Regional Heterogeneity and Complementation in the Expression of the Tumor-associated Glycoprotein 72 Epitopes in Colorectal Cancer

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ABSTRACT

We analyzed the immunohistochemical expression of three epitopes of the tumor-associated glycoprotein 72 (TAG-72) in whole cross-sections of primary colorectal carcinomas and in regional lymph node metastases using monoclonal antibodies (MAbs) B72.3, CC-49, and CC-83, which recognize distinct carbohydrate antigenic determinants. B72.3, CC-49, and CC-83 reacted with 13 of 27 (48%), 25 of 27 (92%), and 21 of 27 (77%) carcinomas, respectively. The immunoreactivity with lymph node metastases followed a similar pattern; MAb CC-49 was again the most reactive of the three antibodies, since it labeled 13 of 15 metastatic lymph nodes. Positive reactions of the MAbs with the primary tumors were not always predictive of the immunorecognition of their metastases. Distinct areas within whole cross-sections of TAG-72-positive primary carcinomas demonstrated marked differences in the expression of the three epitopes. CC-49 tended to react with the highest number of areas and with the highest percentages of carcinoma cells within each area. In no instances did B72.3 demonstrate reactivity superior to that of either CC-49 or CC-83. Tumors negative for the CC-49 epitope in any area also did not express the other two TAG-72 epitopes. However, the comparison of the immunostaining obtained with each MAb in TAG-72-positive primary lesions revealed areas where CC-83 was clearly more reactive than CC-49. Moreover, one lymph node metastasis, negative for CC-83, was recognized by CC-49. Thus, the combined use of MAbs CC-49 and CC-83 resulted in additive immunostaining of primary and metastatic colorectal carcinoma cells. The study provides evidence of intratumoral heterogeneity in the glycosylation pattern of the TAG-72 antigen in colorectal cancer and emphasizes the advantages of cocktails of antitumor-associated antigen MAbs in the immunodetection of colorectal tumor cells.

INTRODUCTION

Several studies documented alterations in the glycosylation processes of carcinoma cells (1–5), reflected in the heterogeneous expression of carbohydrate epitopes of glycoproteins or glycolipids (6, 7). In this respect, several carbohydrate determinants of glycosylated molecules (8–11) should be analyzed to characterize the antigenic phenotype of epithelial neoplasms. MAbs to TAAs were evaluated in several diagnostic and clinical applications (10–13). The availability of several MAbs directed to the same TAA should, therefore, improve the immunorecognition of glycosylated antigens.

The tumor-associated glycoprotein TAG-72 is a well-characterized pancarcinoma antigen, currently evaluated in the management of colorectal cancer patients (11, 14–20). Several MAbs to TAG-72 were developed and shown to react with distinct, but structurally related, carbohydrate epitopes (9, 14, 21). A recent study compared the immunohistochemical staining patterns of three anti-TAG-72 MAbs, B72.3, CC-49, and CC-112, with normal, inflammatory, and neoplastic tissues of various origin (10). MAb CC-49 was shown to be superior in terms of percentages of immunostained neoplastic cells. These results suggested that the CC-49 epitope could be more abundant or more accessible on carcinoma cells, although the affinity constants of the MAbs tested might have also influenced their immunohistochemical reactivities.

In this study we evaluated and compared the expression of the TAG-72 molecule in 27 colorectal carcinomas using MAbs B72.3, CC-49, and CC-83. The purpose was to analyze differences in the immunostaining of specific glycosylated epitopes to: (a) identify MAbs potentially more efficient in the immunohistochemical recognition of primary and metastatic colorectal cancer cell and (b) define whether these MAbs could result in additive immunostaining.

