on ice for 30 min. Then, they were tested for their phenotypes by FACS.

Cytotoxic activity of PBMCs against allogeneic breast cancer cell lines. PBMCs obtained from normal donors were incubated with isotope-labeled breast cancer cell lines at effector/target ratio of 50:1. After 18 hour incubation, the supernatant was collected and measured for the release of 51Cr from the lyzed targets. Whereas the strong lytic activity against all the target cells expressing MUC-1 mucin was found, no activity was against a MUC-1 defficient cell, NZK-K1 (Fig. 2A). The percentage of tumor cell lysis observed did not correlate with the mucin expression level on the cells. To investigate whether NK cells in the PBMCs were involved in the cells lysis observed, cold K562 which is known to be sensitive to NK-mediated cytotoxicity was added to each well containing PBMCs and labeled targets. The cell lysis against ZR75-1 with high levels of the mucin expression was not inhibited by K562. And that against MCF-7 with the

lower expression was inhibited /\to a third of the activity without adding K562 (Fig. 2B). The same results were obtained against T47D and SK-BR3 (data not shown). Whereas added K562 did not affect the cell lysis against T47D, it did that against SK-BR3. Since the effector cells showed approximately 20 % lytic activity against K562, NK cells were suggested to be present in the PBMC prepared (Fig. 2B).

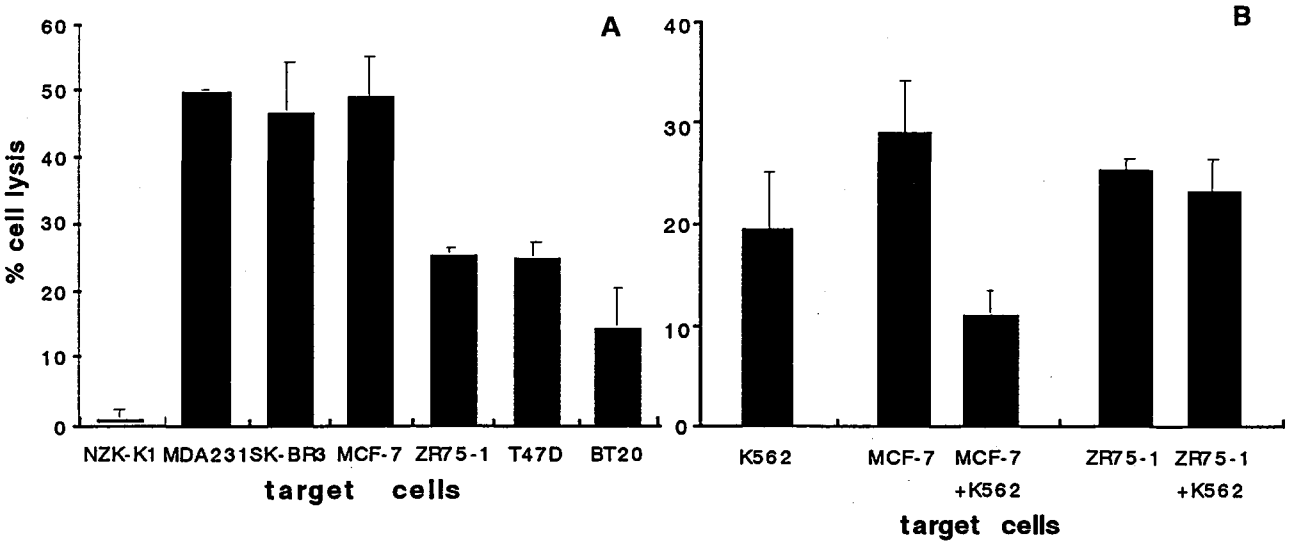
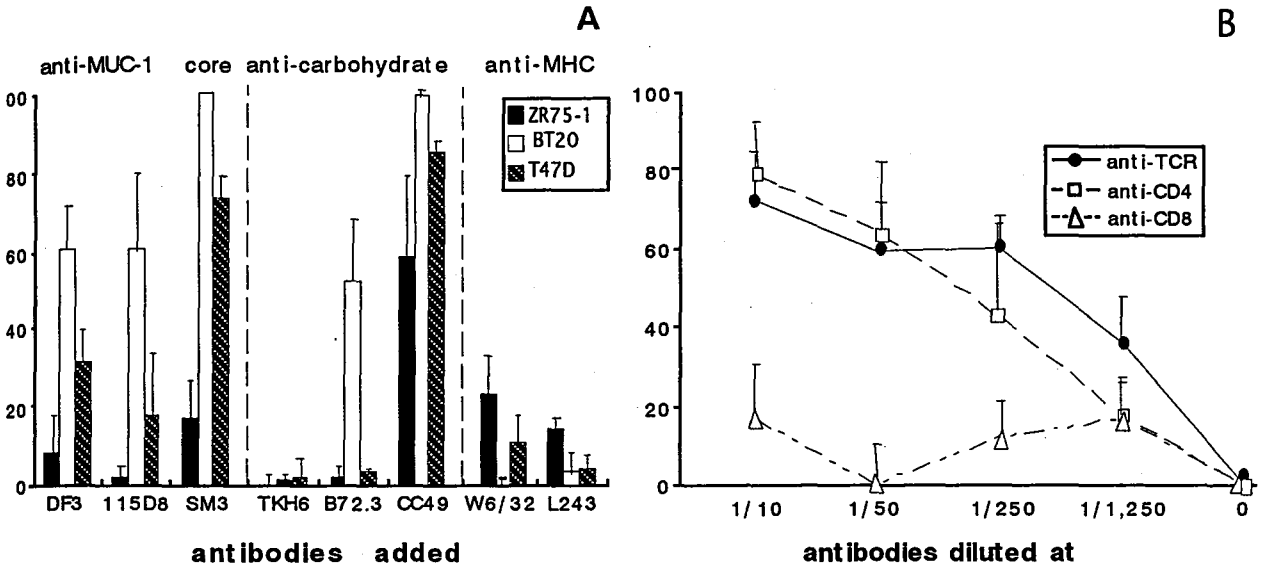


