Antibody Penetration of Tumor GS-7 Xenografts in Nude Mice: A Model for
Mucinous Adenocarcinoma of the Colon

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Abstract

A new cell line derived from a human adenocarcinoma of the colon, GS-7, was propagated as a s.c. tumor in nude mice. This tumor histologically is a mucinous adenocarcinoma (also designated mucoid or colloid) with characteristic large mucin pools that are not lined by an epithelial layer but may contain scattered, randomly distributed cancer cells. Ten to 20% of human colorectal adenocarcinomas are of this histological type, but rapidly growing xenografts with this histology have been rarely used experimentally. This tumor, therefore, constitutes a useful model for similar human tumors. The mucin pools contain large amounts of carcinoembryonic antigen and tumor-associated glycoprotein 72, and the cells express epithelial glycoprotein 2 on their surface. The ability of antibodies injected i.v. to penetrate this tumor was investigated, using both biotinylated and radioiodinated antibodies (Abs). The results demonstrate that Abs can effectively penetrate the mucin pools, and that large amounts of Ab can localize there. This tumor type may have advantages as a target for certain forms of experimental immunotherapy.

Introduction

The ability of human carcinoma xenograft models to predict therapeutic results in humans is limited by the relative lack of differentiation of the most widely used xenograft tumors in comparison to the level of differentiation present in many human adenocarcinomas. For example, most colon carcinomas display a considerable level of glandular organization, whereas the most widely used colon carcinoma nude mouse xenografts are poorly differentiated. This difference seems likely to have an effect on the ability of Abs to penetrate these tumors, because the epithelial cells lining glandular structures are joined by tight junctions, which prevent the passage of macromolecules, and some of the most widely used target antigens are either secreted molecules, which are present at high concentrations in the lumens of malignant glands, or cell surface components present only at the luminal edge of glandular epithelial cells. Such antigens include mucins such as polymorphic epithelial mucin and TAG-72, and CEA. The significance of this factor was demonstrated by Perverz et al., who found that the well-differentiated colon carcinoma HRA-19 expressed high levels of the human milk fat globule-1 antigen, but that Abs to the antigen were unable to localize to the tumor in vivo, presumably due to antigen inaccessibility resulting from the presence of tight junctions. The HRA-19 tumor was reported to grow very slowly and inconsistently, which complicates its use as an experimental model. Other colon carcinoma xenografts have also been described that display relatively developed glandular structures. However, the tumors described by Park et al., although histologically similar to human tumors, have not been widely used, partly because some do not express the target antigens CEA or TAG-72. Lewis et al. described two moderately to well-differentiated colon carcinoma xenografts. Both retained their histological appearance over five to nine passages, but the CEA content seemed to be reduced relative to that in the primary tumor, suggesting the selection in nude mice of a subpopulation of tumor cells expressing less CEA. A third tumor used by Lewis et al. was described as a mucoid adenocarcinoma with signet ring cells. This tumor was used in experiments to determine the microscopic localization site of anti-CEA Abs in nude mouse xenografts by autoradiography. Although some antigen-specific localization to viable tumor cell clusters was detected, Ab localization to antigen present in mucinous areas could evidently not be evaluated, due to the nonspecific localization of a control Ab to this site.

We describe here a new colon carcinoma xenograft, GS-7, which originated from a patient with mucinous adenocarcinoma. This tumor type, which is also called colloid or mucoid adenocarcinoma, represents approximately 10-20% of colon carcinomas in the United States. It is characterized by large mucin pools that are not lined by epithelial cells; it is considered that epithelial cells were originally present and were responsible for mucin secretion but degenerated after formation of the mucin pool. The viable tumor cells typically form glandular structures at the edges of the tumor mass or are found as focal cells lying free within the mucin pool. Mucinous adenocarcinomas are more malignant than the more common moderately differentiated colon adenocarcinomas. Tumors having a similar morphology sometimes occur among carcinomas of the stomach, pancreas, or breast. In this study, we have evaluated the ability of mucinous antigens in tumors of this type to serve as targets for circulating Abs. GS-7 expresses high levels of CEA and TAG-72 in the mucin pools. We report that Abs to these antigens can thoroughly penetrate the mucin pools, and that high levels of Abs can specifically bind. Although this tumor type is not a very common type of colon carcinoma, it may constitute a particularly advantageous target for RAIT, especially using the high-energy B-particle emitters that are most commonly used for this purpose. Radioisotopes loaded into the mucinous centers of such tumors can potentially deliver a high radiation dose to adjacent viable tumor cells.

