Mosaicism in the Expression of Tumor-associated Carbohydrate Antigens in Human Colonic and Gastric Cancers

Hisao Nakasaki, Toshio Mitomi, Takashi Noto, Kyoji Ogoshi, Hitoshi Hanauye, Yutaka Tanaka, Hiroyasu Makuuchi, Henrik Clausen, and Sen-itiroh Hakomori


ABSTRACT

Serial sequential sections from a single tumor were examined by immunohistological staining with several monoclonal antibodies directed, respectively, to different tumor-associated carbohydrate epitopes. Staining patterns were compared with those of conventional staining with hematoxylin-eosin or periodate/Schiff's reagent. Each tumor showed different areas of staining with different antibodies, and the combined staining map shows a clear mosaicism of antigen expression within the same tumor. For example, some areas of a given tumor were stained by FH4 (defining dimeric Le<sup>a</sup>), while other complementary areas were strongly stained, in a mutually exclusive manner, by SH1 (defining Le<sup>a</sup>) and AH6 (defining Le<sup>e</sup>); FH6 (defining sialosyl dimeric Le<sup>a</sup>), or TKH2 (defining sialosyl-Tn). Some areas were stained by two or three of these antibodies. Comparisons of the mosaic-staining patterns with cytohistological properties of tumor cells within specific areas suggested that the pattern of antigen expression is correlated with degree of differentiation; e.g., poorly-differentiated cells with severe dysplasia did not express high levels of Le<sup>a</sup> or Le<sup>e</sup> but did express sialyl-Le<sup>a</sup> or dimeric Le<sup>a</sup>; on the other hand, moderately or well-differentiated tumor cells in some areas expressed high levels of Le<sup>a</sup> or Le<sup>e</sup> but lower levels of sialyl-Le<sup>a</sup>. Areas showing strong expression of sialyl-Tn in their secretions were consistently correlated with presence of well-differentiated tumor cells, whereas secretions from normal mucosa were consistently characterized by lack of sialyl-Tn expression. It is postulated that the original in situ tumors (which had homogeneous glycosylation patterns) evolved into several spatially discrete cell populations displaying different degrees of glycosylation, reflecting stages of tumor cell differentiation and progression.

INTRODUCTION

Increasing numbers of tumor-associated carbohydrate antigens defined by various monoclonal antibodies have been identified in human cancers (1). Among these, lactoseries type 1 or 2 chain antigens with fusocyl or sialosylfusocyl derivatives (2-8), precursors for ganglio-series structures such as GD<sub>3</sub>, GD<sub>2</sub> (9-12), extended globo-series structure (13, 14), and the core structure (precursor chain) of O-linked mucin-type glycoproteins representing Tn and sialosyl-Tn (15-18) are the most common, being highly expressed in a large variety of human cancers. However, the expression of these antigens has been studied separately in different tumors, and we have no systematics knowledge of their expression within a single tumor. We have therefore studied the distribution pattern of individual antigens within a single tumor using sequentially dissected histological sections. Each section was stained by defined reagents, i.e., hematoxylin-eosin, periodate/Schiff's reagent, and antibodies FH6, SH1, TKH2, and AH6. Sections from some tumors were stained with additional antibodies FH4 and KH1.

The overall pattern of staining with these different antibodies showed a clear mosaicism. Some populations showing a defined glycosylation pattern could be correlated with the stage of tumor cell differentiation.

MATERIALS AND METHODS

Monoclonal Antibodies. The following MoAbs<sup>4</sup> were established in this laboratory and utilized after purification of IgG3 antibodies on protein A column and of IgM antibodies by high-pressure liquid chromatography (19). Anti-Le<sup>e</sup> antibody SH1 (IgG3) was prepared after immunization of mice with Le<sup>e</sup> glycolipid (III<sup>i</sup>FucnLC<sub>2</sub>) coated on Salmonella minnesota<sup>e</sup>. MoAb FH4 was previously established as being directed to dimeric Le<sup>a</sup> (V'<FucIII'>FucnLC<sub>2</sub>) (2). MoAb FH6 is directed to sialosyl difucosyl type 2 chain (V'<NeuAcV'<FucIII'>FucnLC<sub>2</sub>) (3). MoAb AH6 was prepared against human gastric adenocarcinoma MKN74 and is directed to the Le<sup>e</sup> determinant (V'<FucIII'>FucnLC<sub>2</sub>) (20). MoAb KH1 is directed to trifucosyl Le<sup>e</sup> (V'<FucV'<FucIII'>FucnLC<sub>2</sub>) (5). MoAb TKH2 is directed to the sialosyl 2→6GalNAc residue (sialosyl-Tn) as described recently (17). The structures of the above antigens are shown in Table 1. The anti-CEA MoAb was purchased from Abbott Laboratories (Abbott Park, IL).