Figure 2. Cytotoxic activity of PBMCs against allogeneic breast cancer cell lines. 1 x 104 breast cancer cells labeled with 51Cr were incubated with 5 x 105 PBMCs obtained from normal donors in each well for 18 hours. The supernatant was collected from the well and counted for the release of 51Cr from the lyzed target cells. (A). 2 x 105 non-labeled K562 were added to a well containing PBMCs and labeled MCF-7 or T47D, to abrogate NK-mediated cytotoxicity. The percentage cell lysis was calculated as described above (B). They were performed in triplicate.

Inhibitory effect of anti-MUC-1 antibodies on the cytotoxicity against MUC-1 expressing target cells. The three cell lines with high levels of MUC-1 expression, ZR75-1, BT-20 and T47D were pre-treated with anti-MUC-1, or anti-mucin-associated carbohydrate antibodies followed by co-incubation with PBMCs. Of the three cells, the cytotoxicity against BT-20 and T47D, both of which showed the strong reactivity to SM3, was inhibited by this antibody (Fig. 3A). In contrast, that against ZR75-1 with a scarcity of the reactivity was not inhibited by it. The cytotoxicity against all of them was vigorously inhibited by CC49, and not by antibodies to MHC molecules (Fig. 3A). Against the targets with lower levels of MUC-1 expression, MCF-7, SK-MR3 or MBA-MD231, the cell lysis was partially inhibited by antibodies to the tandem repeat and the carbohydrate (data not