MATERIALS AND METHODS

The generation and characterization of MAbs B72.3, CC-49, and CC-83 were previously reported (9, 14). Several studies have shown that these MAbs react with formalin-fixed as well as with frozen sections (8–11, 14–18). The antibodies used in this study were kindly supplied by Dr. J. Schlom, Laboratory of Tumor Immunology and Biology, National Cancer Institute, NIH, Bethesda, MD. We analyzed 25 cases of colorectal cancer, pathologically staged and graded according to the method of Payne (22), which included two cases of double synchronous carcinoma and eight cases with metastases to the regional lymph nodes (total number of tumors, 27; total number of regional metastases, 15). All tissues were fixed in 10% buffered formalin within minutes from surgery. Each case examined included a cross-section of the whole primary tumor mass as well as the adjacent colonie mucosa. Cross-sections through all metastatic lymph nodes were also examined. Serial sections from paraffin-embedded blocks were dewaxed in xylene and hydrated through graded ethanol to PBS, pH 7.4. Endogenous peroxidase activities were blocked by immersion in 0.3% (vol/vol) hydrogen peroxide in absolute methanol for 30 min. MAbs (ascitic fluids) were incubated for 30 min at room temperature (CC-49, CC-83) or overnight at 4°C (B72.3). The dilutions used in the study were selected by end point titration on paraffin sections of carcinomas expressing the TAG-72 antigen. These dilutions (B72.3 and CC-49, 1:1000; CC-83, 1:1500) were those which allowed maximal reactivity with carcinoma cells and absence of background on tumor stroma. Immunohistochemistry was performed using a streptavidin-biotin system kit for primary murine antibodies (Zymed Laboratories, San Francisco, CA). The peroxidase reaction was initiated by the addition of 0.06% DAB (Sigma) in PBS containing 0.01% hydrogen peroxide. The DAB reaction time was controlled at 7 min.

Several distinct areas within whole cross-sections of each primary tumor were identified by low-magnification scanning of hematoxylin-eosin-stained sections on the basis of variability in tumor histological grade (well, moderately, or poorly differentiated) (22); pattern of tumor growth (papillary, tubular, solid) (22); and level of intratumoral...
invasion (superficial, i.e., within the upper third of the muscularis, or deep, i.e., in the lower two-thirds of the muscularis or in pericolic soft tissue). These areas were systematically examined on serial sections for their immunohistochemical reactivity with each of the three anti-TAG-72 MAbs at both low and high microscopic magnifications. The degree of staining heterogeneity obtained with each MAb in each of the different areas of the primary tumors and in each individual lymph node metastasis was evaluated by counting DAB-stained tumor cells out of a minimum of 500 cells in 5 microscopic fields per each area at a magnification of x400. The results obtained were consistent with the overall reactivity of each MAb in that particular area, as examined by low magnification. A tumor, or an area within a tumor, was considered positive when at least 5% of the tumor cells were immunostained. Negative controls were performed replacing the anti-TAG-72 MAbs with PBS and with a murine monoclonal immunoglobulin G1 of irrelevant specificity.

RESULTS

MAbs B72.3, CC-49, and CC-83 reacted with different percentages of the primary and metastatic carcinomas tested (Table 1). In this respect, B72.3 detected the lowest number of primary tumors, i.e., 13 of 27 (48%), whereas CC-49 and CC-83 reacted with 25 of 27 (92%) and 21 of 27 (77%) tumors, respectively. The immunoreactivity with regional lymph node metastases followed a similar pattern; B72.3 reacted with 7 of 15 (46%), CC-83 with 10 of 15 (66%), and CC-49 was the most reactive, since it labeled 13 of 15 metastatic lesions (86%) (Table 1).

Several common patterns of staining were detected in the carcinomas studied. Tumors with tubular differentiation displayed apical labeling, often associated with cytoplasmic staining (Fig. 1). Less differentiated adenocarcinomas tended to exhibit a more diffuse cellular labeling (Fig. 2A). In areas with mucinous differentiation, secretion products infiltrating the peritumoral stroma homogeneously expressed all three TAG-72 epitopes (Fig. 2B). Moreover, B72.3 tended to selectively immunoreact with secretion products, while CC-49 and CC-83 were also associated with the cytoplasm and cell membranes. All three MAbs stained the epithelium of the mucosa adjacent to the tumors, which often exhibited goblet cell hyperplasia.

The microscopic examination of whole cross-sections of colorectal tumors revealed marked regional differences in pattern of growth, level of invasion, and histological grade. The percentages of cells stained by each antibody varied in different areas of the same tumor. Fig. 3 shows the reactivities of B72.3, CC-49, and CC-83 with different areas within each of the 27 carcinomas. This intratumoral variability was readily apparent...
Fig. 3. Percentages of cells reactive with B72.3 (A), CC-49 (B), and CC-83 (C) in 25 cases tested. Each case is individually represented (vertical lines). Different areas within each primary tumor are indicated as closed dots (•); metastatic lymph nodes as closed triangles (△). Arrows indicate two cases where complementary reactivity is exemplified. Open symbols differentiate areas in primary tumors (O) or lymph node metastases (A) from second malignancies in two cases with double synchronous lesions.