Immunohistological Staining. Tumors were obtained from surgical operations at the Department of Surgery, Tokai University School of Medicine, Kanagawa, Japan, and prepared as paraffin-embedded sections. Serial consecutive sections (4- to 5-μm thickness) were cut by microtome, placed on objective glass, deparaffinized in xylene for 5 min, dehydrated in graded ethanol, and washed with PBS at 4°C. Eight consecutive sections (in duplicate or triplicate) were stained with hematoxylin-eosin, periodate/Schiff's reagent, and MoAbs SH1, AH6, FH4, FH6, TKH2, and KH1. Some sections were stained with anti-CEA antibody, and occasionally the staining with AH6 and KH1 was omitted. For antibody staining, sections were incubated with primary antibody overnight (20-30 μg/ml) at 4°C in a moist chamber. After being washed 3 times with PBS, sections were incubated with biotinylated secondary antibody diluted 1:100 [biotinylated horse anti-mouse immunoglobulin (both IgG and IgM); Vector Laboratory, Inc., Burlingame, CA] for 1 h at room temperature in a moist chamber. The sections were then incubated for 1 h with avidin-peroxidase conjugate reagent (diluted 1:50; Vector Laboratory), rinsed 3 times with PBS and buffer containing 0.05% 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.03% hydrogen peroxide. Finally, sections were counterstained with methyl green, dehydrated in graded ethanol and xylene, and mounted.

RESULTS

Of 14 tumor specimens examined, all sections expressed more than two antigens and showed spatially discrete cell

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3 The term "mosaicism" is used here to indicate the presence of two or more spatially discrete populations of tumor cells differing in their glycosylation pattern or cytohistologic properties. In contrast, the term "heterogeneity" has a broader definition not requiring the presence of such spatially discrete cell populations.

4 The abbreviations used are: MoAb, monoclonal antibody; PBS, phosphate-buffered saline (140 mM NaCl, 15 mM sodium phosphate buffered as indicated); CEA, carcinoembryonic antigen. The structures termed Le<sup>a</sup> and Le<sup>e</sup> were defined as previously described (6), and the determinant termed sialosyl-Tn was defined as recently published (14).

5 A Singhala, S. Nance, and S. Hakomori, unpublished data.
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Table 1 Monoclonal antibodies used in this study and antigen structures defined by them

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen defined</th>
<th>Structure</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>SH1 (IgG&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>Le&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Galβ1→4GlcNAcβ1→3Galβ1→R</td>
<td>Singhal et al. *</td>
</tr>
<tr>
<td>FH4 (IgG&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>Dimeric Le&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→R</td>
<td>Fukushi et al. (2)</td>
</tr>
<tr>
<td>FH6 (IgM)</td>
<td>Sialyl dimeric Le&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(or sialyl Le&lt;sup&gt;e&lt;/sup&gt;-i) Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→R</td>
<td>Fukushi et al. (3)</td>
</tr>
<tr>
<td>AH6 (IgM)</td>
<td>Le&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Galβ1→4GlcNAcβ1→3Galβ1→R</td>
<td>Abe et al. (20)</td>
</tr>
<tr>
<td>KH1 (IgM)</td>
<td>Trifucosyl Le&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→R</td>
<td>Kaizu et al. (5)</td>
</tr>
<tr>
<td>TKH2 (IgM)</td>
<td>Sialosyl-Tn</td>
<td>NeuAcα2→6GlcNAcα1→O-Ser/Thr</td>
<td>Kjeldsen et al. (17)</td>
</tr>
</tbody>
</table>

populations differing from each other in terms of antigen expression (i.e., mosaicism). Histopathological diagnosis, clinical pathological status, and immunohistological staining patterns for eight cases are summarized in Table 2. Typical examples showing clear mosaicism of expression are illustrated in Figs. 1–4.