ıown). To study what population of effector cells was responsible for the UC-1 specific cytotoxicity obtained, PMBCs were treated with anti-TCRab, D4 or CD8 monoclonal antibody in prior to co-incubation with radio-labeled rgets in the cytotoxicity assay. Anti-TCRab and CD4 antibodies were ıown to inhibit the cell lysis against ZR75-1 by PBMCs (Fig. 3B). Anti-CD8 ıtibody did not affect the cytotoxicity. The lytic activity against T47D was hibited by the treatment of anti-TCRab and CD4 antibodies as well (data ɔt shown).



igure 3. Inhibitory effect of anti-MUC-1 monoclonal antibodies on the ytotoxicity against breast cancer cell lines with high levels of MUC-1 xpression. ZR75-1, BT-20 and T47D, all of which showed high expressions f the mucin, were pretreated with anti-MUC-1 antibodies or anti-MHC ntibodies at 37°C for 45 min in prior to co-incubation with PBMCs.

Immunohistochemical studies of breast tumors from the patients.

wenty-six female patients ranging in age from 32 to 77 and in clinical tage from 1 to 3 were studied. Clinical features of the patients were ummarized in Table 2. Paraffin-embedded or frozen sections of the tumor ssues were stained with anti-MUC-1 antibodies, using an indirect mmunoperoxidase technique. The cancer cells were stained intensely and iffusely in the cytoplasm in the tumor tissues in some cases (data not hown). Cancer cells forming glandular structures were stained in their pical borders in some cases (data not shown). The percentage of positive ells were calculated by the number of positively stained cells out of five undred cancer cells counted. Mean percentages of positive cells for each ntibody-reactivity were shown in Fig. 4. The expression of the tandem peat was particularly higher than that of the carbohydrate in the cancer ells (Fig. 4). Ninety-two percent of breast cancer patients studied were

positive for the mucin expression in the tumors (data not shown).

Table2 Clinical features of breast cancer patients studied

Patient no.	Age	Clinical stage	Histological type	
1	45	t2n1m0	SC	§
2	61	t2n0m0	PT	#
3	39	t1n0m0	PT	
4	64	t2n0m0	SC	
5	50	t2n1m0	SC	
6	77	t2n0m0	SC	
7	68	t3n0m0	SC	
8	61	t3n0m0	SC	
9	67	t3n1m0	Med.	
10	47	t2n0m0	PT	
11	59	t2n1m0	Med.	*
12	53	t2n0m0	ST	
13	42	t4n0m0	SC	
14	54	t2n0m0	ST	
15	45	t2n0m0	SC	$
16	45	t2n0m0	SC	
17	71	t2n0m0	PT	
18	35	t2n0m0	SC	
19	60	t2n1m0	SC	
20	67	t3n0m0	SC	
21	46	t2n0m0	SC	
22	44	t1n0m0	Med.	
23	38	t3n2m0	Med.	
24	47	t2n0m0	PT	
25	32	t2n0m0	ST	
26	56	t2n0m0	Comedo	

