Immunohistological and Biochemical Detection of Fucosylated Polylactosamine Antigen with Monoclonal Antibody ACFH18 in Gastric Cancer and Normal Gastric Mucosa

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A mouse monoclonal antibody ACFH18, produced by immunizing with human gastric cell line MKN-74, recognizes a novel glycolipid antigen, fucosyl-lactoneodecaosylceramide. In the present study, we investigated the immunohistological localization of this antigen and its biochemical detection in glycolipid fraction in normal and malignant human gastric tissue, compared with other tumor-associated type-2 chain glycolipid antigens such as sialyl Le^x, sialyl Le^x-i and Le^y. Reactivity with ACFH18 was detected immunohistologically in the proliferating zone of normal fundic gland region as well as in 38 of 54 cases of gastric cancer, with preferential binding to undifferentiated type cancer. Glycolipids reacting with ACFH18, especially slow-migrating glycolipids on thin-layer chromatography, were accumulated in all of four cancer specimens compared with normal mucosa obtained from the same patients. Many glycolipids with sialyl Le^x or sialyl Le^x-i epitope were detected in normal mucosa and almost all of these glycolipids were accumulated in cancer specimens. Sialyl Le^x-i antigen was increased more specifically in cancer than sialyl Le^x was. A Le^y antigenic glycolipid was considerably decreased in three of four cancer cases and increased in one case. From these results, glycolipid detected by ACFH18 appeared to be a tumor-associated antigen comparable to sialyl Le^x and sialyl Le^x-i antigens, but with a unique character as an undifferentiated-type tumor-associated antigen.

Key words: Human gastric cancer — Monoclonal antibodies — Glycolipid

So far, several monoclonal antibodies against cancer-associated carbohydrate antigens have been applied for clinical use. In gastrointestinal carcinoma, type-2 chain carbohydrate antigens such as Le^x, sialyl Le^x, and Le^y have been regarded as tumor-associated antigens. It is accepted that the presence of these antigens in cancer tissue is an anachronic expression of development-associated antigens.^{1,2)}

Several monoclonal antibodies have been raised against sialyl Le^x or sialyl Le^x-i antigens.³⁻⁵⁾ Using these antibodies, sera from patients with adenocarcinoma or cancer tissue have been reported to show specific binding.^{4,6,7)} Le^y antigen^{8,9)} was found preferentially in colorectal malignant and premalignant lesions.¹⁰⁾ As for gastric tissue, it has been reported that sialyl Le^x-i and Le^y antigens were expressed in 70-80% of gastric cancer immunohistologically.¹¹⁾ In normal gastric mucosa, Le^y antigen was strongly expressed in all layers of gastric mucosa, while sialyl Le^x antigen was not found in the fundic gland region but was found sporadically in the pyloric gland region.^{11,12)}

A mouse monoclonal antibody, ACFH18, was produced by immunizing with human gastric cancer cell line MKN74. The glycolipid reacting with this monoclonal antibody was firstly isolated from human colonic cancer, and the minimum requirement for the maximal reactivity of ACFH18 was identified as fucosyllactoneodecaosylceramide (VII³Fuc-nLc₁₀), which possesses an internal repeating N-acetyllactosamine with a fucosyl residue with or without terminal sialic acid.¹³⁾ In this study, we intended to characterize the glycolipids reacting with ACFH18 as tumor-associated antigens, especially in gastric cancer. Firstly, the immunohistological localization of the antigen was studied on a number of gastric tissue specimens, revealing the antigen expression in a high percentage of undifferentiated type gastric cancer. Secondly the features of the glycolipids antigen were assessed using glycolipid extracted from both malignant and non-malignant gastric mucosa of each patient simultaneously. The alterations of sialyl Le^x, sialyl Le^x-i, and Le^y concomitantly with that of VII³Fuc-nLc₁₀ related antigens in glycolipid fraction were studied and compared. In this study on the glycolipid fraction the difference of cancer tissue and normal tissue was demonstrated very clearly. This is the first study in which the alteration of

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glycolipids carrying oncofetal antigen has been biochemically evaluated in gastric cancer in comparison with the normal tissue.