Materials and Methods

Tumor Propagation. Tumor GS-7 originated in 1990 from a patient diagnosed with mucinous adenocarcinoma of the colon. The tumor was minced by pushing through a steel screen (E-C Apparatus Corp., St. Petersburg, FL), and 0.5 ml of a 10% suspension (packed cell v/v) was injected s.c. into outbred nude mice (Harlan Sprague-Dawley, Indianapolis, IN) as described previously. It was passaged eight times, over a 2.5-year period, before being used in the experiments described. At each passage, paraffin sections were stained with H&E, and the morphology of the tumors did not change significantly with passage number. Tumors were used at a size of 0.5-1.0 g, which required 3-4 months of growth.

Ab Penetration and Localization. The Abs used include NP-4 and MN-14, both Abs to CEA (13); B72.3 (5), reacting with the TAG-72 mucin antigen that has been characterized as the carbohydrate epitope sialyl-Tn (6, 14); M337 and MH99, which react with the epithelial differentiation antigen designated...
epithelial glycoprotein 2 (15, 16); and MA103, which reacts with a widely distributed cell surface antigen (17). The negative control Ab used was the mouse MOPC-21 myeloma protein, of unknown specificity, produced by the cell line P3 × 63Ag8 (American Type Culture Collection, Rockville, MD), which we designate Ag8 (18). All of these Abs are mouse IgG1s, except for MA103, which is an IgG2, Ab. Abs were biotinylated and used in penetration experiments in vivo as described previously (17), with i.p. injection of 1.0 mg Ab/mouse. Three days after Ab injections, a period that was determined previously to allow maximum tumor penetration, mice were sacrificed, tumors were removed and frozen, and 8-μm cryostat sections were analyzed for the distribution of the biotinylated Ab. Adjacent sections were reacted with the same biotinylated Ab in vitro, to estimate whether the majority of the antigen was saturated in vivo. Briefly, ethanol-fixed sections were treated with medium or biotin Abs at 20 μg/ml, then with the ABC reagent (Vector Laboratories, Inc., Burlingame, CA) at 1:150, followed by freshly prepared diaminobenzidine (Sigma Chemical Co., St. Louis, MO) at 0.5 mg/ml for 15 min. Sections were counterstained with hematoxylin. All sections were stained in duplicate, and each experiment was performed at least twice. As an additional and more sensitive test to determine whether antigen saturation occurred in vivo, mice were injected with large amounts of unconjugated Abs to determine whether the subsequent binding of biotinylated Abs in vitro could be effectively inhibited. Radioimmunolocalization experiments were performed as described previously (19). Briefly, tumor-bearing nude mice were injected with 10 μCi 131I-labeled Abs. At various times, mice were sacrificed, and the dissected organs were weighed and counted for radioactivity.

Other Methods. The CEA content of tumor extracts was measured by a peroxidase-linked immunoassay as described (20). Briefly, wells were coated with NP-1, an Ab to CEA. After incubation with cell extracts or a standard solution of CEA, wells were incubated with a horseradish peroxidase conjugate of NP-3, an Ab to a different epitope of CEA, followed by the peroxidase substrate tetramethylbenzidine (Sigma).

RESULTS

Propagation, Histology, and Antigen Expression in Tumor GS-7. Tumor GS-7 has been passaged 16 times in nude mice, over a period of >4 years. It grows consistently, with tumors appearing in virtually all of the mice. However, its growth rate is relatively slow, with 3–4 months often required until a tumor size of approximately 0.5 g is obtained. In addition, the growth rate is often variable in the same batch of mice. The histological appearance of tumor GS-7 is shown in Fig. 1. This appearance has remained quite consistent over time, and the original tumor specimen was very similar histologically to the first transplant generation, which is shown in Fig. 1. The central part of the tumor is primarily made up of mucin pools, which often are subdivided by septa of connective tissue. Viable tumor cells form glandular structures at the edges of the tumor mass. These glandular structures range in appearance from glands lined by tall columnar epithelial cancer cells and having small lumina, to large mucin-filled glands lined by goblet cells, to mucin-filled glands in which the epithelia are in various stages of degeneration.