§ Scirrhous type

Papillo-tubular type

* Medullary type

$ Solid tubular type

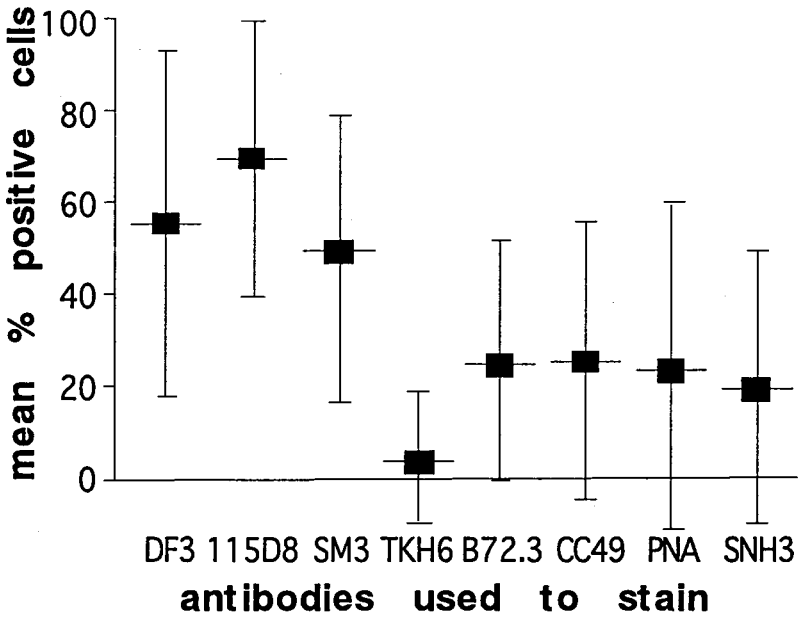


Figure 4. Mean percentages of cancer cells positively stained with anti-MUC-1 antibodies in the breast tumors. The percentage of cancer cells positive for the antibody were calculated by the number of positive cells out of 500 cancer cells counted. Mean percentages of positive cancer cells for each antibody were shown.

Cytotoxic activity of PBMCs from patients with breast cancer against autologous cancer cells. Freshly isolated PBMCs from the patients were co-incubated with 51Cr-labeled autologous cancer cells for 18 hours. The percentage of tumor cell lysis in each case was plotted (Fig. 5). The percentage of more than 6.3% was statistically significant ($p<0.05$). Seven (27%) out of twenty-six cases showed positive for the cytotoxic activity.

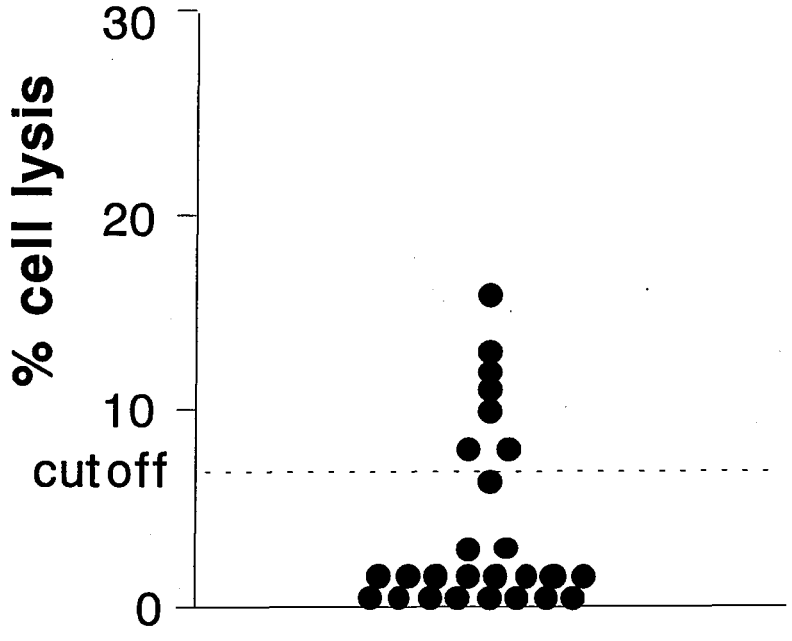


Figure 5. Cytotoxic activity of PBMCs from patients with breast cancer against autologous cancer cells. Breast cancer cells prepared as described in 'Material and Methods' from the patients were incubated with freshly isolated PBMCs following radio-labeling for 18 hours. The percentage of tumor cell lysis in each case was plotted. 6.3 % lytic activity was statistically significant ($p < 0.05$).

Correlation between MUC-1 expression in cancer cells and their susceptibility to tumor cell lysis by PBMCs. The patients studied were divided into two groups, the positive and negative cases for the anti-tumor cytotoxicity. The reactivity of tumor cells to each anti-MUC-1 antibody was compared between the two groups, by the immunohistochemical study (Fig.6). The percentage of cancer cells positive for DF3, 115D8 and CC49 was significantly higher in the positive cases comparable with that in the negative cases. All of the 7 positive cases showed a strong MUC-1 expression in their tumors (data not shown). Of the 19 negative cases, however, only 2 cases showed lack of the expression, and 2 were the low expression cases (% positive cells < 40 %). Although the remaining 15 negative cases showed a strong expression of MUC-1 tandem repeat, 7 of them had lack or a scarcity of CC49-reactivity in their tumors (data not shown).