MATERIALS AND METHODS

Tissue The tissues used in this study were surgical specimens from patients treated at Ohno Hospital (Osaka). Glycolipid fractions prepared from four cases of advanced-stage gastric carcinoma, one case of colonic carcinoma and eight cases of peptic ulcer were tested. All four gastric cancer cases were classified as Borrmann type III and undifferentiated type adenocarcinoma. The cancer tissue was macroscopically separated from the intact mucosa. In ulcer cases, gastric mucosa free from lesion was used. For the immunohistological study, formalin-fixed paraffin sections fifty-four cases of gastric cancer and five cases of peptic ulcer were used. Twelve biopsy samples from various locations in the colon as described previously¹⁰⁾ and intact duodenal mucosa from three cases of ulcer were also examined.

Monoclonal antibodies All monoclonal antibodies were derived from mouse hybridomas and IgM type (Table I). Monoclonal antibodies AH6 directed to Le^y,⁹⁾ and ACFH18 directed to VII³Fuc-nLc₁₀,¹³⁾ were obtained as hybridoma supernatant. Hybridoma supernatant of FH6 directed to sialyl Le^x-i³⁾ was the gift of Dr. Y. Fukushi (Tohoku University, School of Medicine). Purified monoclonal antibody KM93 directed to sialyl Le^x⁵⁾ was provided by Kyowa Hakko Tokyo Research Laboratories. The epitope specificity of this antibody will be described elsewhere.

Preparation of glycolipid fraction The upper neutral glycolipid fraction and upper acidic glycolipid fraction were prepared after extraction with 20 volumes of chloroform/methanol 2:1 (v/v) and 5 volumes of isopropyl alcohol/hexane/water 55:25:20 (v/v/v), followed by Folch's partition and DEAE-Sephadex chromatography, as described previously.¹⁴⁾

Thin layer chromatography (TLC) and immunostaining Detection of glycolipid antigens on TLC plates was performed by a modified version of the procedure described by Magnani *et al.*¹⁵⁾ Glycolipid fractions were applied to high-performance TLC (HPTLC) plates (J. T. Baker Chemical Co., Phillipsburg, NJ) for chromatography using an indicated solvent system. After incubation of the plates with monoclonal antibodies, bound antibodies were detected using a Vectastain ABC Kit (Vector Laboratories Inc., Burlingame, CA) according to the vendor's protocol. As a substrate of peroxidase, 4-chloro-1-naphthol was used.

Immunohistological analysis with monoclonal antibody ACFH18 Formalin-fixed paraffin sections were tested with monoclonal antibody ACFH18 and a Vectastain ABC Kit (Vector), as described before.¹¹⁾ Diaminobenzidine tetrahydrochloride was used as a substrate of peroxidase. Sections were counterstained with Meyer's hematoxylin.

RESULTS

The immunohistological detection of VII³Fuc-nLc₁₀-related antigens is shown in Fig. 1. As shown in panel A, in the fundic gland region of all non-cancerous specimens

Table I. Monoclonal Antibodies Used in This Study

Monoclonal antibody	Directed to	Antigenic structure
KM93 (IgM)	Sialyl Le ^x	NeuAcα2→3Galβ1→4GlcNAc→R ^{a)}
		$\begin{array}{c} \uparrow 3 \\ \text{Fuca1} \end{array}$
FH6 (IgM)	Sialyl Le ^x -i	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc→R
		$\begin{array}{c} \uparrow 3 \\ \text{Fuca1} \end{array} \quad \begin{array}{c} \uparrow 3 \\ \text{Fuca1} \end{array}$
AH6 (IgM)	Le ^y	Galβ1→4GlcNAc→R
		$\begin{array}{cc} \uparrow 2 & \uparrow 3 \\ \text{Fuca1} & \text{Fuca1} \end{array}$
ACFH18 (IgM)	VII ³ Fuc-nLc ₁₀	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3
		$\begin{array}{c} \uparrow 3 \\ \text{Fuca1} \end{array}$
		Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer

a) R, core glycolipid.

Fig. 1. Examples of the immunohistological staining of normal gastrointestinal mucosa and gastric cancer with monoclonal antibody ACFH18. Panel A, normal fundic mucosa obtained from a case with peptic ulcer ($\times 120$); panel B, a section from the same specimen as panel A immunostained with FH6 showing negative staining ($\times 120$); panel C, undifferentiated type gastric cancer immunostained with ACFH18 ($\times 150$); panel D, differentiated type gastric cancer immunostained with ACFH18 ($\times 298$); panel E, biopsy sample from intact cecum immunostained with ACFH18 ($\times 414$); panel F, biopsy sample from intact rectum immunostained with ACFH18 ($\times 118$).