Immunohistochemistry demonstrated that Abs MH99, NP-4, and B72.3 reacted strongly with GS-7 but with markedly different patterns of distribution (Fig. 2). MH99 reacted homogeneously with the surface of viable cells and with cells that were partially degenerated but still...
Fig. 2. Antigen expression in GS-7 xenografts. Frozen sections were stained with biotinylated Abs, followed by the ABC reagent and the substrate diaminobenzidine. All photographs were taken with a ×4 objective, except C, which was taken with a ×10 objective. Bars, 0.25 mm. A, H&E stained for reference. A large, acellular, mucin-filled area is shown on the right. B and C, MH99, showing staining of the cell surface. D, B72.3, showing granular staining of secretions. E, anti-CEA NP-4, showing staining of secretions and some staining of the luminal edge of epithelial cells. F, negative control Ab Ag8. Note that B72.3 and NP-4 both stain the contents of the lumina and mucin pools but with slightly different patterns.

recognizable. It also reacted with material in some of the mucin pools, which could be cell debris. Both NP-4 and B72.3 reacted strongly and predominantly with the mucin pools; in the viable areas of the tumor, these two Abs reacted with the secretions and/or the luminal edges of the glands, but basolateral surfaces of the tumor cells appeared negative. Despite the generally similar pattern of distribution, NP-4 and B72.3 consistently had significantly different microscopic patterns of reactivity, as shown in Fig. 2. The B72.3 reactivity appeared more fibrous, whereas the NP-4 reactivity was more granular. Also, NP-4 produced dark staining of the luminal edges of glands that was not seen so prominently with B72.3. The CEA content of tumor extracts, determined by a peroxidase-linked immunoassay, was $297 \pm 121 \mu g/g$ tumor, which is considerably higher than values obtained with other colon carcinomas that are high producers of CEA (20).

**Penetration of Tumor GS-7 by Biotinylated Abs.** To determine whether antigens within tumors were accessible to circulating Abs, biotinylated Abs were injected into tumor-bearing nude mice, and 3 days later, tumors were collected and frozen. The penetration of GS-7 tumors by biotinylated MJ37 (similar to MH99), B72.3, and NP-4 is shown in Figs. 3 and 4. In all experiments, adjacent sections were stained with either the ABC reagent only, to detect Ab bound in vivo, or with the same biotinylated Ab in vitro (followed by the ABC...
Fig. 3. Penetration of GS-7 tumor xenografts by biotinylated Abs injected into tumor-bearing mice. A tumor from a mouse injected with biotin M37 was stained with H&E (A and B) or ABC reagent (C) to detect Ab localized to the tumor. A, area toward the center of the tumor, with an acellular, mucin-filled region in the upper right. B, area near the edge, which contains healthier, more glandular structures. Cell membranes were stained with biotin M37. A tumor from a mouse injected with biotin B72.3 was stained with H&E (D) or with ABC reagent (E). Secretions were stained by biotin B72.3. A tumor from a mouse injected with the negative control Ab biotin Ag8 was stained with H&E (F) or ABC reagent (G). With all tumors, adjacent sections were stained with the same biotinylated Ab in vitro, and little if any additional staining was produced. Bar, 0.25 mm.
reagent), to determine the total distribution of the antigen. All three Abs thoroughly penetrated large, solid tumors. The tumor size averaged 0.5–1.0 g, but some tumors of nearly 2.0 g were used with similar results. Strong staining was obtained throughout the tumors, with no indication of areas that were inaccessible. For Abs NP-4 and B72.3, which stained secretions and/or the luminal edges, we examined particularly the healthy glands, at the edges of the tumor masses (exemplified by Fig. 3B), to determine whether antigen at these sites might be inaccessible to circulating Ab but did not detect evidence for this. If Abs were blocked by an “antigen barrier” (21), which also would create certain inaccessible sites, this fact also should have been revealed in our studies but was not. However, the antigen barrier may operate primarily at low Ab doses, which were not tested in these studies.