Fig. 4. Heterogeneous and uncoordinated expression of TAG-72 epitopes in Cases 70 (A) and 127 (B). △, superficial area; •, invasive area.

in several cases, where the same tumor included areas which contained 100% positive cells and areas which were negative or contained less than 10% positive cells. It was not possible to correlate the immunostaining with histological differentiation or depth of invasion.

MAb CC-49 reacted with the highest number of areas and with the highest percentages of tumor cells within each area.

Notwithstanding this trend, in some instances CC-83 and B72.3 reacted with more carcinoma cells than did CC-49, as exemplified by two cases indicated in Fig. 3, A to C, arrows, and illustrated in detail in Fig. 4. The analysis of the distinct areas within these two tumors revealed complementary reactivities of anti-TAG-72 MAbs. Table 2 shows that anti-TAG-72 MAbs may cooperate in the immunodetection of distinct areas within each primary tumor. The addition of CC-83 to CC-49 resulted in an increase of immunodetectable areas, while no increase was observed adding B72.3 to either CC-49 or to CC-49 and CC-83. Similarly, an additive immunostaining of metastatic lesions was obtained complementing CC-49 with CC-83. One lymph node metastasis which was negative with CC-49 reacted with CC-83 (Table 2). Furthermore, different reactivities of the three MAbs were highlighted by the analysis of double synchronous primary lesions and their distinct regional lymph node metastases. Figs. 5 and 6 illustrate the immunohistochemical

Table 2 Complementary reactivities of monoclonal antibodies with distinct areas within primary carcinomas and with regional lymph node metastases

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<tr>
<th>MAb alone</th>
<th>Additive reactivity with</th>
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<tr>
<td></td>
<td>CC-83</td>
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<tr>
<td>B72.3</td>
<td>21/59*</td>
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<tr>
<td>CC-49</td>
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* Number of positive areas/total number of histologically defined areas.
* Number of positive lesions/total number of lesions tested.

Fig. 5. Heterogeneous reactivities of anti-TAG-72 MAbs with distinct areas from the double synchronous carcinomas of Case 460 (A, Tumor A; B, Tumor B). △, superficial area; •, invasive area; □ and □, metastatic lymph nodes.

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reactivities observed in primary and metastatic lesions from Patient 460, who had double primary carcinomas, Tumor A, at the rectal-sigmoid junction (Figs. 5A and 6A), and Tumor B, at the rectal ampulla (Fig. 5B). The superficial area from Tumor A was distinctly more reactive with CC-83 than with CC-49, while the number of carcinoma cells reactive with B72.3 was negligible. The invasive area from this primary tumor demonstrated a marked loss of immunoreactivity with CC-83, while the percentages of cells reactive with CC-49 and B72.3 did not vary relative to the superficial area (Figs. 5A and 6, A1 to A3). The two lymph node metastases from Tumor A demonstrated enhanced immunostaining, relative to the primary lesion. This was particularly remarkable for CC-49 which stained, respectively, 80% and 100% of metastatic cells, compared with 20% positive primary carcinoma cells (Fig. 5A). In primary Tumor B there was no significant variability in the immunostaining observed with the three antibodies in the superficial versus the invasive area, and CC-49 identified the highest percentages of carcinoma cells (Fig. 5B). The single lymph node metastasis from Tumor B demonstrated a loss of immunoreactive cells relative to its primary lesion. CC-49 reacted with 40% of metastatic tumor cells (as compared with 80% in the primary lesion), and the immunostaining obtained with CC-83 and B72.3 was either negligible or undetectable (Fig. 5B). Thus, only CC-49 clearly immunodetected this metastasis.

Another case with double primary carcinomas also demonstrated complex patterns of immunoreactivity (Fig. 7). Two areas, superficial and invasive, both Grade 2, could be identified in Tumor A. The superficial area was diffusely reactive with CC-83 (80% positive cells) and focally reactive with CC-49, while in the invasive area there was no significant difference between the immunostaining obtained with the two MAbs. In Tumor B, the superficial area diffusely reacted with both CC-49 and CC-83 (80% positive cells). In contrast, CC-49 identified a significant number of cells (30%) in one of the two invasive areas, while CC-83 was either unreactive or focally positive (Fig. 7B). The two regional lymph node metastases from Tumor B were equally positive with CC-49, while only one was immunodetected by CC-83 (Fig. 7B). B72.3 did not display significant immunostaining with either of these two tumors (Fig. 7).