tested, the cells lining the proliferating zone reacted strongly with ACFH18, and some cells in deeper glands also reacted. It was not expressed in the pyloric gland region (data not shown). The reactivity of cancer tissue is shown in Fig. 1C and 1D. As shown in Table II, ACFH18 reacted with 70% of cancer tissue and had preferential reactivity with undifferentiated-type adenocarcinoma. The reactivity of early-stage cancer did not differ from that of advanced stage cancer. VII³Fuc-nLc₁₀-related antigen was also found in some cells of the intact duodenal epithelium, and throughout the terminal ileum to sigmoidal colon in a similar manner to that shown in Fig. 1E. In rectal mucosa, the staining was limited to the crypt cells and was much stronger than in other parts of the intestine (Fig. 1F).

The presence of sialyl Le^x and sialyl Le^x-i antigens in acidic glycolipid fraction of cancer and normal mucosa is shown in Fig. 2. The immunostaining with monoclonal antibody KM93, directed to sialyl Le^x, showed many positive bands in both normal and cancer tissues. In cases 2, 3 and 4, total KM93-reacting glycolipids were increased in cancer tissue, with some bands being more strongly stained. With FH6, directed to sialyl Le^x-i, normal mucosa showed only faint staining, while the cancer tissue showed evident staining of several bands in all cases. The bands detected with FH6 in cancer tissue seemed to be identical to those strongly stained with KM93. On the other hand, as shown in Fig. 3, concentrated acidic glycolipid fractions extracted from normal gastric mucosa of peptic ulcer cases showed similar reactivity with KM93 and FH6 to those from cancer specimens.

The presence of Le^y antigen in upper neutral glycolipid fractions is shown in Fig. 4A. In cancer cases 1, 2 and 4 decrease of staining in cancer tissue was observed, while in case 3 increased staining in cancer tissue was seen, at the positions indicated with large arrowheads. In case 5, which is a case of colonic cancer, a band which could not be seen in normal tissue appeared in cancer tissue.

Reactivity of the acidic glycolipid fraction with ACFH18 was examined, as shown in Fig. 4B. Glycolipids reacting with ACFH18 were detected in the mucosa from peptic ulcer cases (lanes 6–13), but were evidently increased in gastric cancer (lanes 1c–4c), showing especially strong reactivity of the glycolipids migrating very close to the origin on TLC, as indicated with small arrowheads, compared with the staining pattern of the ulcer cases. Staining of colonic cancer was not different from that of normal mucosa (lanes 5). The reactivity of the upper neutral fraction with ACFH18 on the TLC plate was very weak (data not shown).

Table II. Reactivity of Monoclonal Antibody ACFH18 with Gastric Carcinoma Determined by Immunohistochemical Analysis

Histological type ^{a)}	Number of positive cases ^{b)} /Total number of cases (%)			
	Early stage ^{c)}	Advanced stage	Total	
Differentiated type	4/7 (57.1)	11/20 (55.0)	15/27 (55.6)	] $P < 0.025^d)$
Undifferentiated type	7/8 (87.5)	16/19 (84.2)	23/27 (85.2)	
Total	11/15 (73.3)	27/39 (69.2)	38/54 (70.4)	

a) Cases were classified histologically according to Nakamura *et al.*¹⁷⁾

b) Each case was determined as positive when more than 10% of the cancer cells in the section exhibited positive staining.

c) Invasion of cancer is limited to within the mucosa or submucosa.

d) Significance was evaluated using chi-square analysis.

Fig. 2. Sialyl Le^x and sialyl Le^x-i antigens detected in normal gastric mucosa and cancer from gastric cancer cases by TLC immunostaining with monoclonal antibodies KM93 (anti-sialyl Le^x, panel A) and FH6 (anti-sialyl Le^x-i, panel B). Acidic glycolipid fraction from 100 mg of wet tissue was spotted on TLC plates for chromatography using a solvent system of chloroform/methanol/water 50:40:10 (v/v/v) containing 0.05% CaCl₂. The lane number indicates each of four cases with gastric cancer. Lanes n, normal gastric mucosa; lanes c, cancer tissue. The immunostaining procedure is described in "Materials and Methods."

Fig. 3. Sialyl Le^x and sialyl Le^x-i antigens detected in normal gastric mucosa from ulcer cases by TLC immunostaining with monoclonal antibodies KM93 (anti-sialyl Le^x, panel A) and FH6 (anti-sialyl Le^x-i, panel B). Acidic glycolipid fraction from 300 mg (panel A) and 400 mg (panel B) of wet tissue was spotted on TLC plates. Conditions of TLC and immunostaining were the same as in Fig. 2. The lane numbers indicate the eight cases with peptic ulcer. The immunostaining procedure is described in "Materials and Methods."