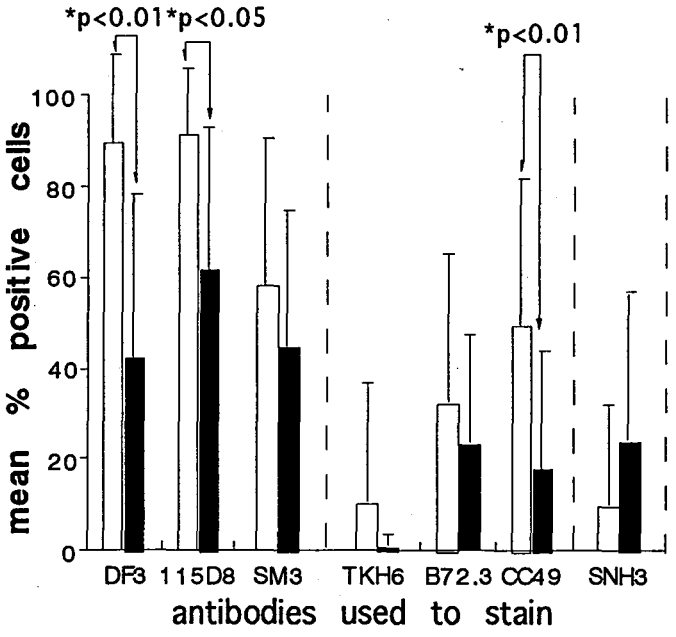


Figure 6. Relationship between MUC-1 expression in cancer cells and its ability to elicit anti-tumor immunity. The patients studied were divided into two groups, positive and negative cases, according to the result of the autologous cytotoxicity assay performed. Mean percentages of cancer cells positive for the reactivity to each anti-MUC-1 antibody was compared

between the two groups.

MUC-1 Expression and Antigenicity of A Transduced Breast Cancer Cell Line. A hundred and nineteen MUC-1 cDNA transduced clones were obtained, and they were screened for their MUC-1 expression by FACS. As the result, six clones showing the strong expression were obtained. By FACS, one of the six clones showed obvious levels of MUC-1 core expression (Fig. 7). Treatment of this transfectant with sialydase increased the all levels of the core expression and of CC49-reactivity on it (Fig. 7).

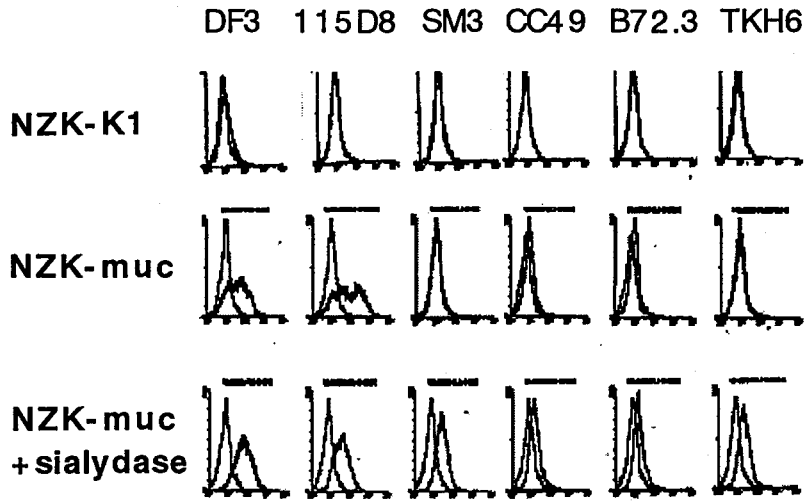


Figure 7. Phenotypic analysis of MUC-1 transfectants by FACS. A MUC-1 defficient parent cell line, NZK-K1, a MUC-1 transfectant, NZK-muc and a sialydase-treated NZK-muc were stained with anti-MUC-1 antibodies. Phenotypes of these cell lines were studied as described in 'Materials and Methods'.