In a section from case 1 (Table 2; figure not shown), one area (area a) was positively stained by SH1 but was not stained by TKH2. Tumor cells in this area were histologically moderately differentiated adenocarcinoma in which approximately 30% of tumor cell membranes were stained and approximately 70% of mucous secretions from tumor cells were stained by SH1. Neither tumor cell membranes nor mucous secretions in this area were stained by TKH2. In contrast, in the high-differentiated area (area c), tumor cell membranes were strongly stained by SH1 but only weak staining was found in mucous secretions. The mucous secretions in this area were strongly stained by TKH2. The area (area b) of normal mucosa showed a strong dysplasia, although the glandular structure was maintained. Cell membranes in this area were not stained by SH1. Its secretions, however, showed a weak staining with SH1 and strong staining with TKH2. The degree of tumor differentiation (area c > area a) seems to correlate with the expression of Le<sup>e</sup> defined by SH1 at the tumor cell membrane (area c > area a); on the other hand, the epitope sialyl-Tn defined by TKH2 was mainly expressed in mucous secretions from tumors and from dysplastic normal mucosa. Sialyl-Tn expression in tumor cell secretions seems to be correlated with degree of differentiation; i.e., secretions of more-differentiated tumors express more sialyl-Tn than do secretions of less-differentiated tumors. In another case (case 2, Table 2; figure not shown) of a moderately differentiated adenocarcinoma, cell membranes were moderately stained by SH1, whereas the area with a high quantity of secreted mucin was strongly stained by TKH2. In both cases, staining with SH1 was associated with cell membranes, while TKH2 stained mucous secretions from tumor cells, although neither antibody stained adjacent normal mucosa epithelia of colon or secretions from normal colonic epithelia. In the section from case 3 (Table 2), as shown in Fig. 1, a complex mixture of differentiated and undifferentiated areas was apparent. Areas a and c were well differentiated and showed a glandular structure, while area b was undifferentiated with dysplasia. Area a showed a high level of mucinous secretions, while area c had no secretions. Both areas a and c were stained by TKH2, particularly the mucinous secretions in area a, although cancer cell membranes in area c were also stained by TKH2. In contrast, tumor cell membranes as well as cytoplasm in area b were strongly stained by SH1, although epithelial cell structure in area a was also stained by SH1. All areas were weakly and diffusely stained by FH6, although the degree of staining was slightly higher in area b (Fig. 1). Anti-CEA did not stain tumor cells but stained mucous secretions regardless of the area (areas a, b, or c). It should be noted that less-differentiated area b was more strongly stained in both membranes and cytoplasm by SH1 and FH6, and as in cases 1 and 2, mucous secretions from tumor cells were strongly stained with TKH2, although the degree of staining was stronger in well-differentiated areas as compared with less-differentiated areas. This pattern was consistent in cases 1, 2, and 3.

In a section from a similar well-differentiated tubular adenocarcinoma (case 4, Table 2; figure not shown), the area (area a) displaying severe dysplastic atypical cells was not stained by SH1 nor by AH6, although its mucous secretions were stained by TKH2. In contrast, the major area (area b), showing well-differentiated tubular morphology, was strongly stained by AH6 but not by SH1 or TKH2. In contrast, the area showing the best-differentiated adenocarcinoma (area c) was stained by SH1, TKH2, and AH6. Mucous secretions present in this area were intensely stained by TKH2. These sections showed a close correlation between the degree of differentiation within a tumor and expression of Le<sup>e</sup>, Le<sup>+</sup>, and sialyl-Tn. In the section of another case of well-differentiated tubular adenocarcinoma (case 5, Table 2; figure not shown), three distinct areas (areas a, b, and c), were clearly distinguishable by their stainability with antibodies and by degree of differentiation. Area a was a less-differentiated area in which the cells were not stained by TKH2, but were weakly stained by SH1 in tumor cell membranes and cytoplasm. Area b was the most well-differentiated area showing mucous secretions. Area c also represents a well-differentiated area, slightly less differentiated than area b but more so than area a. Both areas (areas b and c) were stained well by SH1 and TKH2. However, area b was stained by FH6 in stroma and in mucous secretions, while area c was not.