DISCUSSION

In the immunohistochemical study, VII³Fuc-nLc₁₀-related antigens reacting with ACFH18 were present in the proliferating zone in normal mucosa, and were also detected in 70% of cancer specimens with preferential reactivity in undifferentiated-type carcinoma. Glycolipids reacting with ACFH18 were detected in normal mucosa by TLC immunostaining and the accumulation of very slowly migrating glycolipid in cancer tissue was shown. These changes in cancer detected with ACFH18 were

compared to those of already established tumor-associated antigens as follows.

Monoclonal antibody KM93, which recognizes sialyl Le^x antigen, binds the terminal four sugars common to sialyl Le^x-i antigen recognized by monoclonal antibody FH6. With monoclonal antibodies KM93 and FH6 it was shown that most of the sialyl Le^x antigenic glycolipids were increased in cancer tissue, and among them, sialyl Le^x-i antigenic glycolipids showed evident accumulation. The accumulation of Le^y antigen in colonic cancer observed in lane 5 of Fig. 4 confirmed the finding obtained before by immunohistochemistry that expression of Le^y

Fig. 4. Le^y antigen and $\text{VII}^3\text{Fuc-nLc}_{10}$ -related antigen detected in cancer and normal gastric mucosa from cancer and ulcer cases by TLC immunostaining with monoclonal antibodies AH6 (anti- Le^y , panel A) and ACFH18 (anti- $\text{VII}^3\text{Fuc-nLc}_{10}$, panel B). The arrows indicate the origin of chromatography. Panel A, neutral glycolipid fraction from 80 mg of wet tissue was spotted on a TLC plate for chromatography using a solvent system of chloroform/methanol/water 55:40:5 (v/v/v). Panel B, acidic glycolipid fraction from 200 mg of wet tissue was spotted on a TLC plate for chromatography using a solvent system of chloroform/methanol/water 50:47:13 (v/v/v) containing 0.05% CaCl_2 . The lane numbers correspond to the cases in Figs. 2 and 3. Lane 5 is the sample from a case of colonic cancer. Lanes n, normal mucosa; lanes c, cancer tissue. The immunostaining procedure is described in "Materials and Methods."

antigen was associated with malignant change of colorectal mucosa.¹⁰⁾ In gastric cancer, it was notable that an AH6-positive band was almost completely lost in three of four cases, while all cases with ulcer had Le^y glycolipids. It was proved that Le^y antigen was commonly present in normal gastric mucosa, and the decrease of Le^y could represent one of the malignant changes in gastric mucosa. Thus, the increase of sialyl Le^x and sialyl $\text{Le}^x\text{-i}$ antigens and the decrease of Le^y antigen were considered to be general alterations of glycolipids in gastric cancer.

From the immunohistological study, no difference of the cancer-associated change of Le^y antigen from that of $\text{VII}^3\text{Fuc-nLc}_{10}$ related antigens was apparent; both of them were widely expressed in normal mucosa as well as in 70–80% of the cancer specimens. However, the biochemical study on glycolipid performed here revealed that their alterations in cancer were different. The distinct increase of the $\text{VII}^3\text{Fuc-nLc}_{10}$ -related glycolipids in cancer was comparable to that observed with sialyl Le^x and sialyl $\text{Le}^x\text{-i}$ antigenic glycolipids, but not with Le^y antigenic glycolipids. Although the immunohistochemical study had the advantage that reactivity could be compared with the pathological characteristics of the cancer in detail, the results may be ambiguous because

expression of antigens in one slice of a cancer mass was never homogenous, as reported recently in a substantial immunohistological study.¹⁶⁾ In the present study, the biochemical detection of antigenic glycolipids gave clear results which allowed us to evaluate the cancer-associated change.

Monoclonal antibody ACFH18 can detect glycolipids which accumulate in cancer tissue. Besides, it has the unique feature that it reacts preferentially with undifferentiated cancer, while FH6 (directed to sialyl $\text{Le}^x\text{-i}$) was reported to bind more frequently in differentiated-type cancer.¹¹⁾ Monoclonal antibody ACFH18 will be useful to detect cancer-associated antigens, especially in undifferentiated-type carcinoma.

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