To test the antigenicity of the MUC-1 transfectant, radio-labeled NZK-K1, NZK-muc or sialydase-trreated NZK-muc were co-incubated with autologous PBMCs at E/T ratio of 50 for 18 hours. After the incubation, the supernatant was colected and measured for the amount of isotopes released from the lyzed targets. Although the parent cells were scarcely killed by the PBMCs, the transfectants and T47D were readily lyzed (Fig. 8).

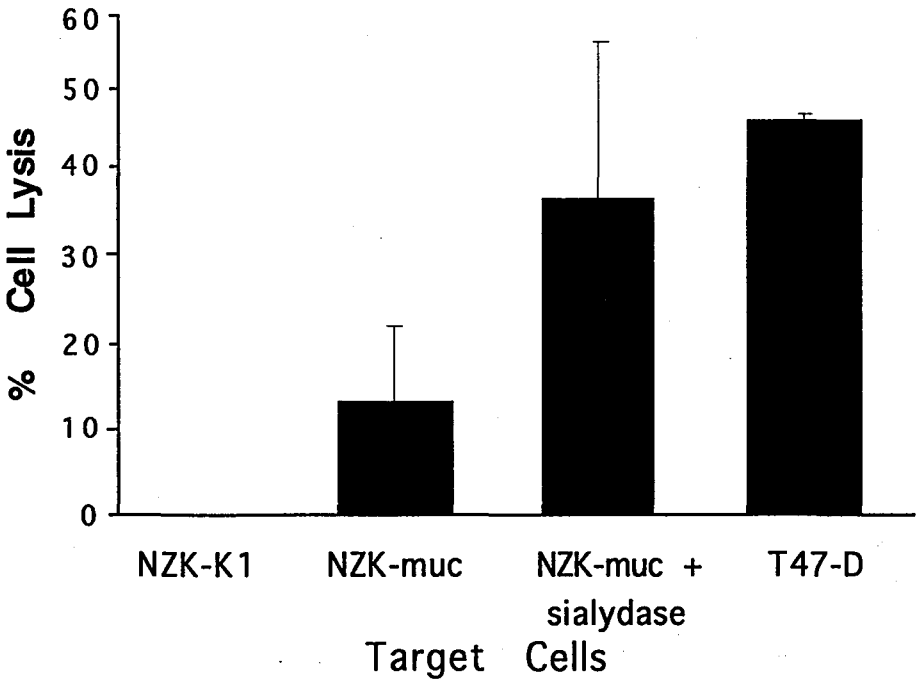


Figure 8. Analysis of the antigenicity of MUC-1 transfectants. Radio-labeled targets, NZK-K1, NZK-muc and sialydase-treated NZK-muc were incubated with autologous PBMCs for 18 hours. The amount of isotope released from lyzed targets were calculated as described in 'Materials and Methods'.

DISCUSSION

In this study, breast cancer cell lines with MUC-1 mucin expression on the surface were demonstrated to be readily killed by PBMCs from normal donors in the allogeneic cytotoxicity assay (Fig. 2A). On the other hand, NZK-K1 lacking the expression was not lyzed. It suggested that MUC-1 mucin expressed on the cancer cells were specifically recognized and lyzed by PBMCs from normal donors. To comfirm that it was a specific anti-MUC-1 immune response, inhibitory effects of anti-MUC-1 antibodies on the cytotoxicity against /\the cancer cells was tested. The cytotoxicity observed against three targets with the high expression, T47D, ZR75-1 and BT-20 was vigorously inhibited by anti-MUC-1 antibodies, whereas the inhibitory effect against targets with lower expressions was partial (Fig. 3A). In particular, against BT-20 and T47D, both of which had SM3-reactive determinants, PDTRP within the tandem repeat domain, the cytotoxicity was inhibited by this antibody. However, that against ZR75-1 with a scarcity of the SM3 reactivity was not inhibited by it. Against the all three targets, it was greatly inhibited by CC49 reactive with mucin core 2 trisaccharide structures, Galb1,3(NeuAca2,6)GalNAc-o-Ser/Thr. These data suggested that not only the tandem repeat domain containing the antigenic epitope as previously reported (11) but also three dimensional structures including the