Adjacent sections were stained with the same biotinylated Ab in vitro, to provide an indication of whether the antigen within the tumor was saturated in vivo. Increased staining intensity would demonstrate that antigen was not saturated in vivo. A lack of increased staining intensity suggests that saturation may be attained, but such data are not conclusive, because dark staining by biotin Abs may not neces-
MUCINOUS COLON ADENOCARCINOMA GS-7

Fig. 5. Biodistribution of 125I-labeled Abs in nude mice bearing GS-7 xenografts: percentage of injected dose/g. The Abs tested were: A, anti-CEA MN-14; B, B72.3; and C, Ag8, a negative control Ab. Means are shown for tumor (○), blood (□), liver (□), spleen (■), kidney (△), and lungs (▲). Bars, SD. There was significant tumor uptake of the nonreactive Ab, relative to normal organs, but much higher uptake of the specific Abs.

obtained with the best tumor targets for these Abs, namely, GW-39 and LS174T (19, 22, 23). The nonreactive Ab Ag8 localized relatively strongly to GS-7 tumors, although similar nonspecific uptake of IgG occurs with many other nude mouse xenografts (18, 24). This nonspecific uptake might be related to the presence of large mucin pools. However, the uptake of specific Abs by GS-7 was markedly greater.

DISCUSSION

An important feature of this study is the description of the GS-7 tumor, which appears to be a rare example of a human mucinous colon adenocarcinoma xenograft. This tumor has been passaged 16 times in nude mice over a period of >4 years, and it grows reproducibly, with a consistent morphology. This tumor may be useful not only for Ab-targeting experiments but also for other studies in which tumor architecture may be a significant factor. A key feature of this tumor is that it expresses high levels of many of the antigens that are widely used as targets in patients, namely, TAG-72, CEA, and epithelial glycoprotein 2 (the antigen recognized by 17-1A, KS1/4, AUA1, and many other Abs).

Although a few earlier nude mouse xenografts of human colon carcinomas have been described as mucinous (8, 9), it is undocumented and unclear whether they form large mucin pools not surrounded by epithelial cells, which is a key characteristic of most human tumors of this type. Fu et al. (25) used orthotopic transplantation of histologically intact human colon carcinoma specimens into nude mice in an attempt to preserve the original characteristics of the tumors; although the tumors produced were indeed similar to the primary tumors in many respects, they had lost their glandular morphology.

The main purpose of this study was to determine whether the antigens within mucin pools would be accessible to circulating Ab. Given the absence of an epithelial layer, with its associated tight junctions, surrounding the mucin pool, it might be predicted that there should not be a major barrier to tumor penetration. However, it was not clear what level of fluid flow could be expected in these areas. Our data show that Ab does effectively penetrate such areas. These results should be compared with those of Pervez et al. (3), who used a glandular tumor in which the epithelial layer remained intact and found that circulating Ab did not reach antigen inside the glands. These data, together, suggest that it is the intact epithelial layer that represents a major barrier to Ab penetration. Another relevant study is that of Lewis et al. (10), which was described in “Introduction.” Although the tumor used, MAWI, was described as mucinous, it is not clear whether it contained large mucin pools and/or intact glandular structures, and whether antigen present in the mucin pools could be targeted by circulating Ab. Most of the experiments described herein

Table 1 Tumor:nonmucous localization ratio for tumor xenograft GS-7 grown in nude mice

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Blood 0.8 ± 0.2</th>
<th>Liver 2.6 ± 0.8</th>
<th>Spleen 3.3 ± 0.4</th>
<th>Kidney 2.3 ± 0.4</th>
<th>Lung 1.4 ± 0.4</th>
<th>Blood 1.0 ± 0.2</th>
<th>Liver 3.0 ± 0.5</th>
<th>Spleen 3.6 ± 0.4</th>
<th>Kidney 2.7 ± 0.3</th>
<th>Lung 1.9 ± 0.2</th>
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<tr>
<td>MN-14</td>
<td>2.0 ± 0.5</td>
<td>7.6 ± 1.1</td>
<td>13.5 ± 2.8</td>
<td>9.3 ± 1.8</td>
<td>5.4 ± 1.3</td>
<td>5.2 ± 2.6</td>
<td>17.8 ± 8.7</td>
<td>19.8 ± 9.5</td>
<td>15.0 ± 6.3</td>
<td>10.1 ± 5.9</td>
</tr>
<tr>
<td>B72.3</td>
<td>1.2 ± 0.2</td>
<td>3.9 ± 1.3</td>
<td>5.8 ± 1.0</td>
<td>4.1 ± 1.0</td>
<td>2.4 ± 0.7</td>
<td>2.0 ± 0.6</td>
<td>6.0 ± 2.3</td>
<td>8.1 ± 2.5</td>
<td>6.2 ± 2.1</td>
<td>4.1 ± 1.1</td>
</tr>
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Values are means ± SDs of the percentage of injected dose/g tumor/percentage of injected dose/g tissue. The antigens recognized are CEA for MN-14 and TAG-72 for B72.3. Ag8 is a nonreactive control Ab.
used high protein doses, 1.0 mg/mouse, to facilitate detection by immunohistology. It is not likely that such high doses would be optimal for RAIT, because tumor:nontumor localization ratios must decrease as antigen saturation is approached, although comparatively high doses of 700 mg Ab/patient have been found to be optimal in lymphoma patients administered certain Abs (26). However, our RAID results demonstrate that antigen-specific localization does occur at low doses of approximately 1 μg/mouse. It is possible that at lower Ab doses, Ab deposition within the tumor would be less homogeneous, due to the preferential binding of Ab to the first antigen molecules encountered (21, 27). The immunohistological method used here is not always useful to resolve such questions, because the staining reaction becomes gradually weaker and difficult to evaluate at lower Ab doses (17).4 Autoradiography, with radiolabeled Abs, is more sensitive at low Ab doses, although it does not allow the same level of resolution. In any case, RAIT with high-energy β-particle emitters does not require every cell to be reached by the Ab (28), and the optimal protein dose for RAIT may be at a level that does not produce homogeneous tumor penetration.