The analysis of distinct areas within whole cross-sections of primary carcinomas and of their autologous metastases indicated variability in not only the expression of the TAG-72 antigen but also its distinct glycosylated epitopes. MAb CC-49 detected the majority of primary and metastatic tumors and of areas within tumors. However, in some instances, the combination of CC-49 with CC-83 resulted in an increase in the detection of immunopositive areas or metastases and/or in an increase of the percentages of tumor cells immunostained.

**DISCUSSION**

TAG-72 is a mucin expressed at high levels in several types of carcinoma and in fetal gut epithelium, but also detectable in normal adult colonic mucosa (7, 8, 11, 13, 15–20, 23, 24). Monoclonal antibody B72.3 was proved to be useful for several applications in clinical oncology, including the serological monitoring of colorectal and ovarian cancer patients, the diagnostic evaluation of neoplastic effusions by immunocytochemistry, and the in vivo detection of metastases by radioimmunospectography (12–14, 25–27). Several studies indicated that distinct carbohydrate epitopes of a tumor-associated antigen may not be coordinately expressed (8–11, 13, 16). Thus, the immunological recognition of colorectal carcinoma cells could be enhanced using combinations of MAbs (8–11, 28). Second generation MAbs were developed to allow the serological mapping of the TAG-72 molecule on the basis of the expression of epitopes more representative of the TAG-72 glycoprotein than the O-linked sialosyl-Tn B72.3 epitope (9, 13, 21, 27).

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**Fig. 6.** Regional variability of TAG-72 expression in Case 460, Carcinoma A. Whole cross-section through the maximum diameter of the lesion (A1); arrows indicate selected fields magnified in A1, A2, and A3. Heterogeneous expression of CC-49 (A1) and CC-83 (A2) in the superficial area. Negligible reactivity of CC-83 in the invasive area (A3). Immunoperoxidase counterstained with hematoxylin; A: bar, 2000 μm; A1 to A3: bar, 1000 μm.
epitopes in the regional lymph node metastases. Paralleling the reactivity with primary carcinomas, CC-49 was the most reactive antibody, since it labeled 13 of 15 metastatic lesions. However, one lymph node metastasis, negative with CC-49, was positively immunorecognized by CC-83.

These observations, focusing on the expression of three distinct TAG-72 epitopes in colorectal cancer as a model, provide further evidence of intratumoral heterogeneity in glycosylation patterns, as indicated by the not coordinate expression of TAG-72 epitopes. This suggests that, notwithstanding the prevalent expression of a specific epitope, combinations of MAbs may cooperate in the immunodetection of primary and metastatic carcinoma cells.

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REFERENCES


In this study, we evaluated and compared the immunohistochemical distribution of TAG-72 in 27 colorectal carcinomas using MAbs B72.3, CC-49, and CC-83, which recognize distinct carbohydrate epitopes and have different affinity constants ($K_a$ 2.5, 16.2, and $27.7 \times 10^7$ M$^{-1}$, respectively) (9, 21). Our results revealed intratumoral heterogeneity in the glycosylation pattern of TAG-72, not only, as anticipated, among tumors, but also in morphologically distinct areas within whole cross-sections of a lesion. MAb CC-49 reacted with the highest number of primary and metastatic tumors, the highest number of areas within each tumor, and the highest percentages of neoplastic cells. The superior reactivity of CC-49 could not be entirely due to its high affinity constant, since CC-83 had the highest affinity constant among these MAbs. It would rather appear that the CC-49 epitope was either more accessible or more frequently mounted on colorectal cells (10). However, in some areas within lesions, CC-83 was clearly more reactive than CC-49. MAb B72.3 did not contribute to the immunological recognition of tumor cells in areas which were negative or heterogeneously positive with the other two MAbs. These observations indicated that, among the MAbs tested, CC-49 was the anti-TAG-72 MAb of choice; however, the addition of CC-83 resulted in additive immunostaining of primary colorectal carcinoma cells.

One of the most promising clinical applications of MAbs in the management of colorectal cancer patients is the possibility to radioimmunodetect occult metastases identified by a particular antigenic phenotype. The regional variability of the TAG-72 antigenic profile within a primary carcinoma was reflected also in the heterogeneous and not coordinate expression of its
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