carbohydrate side chains may determine the susceptibility of the cancer cells to MUC-1 specific anti-tumor immunity.

The cytotoxicity observed agaist ZR75-1 and T47D was inhibited by antibodies to TCRab and CD4 (Fig. 3B) and not inhibited by adding cold K562 (Fig. 2B). It was suggested that effector cells responsible for the tumor cell lysis were TCRab/CD4 positive T lymphocytes, and that these cells specifically recognized and lyzed MUC-1 expressing cells without a MHC restriction. The cell population characterized as large granular lymphocytes which can kill K562, like NK cells or NKT cells, was not involved in the cytotoxicity against these cells with the high expression. It was unlikely that some populations of T lymphocytes in freshly isolated PBMCs from normal donors could recognize /\the tumor antigen without prior priming in vitro or in vivo. It might be possible, however, that naive T lymphocytes already encountered the mucin, which was shed from glandular epithelial cells, or which was processed and presented by phagocytes following apoptotic death of the epithelial cells in normal individuals. In fact, there has been several reports that less than 10 % of normal individuals as well as 10 % to 60 % of cancer patients showed the existance of soluble MUC-1 mucin in their sera (25-28). It indicates that some populations of T lymphocytes have already been primed with MUC-1 mucin and can be re-activated immediately in response to cancer cells expressing the mucin. Also, there has been a reported data of approximately 15 % of cancer patients with antisera to MUC-1 mucin (29). It is possible that normal individuals as well as cancer patients may have some already-primed T lymphocytes specific for MUC-1 mucin in their PBMCs. Other populations of effector cells responsible for the cell lysis was NK cells, because the cytotoxicity observed against MCF-7 expressing lower level of the mucin was inhibited by adding cold K562 in the cold target inhibition assay (Fig. 2B). It was an unexpected result that the recognition of target cells with the low expression by the effector cells, one population of which was a NK cell, was blocked by anti-MUC-1 antibodies (data not shown). Recently, Adachi et al. (30) reported that the increase of MUC-1 core expressions by blocking GalNAc transferase activity resulted in inducing MUC-1-specific cytotoxicity mediate by NK and LAK cells. Taken together, it was indicated that both of the two populations, CD3/CD4 positive T lymphocytes and NK cells were involved in the specific recognition and the cell lysis of cancer cells expressing MUC-1 mucin.

We underwent analysis of the antigenicity of MUC-1 mucin expressed on breast cancer cells obtained from patients with breast cancer. In the autologous cytotoxicity assay, an apparent cytotoxic activity of PBMCs from

the patients was observed against autologous cancer cells expressing high levels of MUC-1 tandem repeats, detected with either DF3 or 115D8, or of CC49-reactive structures. The result obtained was compatible with that in the allogeneic cytotoxicity assay, in terms that the ability to elicit a strong anti-tumor immunity was affected by not only tandem repeat expression but also carbohydrate side chains bound on the core protein. Taken together, we comfirmed that the expression of the tandem repeat as well as core 2 trisaccharide strucures were impotant in the autologous response inducing a strong anti-tumor immunity. Considering the unique structure and characteristics described above, we are thinking of some possibilites to explain the results obtained. Immune effector cells involved in the tumor cell lysis may recognize three dimensional structures formed by the core peptides and the carbohydrates, reffered to as a molecular mimicry, in stead of antigenic peptides in the context of MHC molecules. Or the carbohydrate structures detected with CC49 bound on the core protein may cause the conformational change of the whole mucin, leading to the increase of exposure of the epitope in the tandem repeat.