A section of liver metastatic lesion from colon cancer (case 6, Table 2) was characterized by the presence of a large area (area c) of clear cell carcinoma (i.e., cells containing clear structures...
cytoplasm); this type of cell was absent in the original colonic cancer. This area was strongly stained by FH6, but was not stained by SH1 (Fig. 2). Areas representing well-differentiated carcinoma (areas a and b) were weakly stained by FH6 and strongly stained by SH1. Areas a and b were morphologically very similar and had similar degrees of differentiation.

The staining pattern of a section from another case of differentiated tubular adenocarcinoma of stomach (case 7, Table 2) is shown in Fig. 3. Three distinct areas (areas a, b, and c) were observed. Area a, a mixture of inflammatory cells and undifferentiated cancer cells, was stained by SH1 and strongly stained by FH6 but not by TKH2. Areas b and c were identical in their histological pattern, showing typical moderately differentiated tubular adenocarcinoma. Area b (but not area c) was strongly stained by TKH2; both areas were weakly stained by SH1.

The clearest example of mosaicism reflecting degree of differentiation is shown in Fig. 4. The section is a gastric adenocarcinoma derived from a 52-year-old man (case 8, Table 2). Area a represents poorly differentiated adenocarcinoma, which was strongly stained by FH4 as well as SH1 but poorly by FH6. Area b represents moderately differentiated adenocarcinoma, which was stained strongly by SH1 but poorly by FH6. Areas a and b were not stained at all by TKH2. Area c, representing well-differentiated adenocarcinoma, was minimally stained by SH1 but strongly stained by TKH2. In particular, those cells secreting mucinous material were characterized by intense staining by TKH2. It should be mentioned that both gastric adenocarcinoma and colorectal adenocarcinoma that secrete mucinous material are stained by TKH2, including the mucinous secretion of area c, which shows strong expression of sialyl-Lea and sialyl-Leb in both membrane and cytoplasm.

DISCUSSION

In previous studies from this laboratory and others, two classes of carbohydrate antigens defined by MoAbs have been found most frequently and most intensely expressed in a variety of common human cancers derived from gastrointestinal, bronchopulmonary, and mammary epithelia. They are fucosyl or sialosyl-fucosyl type 2 chain antigens (2–8, 21), and Tn and sialyl-Tn, the core structure of O-linked mucin-type glycans (15–18). Although each of these structures is clearly defined by specific MoAbs, patterns of their expression in human cancer and in normal tissue have not been studied previously with combinations of these MoAbs. In the present study, distribution patterns of various tumor-associated carbohydrate antigens in...
Fig. 1. Immunohistological staining patterns (top) and corresponding sketches (bottom) for case 3. Table 2 (primary colonie cancer). A, H & E; B, SH1; C, FH6; D, TKH2. The entire tumor section was stained by SH1 (top, B); some areas were stained strongly (area b) and others relatively weakly (area a) (bottom, B). Some areas (area a) weakly stained by SH1 were strongly stained by TKH2, whereas some areas (area b) strongly stained by SH1 were not stained by TKH2 (top and bottom, D). Diffuse positive staining with sporadic strong staining at membranes with FH6 was observed (top, C). Anti-CEA stained areas different from those stained by FH6 and SH1 (data not shown). FH4 did not stain at all (data not shown). For the relationship between pattern of carbohydrate antigen expression and degree of differentiation, see text.
Fig. 2. Immunohistological staining patterns (top) and corresponding sketches (bottom) for case 6, Table 2 (liver metastasis from colon cancer). A, periodate/Schiff reagent; B, SH1; C, FH6; D, anti-CEA. Sketches (bottom) show staining patterns of SH1 and FH6, defining areas a, b, and c. The entire tumor was strongly stained by periodate/Schiff reagent (A), SH1 (B) and FH6 (C), although a detail of staining pattern indicated a clear complementarity between SH1 and FH6. Area b was strongly stained by SH1 but weakly stained by FH6. In contrast, area c was strongly stained by FH6 and weakly stained by SH1 (top and bottom, B and C). A weak, diffuse staining was observed with anti-CEA.
Fig. 3. Immunohistological staining patterns (top) and corresponding sketches (bottom) for case 7, Table 2 (primary gastric cancer). A, H & E; B, periodate/Schiff reagent; C, SH1; D, FH4; E, FH6; F, TKH2. Sketches (bottom) show staining patterns of FH6 and TKH2, defining areas a, b, and c. The entire tumor was strongly stained by periodate/Schiff reagent (B) and by antibody SH1 (C). A clear complementarity of staining was found between FH6 (E) and TKH2 (F), i.e., area a (see bottom) was strongly stained by FH6 but not stained by TKH2, while areas b and c (see bottom) were strongly stained by TKH2 and not stained by FH6. There was weak, diffuse staining by FH4.
Fig. 4. Immunohistological staining patterns (top) and corresponding sketches (bottom) for case 8, Table 2 (primary gastric cancer). A, H & E; B, periodate/Schiff reagent; C, AH6; D, SH1; E, FH6; F, FH4; G, TKH2; H, anti-CEA. Sketches (bottom) show staining patterns of SH1, FH4, and TKH2, defining areas a, b, and c. The entire tumor section was strongly stained by periodate/Schiff reagent (B), AH6 (C), and SH1 (D). Area a was strongly stained by SH1 but also stained by FH4 and FH6, whereas area c, which was not stained by FH6 or weakly stained by SH1 was strongly stained by TKH2 (see bottom). Anti-CEA antibody did not produce good staining.
a single tumor have been examined utilizing serial sections of tumor tissue stained with specific monoclonal antibodies. We used MoAbs defining, respectively, Le', Le, dimeric Le', sialosyl dimeric Le', sialosyl-Tn, and CEA, applying each to consecutive serial tumor sections, and compared the antigen staining patterns thus obtained with those from hematoxylin- and peroxidase/antiperoxidase reagent. Only patterns from colorectal and gastric tumor samples are reported here.