Although only 10–20% of human colon adenocarcinomas are classified as mucinous, the more common differentiated adenocarcinomas also sometimes have areas in which the epithelial layer has degenerated. In GS-7, we found that even in the healthy, glandular areas of the tumor, circulating Ab was able to reach the intraluminal antigen. This may possibly be explained by the fact that healthy glands are continuous with glands in which the epithelia have degenerated. Hence, Abs may enter glands in areas in which the epithelia are degenerated and then move within the lumen to areas that have intact epithelia.

The delivery of large amounts of Ab to the mucin pools within certain tumors may be advantageous for certain forms of immuno-therapy and disadvantageous for others. One potential advantage is that Ab catabolism may not be a factor limiting Ab retention in the tumor. We have found that in vitro, most Abs binding to the surface of solid tumor cells are internalized and degraded, with a half-life of 1–2 days (29, 30). Similar Ab catabolism occurs in vivo in tumor-bearing animals and significantly affects radioisotope retention in the tumor (28, 30). With GS-7, in contrast, Abs binding to antigen in mucin pools would not be susceptible to intracellular catabolism and might be retained at the tumor site for prolonged periods. We note, however, that lack of Ab catabolism appears to be more dependent on the particular antigen than on the particular tumor model, in that B7.23 and anti-CEA Abs show little evidence of catabolism after targeting other colon carcinoma xenografts, probably because much of the Ab is not binding to the cell surface (discussed in Ref. 30).

The RAID results with GS-7 described are, in fact, quite similar to results obtained with the same Abs using other colon carcinoma cell lines as targets, in terms of percentage of injected dose/g tumor (19, 22, 23). The most widely used colon carcinoma cell lines, such as LS174T and LoVo, are relatively undifferentiated tumor cells, which do not form well-developed glandular structures. GW-39, also widely used, is a signet ring carcinoma (31), which also does not form glandular structures but which expresses large amounts of intracellular mucin. Signet ring carcinomas are classified as a subset of mucinous carcinomas and account for <1% of total colon carcinomas (12). These tumors also express large amounts of the antigens CEA and TAG-72 (20, 23), but anti-CEA Ab localization in vivo sometimes does not correlate with CEA content, as measured in cell extracts (20). The site at which Ab localizes in these tumors has been investigated by several laboratories by autoradiography (27, 32–35). Such results, however, are not directly relevant to the present investigation, due to the absence of well-developed glandular structures and mucin pools. Ab B72.3 was found to localize to mucin globules in LS174T xenografts (32), and anti-CEA was found to localize to clusters of tumor cells in LS174T (33, 34) and GW-39 (35) xenografts. Pimm et al. (33) also reported anti-CEA Ab localization to the lumina of pseudoacini in LS174T and HRVB colon carcinoma xenografts, but such pseudoacini are, at most, very poorly developed glandular structures, and their experiments lacked a negative control Ab to determine sites of non-specific Ab localization. Variation in antigen localization related to histological morphology, as discussed above, may affect the results of RAIT experiments, and this possibility is presently being evaluated.

ACKNOWLEDGMENTS

We are grateful to Rosario Aninpat, Susan Chen, Jamie Sargeant, and Philip Andrews for technical assistance.

REFERENCES

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