Jerome et al. reported that MUC-1 expressing target cells were lyzed by MUC-1 specific CTL established after stimulation of PBLs with EBV-transformed B cells transfected with MUC-1 cDNA, whereas not killed were MUC-1 defficient cells (31). In addition, the mucin expressing target cells which were pretreated with glycosylation inhibitors to increase the tandem repeat expression were reported to be lyzed more efficiently by the CTL. The tumor cell lysis shown could be blocked by adding anti-MUC-1 monoclonal antibodies to the tandem repeat such as SM3 and HMFG-1 and 2 (32-33). The data suggested that there might be antigenic epitopes recognized by CTL, PDTRP in the repeat domain was the most likely, on the MUC-1 core and that they could induce a cell-madiated immune response to tumor cells expressing the mucin. The epitope responsible for the induction was reported to be ordinarily masked on normal mammary epithelial cells or some breast cancer cells, with sialic acids or fucoses which terminates carbohydrate side chains (29). All of the cancer patients with lytic activity in the autologous cytotoxicity assay demonstrated a strong MUC-1 expression in the cancer cells (data not shown). On the other hand, of 19 patients without the activity, only 2 were negative for MUC-1 expression in their tumors and 2 showed a scarcity of the expression (% positive cancer cells < 40%). Although the remaining 15 patients showed strong MUC-1 core expressions in the tumors, 7 of them were negative for CC49-reactivity (data not shown). These data suggested that at least a strong expression of MUC-1 mucin on cancer cells was required for the specific tumor cell lysis

by PBMCs, but it was not sufficient. Modulation of carbohydrate structures as well as loss of expression of the molecule could be an escape mechanism of cancer cells from being recognized by immune effector cells. The unresponsiveness of PBMCs to the autologous cancer cells observed was not caused by dysfunction of the effector cells in the patients, because the cytotoxic activity of PBMCs from a cancer patient showing no autologous tumor cell lysis was observed against the autologous MUC-1 transfectant in the cytotoxicity assay (Fig.8). Treatment of the transfectant with sialydase to modify the carbohydrate structure of the mucin led to the restoration of anti-tumor immunity by the PBMCs.

MUC-1 mucin has been thought to be important not only as tumor markers in diagnosis and in evaluation of the effect of anti-cancer treatment to carry out but tumor antigens that can be pilloried for immunotherapy against cancer. We had a supportive data that 92% of patients with breast cancer studied were shown to be positive for MUC-1 mucin in their tumors by an immunohistochemical study (data not shown). Since the mucin can elicit an anti-tumor immunity in MHC unrestricted manner, it is easy to obtain or activate tumor-specific T lymphocytes or NK cells from the patients' PBMCs, using allogeneic cancer cell lines expressing a favorable MUC-1 mucin ex vivo as stimulators, for the purpose of adoptive immunotherapy. Or transfection of glucosyltransferase cDNA or its antisense nucleotide as well as MUC-1 mucin cDNA into cancer cells to modify the antigenicity in vivo can be employed for the treatment, so that the transduced cells can be efficiently recognized by MUC-1 specific CTL. Taking into account of the escape mechanism of the cancer cells observed, however, it should be concluded that targetting the natural MUC-1 mucin expressed on cancer cells as a tumor antigen is not expected to obtain successful effects for anti-cancer immunotherapy. Further investigation is necessary to generate or increase the antigenicity of this molecule.

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PREFACE

CONTRIBUTORS

Head Investigator

Shuhei Inoue, Associated Professor, Second Department of Surgery, Shiga University of Medical Science

Investigators

Yoshio Okada, Principal, Shiga University of Medical Science

Hirofumi Kato, Associated Professor, Second Department of Surgery, Shiga University of Medical Science

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