Expression of Le' and Le' antigens, defined by MoAbs SH1 and AH6, respectively, was found to be lower in less-differentiated areas. Expression of these antigens was maximal in cell membranes of moderately to highly differentiated tumors. The antigens were also expressed in the crypt areas of normal mucosa and were strongly expressed in mucosa epithelia showing strong dysplasia, in agreement with previous observations (8, 21). Sialyl-Le' and dimeric Le', defined by MoAbs FH6 and FH4, were highly expressed in less-differentiated areas within the same tumors. All these type 2 chain-derived antigens were only weakly expressed in secretions derived from tumor cells, but highly expressed in plasma membranes or cytoplasm of tumor cells. Those type 2 chain-derived structures were also expressed in the crypt area and in a limited number of normal cells (parietal cells of gastric mucosa, Panet cells of intestine, etc.) (2, 8, 21). Expression of sialyl-Tn was not observed in less-differentiated tumors, but was generally associated with secretions from highly differentiated tumor cells. Intense staining by TKH2 was consistently associated with highly differentiated areas where tumor cells were secreting mucinous material. Secretions from normal epithelia or adjacent normal mucosa were not stained by TKH2. Interestingly, one section showing clear cell carcinoma was characterized by intense staining with FH6, indicating the presence of sialyl-Le', which was absent in the original tumor. Recently, metastatic lesions have been found to be associated with strong staining by FH6.7

Diversity of aberrant glycosylation, particularly fucosyl or sialosyl-fucosyl in type 2 chain and Tn/sialyl-Tn antigens, may be closely correlated with the degree of differentiation of cell populations within a single tumor. Such diversity may be lacking in the original in situ tumor, but develop gradually during tumor progression, resulting eventually in a complex mosaic pattern of antigen expression as exemplified by case 7. Some groups of cell populations (e.g., those expressing sialyl-Le') may have greater metastatic potential, while other populations (e.g., those secreting mucinous material which expresses sialyl-Tn) are found only in highly differentiated tumors and have much less metastatic or invasive potential.

The present study also indicates that no single antibody can detect 100% of tumors with respect to either immunohistology or immunomaging of tumors in vivo, although immunomaging techniques have been increasingly utilized (22-24). The use of an antibody "cocktail" is important in view of the variety of antigens expressed. This is also true and important for delivery of anticancer drugs via antibody-drug conjugates.

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