Human Monoclonal Antibody against a Tissue Polypeptide Antigen-related Protein from a Patient with a Signet-Ring Cell Carcinoma of the Stomach

Martin Pfaff, Rosemary O'Connor, H. Peter Vollmers, and Hans Konrad Müller-Hermelink

Pathology Institute of the University of Würzburg, Josef-Schneider-Str. 2, 8700 Würzburg, Federal Republic of Germany

ABSTRACT

For a comparative study of the humoral immunity of patients with gastric signet-ring cell carcinoma, lymphocytes from spleen and lymph nodes were fused with the heteromyeloma SPM4–0. Immunoglobulin-producing clones were primarily tested in binding assays on autologous and allogeneic tumor cells and tissues. One of the resulting human monoclonal antibodies, designated 56/16 (IgM, λ), was found to be suitable for a detailed biochemical characterization. Immunoblotting and comparative two-dimensional gel electrophoresis on cell and tissue extracts as well as on preparations of the cytoskeleton revealed that the main epitope is not an integral membrane molecule but a degradation product of cytokeratin 8, which is a main component of the tumor marker, tissue polypeptide antigen. The M₈, 38,000/45,000 antigen could be identified in tumor and normal tissues, with highest expression in secretory cells and organs. Thus, the human monoclonal antibody 56/16 might represent an immune response in the patient against breakdown products of cytokeratin 8, which are released from the tumor cells during cell division, secretion, or cell death. A possible association of the antibody with the secretory activity of signet-ring carcinoma cells is discussed.

INTRODUCTION

humAb₄ technology has offered a unique opportunity to study the human B-cell repertoire and to recognize anti-tumor humoral immune reactions in cancer patients. Several authors have described the isolation of humAbs from patients with melanoma (1–3) or with carcinomas of the breast (4, 5), cervix (6), lung (7), colon (8, 9), and stomach (10, 11). Most humAbs are of the IgM isotype, react or cross-react with intracellular components, and show a broad distribution in malignant and normal tissues (12). The apparent multispecificity of these antibodies is the subject of several reviews (13, 14), and it has been attributed to their possible function in the clearing of autoantigens from the circulation or in the constitution of a rapid first line of defense against foreign invaders (15, 16).

The broad pattern of cross-reactivity and low affinities of the IgM antibodies for the corresponding antigens makes a specific epitope analysis of the humAbs difficult and might lead to errors in the evaluation, e.g., in immunohistochemical analysis. Reports of humAbs that are susceptible to a detailed biochemical analysis of their recognized epitopes are more limited and often of a preliminary character (1–6, 10, 12, 17). Therefore, conclusions concerning the humoral immunity of cancer patients can only be made in rare cases and the value of many humAbs remains limited.

In a recent report (10) we presented an alternative strategy to isolate humAbs against functional cell surface antigens on tumor cells. This strategy was based on an initial selection of mAbs for their ability to inhibit adhesion and movement of stomach carcinoma cells (10, 18, 19). It led to the identification of SC-1 (IgM, λ), a humAb that specifically interacts with autologous and allogeneic tumor cells in immunohistochemical, immunocytochemical, and functional attachment and motility assays. The antibody was shown to immunoprecipitate a M₈, 50,000 protein from [³⁵S]methionine-labeled autologous tumor cells (10).

Here, we report the isolation of a new humAb, 56/16, which has been selected according to the same guiding principles as SC-1. The mAb 56/16 was found to be suitable for a thorough biochemical characterization because of its strong reactivity in Western blot analysis. By one- and two-dimensional immunoblotting, the antibody could be shown to selectively interact with degradation products of cytokeratin 8 and not with the undegraded variant of this molecule. Proteolytic fragments of cytokeratin 8 have recently been shown (20, 21) to be the major constituents of human TPA, a widely used serodiagnostic carcinoma marker (22). Serum TPA levels have been correlated with the proliferative activity of a carcinoma and an externalization of TPA from cultured tumor cells into the culture medium is described (23–25).

Therefore, we conclude that the humAb 56/16 is the result of an immune response in the patient against TPA-like components released during tumor progression.

MATERIALS AND METHODS

Cell Lines. The heteromyeloma cell SPM4–0 was kindly provided by Hoffman La Roche (Basel, Switzerland). Primary cell lines from stomach carcinoma tumors were established as previously described (10). MECEM and HT29 cells were kindly provided by Dr. F. A. Anderer (Tübingen, FRG). The cells were maintained in RPMI 1640–10% fetal calf serum–1% penicillin and streptomycin at 37°C and 5% CO₂.

Monoclonal Antibodies. Procedures for derivation and preselection of human monoclonal antibodies are described in a previous report (10). Briefly, lymphocytes were prepared by mechanical means from lymph nodes and spleens. Subsequently, they were fused at a ratio of 1:1 with the heteromyeloma cell SPM4–0 using 40% polyethylene glycol. After 4 weeks, hybridoma supernatants were screened for antibody production by an enzyme-linked immunosorbent assay (10). Positive clones were then tested in attachment inhibition and immunohistochemical assays and cloned by limiting dilution using irradiated nude mouse lymphocytes as feeder layers. Mouse monoclonal antibody 35/H11 (IgM) was kindly provided by Dr. A. M. Gown (Seattle, WA).

Cell and Tissue Extracts. Tissue extracts were prepared by first sectioning with a cryostat and then homogenizing in PBS containing 0.5% NP-40 and 1 mM phenylmethylsulfonyl fluoride with a Potter homogenizer (Braun, Melsungen, FRG). After 30 min on ice, homogenates were cleared from nuclei and cell debris by centrifugation at 14,000 × g for 10 min at 4°C. Cultured cells were washed with cold PBS and then homogenized and centrifuged in an analogous manner.

Whole cell extracts were prepared by directly lysing cells with Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and immediate heating to 96°C. The viscosity of the lysate was reduced by ultrasonication (Bandelin, Sonopuls, FRG).

Immunoperoxidase Staining on Tissues. Frozen sections, 5 μm thick, were prepared, air dried, and fixed in acetone. Slides were washed in
Tris buffer (pH 7.4) and incubated with normal swine serum for 15 min. After 3 wash steps, tissues were incubated with hybridoma cell supernatant for 2 h at room temperature. Peroxidase-conjugated rabbit anti-human IgM (Dianova, Hamburg, Federal Republic of Germany) diluted 1:250 was applied for 30 min at 20°C. Slides were then washed 3 times with Tris (pH 7.4) and once with Tris (pH 7.6) before incubation with substrate (Tris, pH 7.6, containing 0.006% diamobenzidine and 0.015% hydrogen peroxide) for 10 min. Sections were then counterstained with hematoxylin.

Indirect Immunofluorescence. Cells grown to subconfluency on microscope slides during an overnight incubation at 37°C were washed with PBS and fixed with methanol/acetone (1:1) for 10 min at room temperature. Thereafter, the samples were washed 3 times with PBS, incubated with hybridoma cell supernatant for 45 min at 37°C, and again washed and incubated with a 1:20 dilution of rabbit anti-human IgM conjugated with fluorescein isothiocyanate for 45 min at 37°C. After washing, the slides were mounted in PBS:glycerol (1:9).

Attachment Assay. The attachment assay was carried out as already described (18). Briefly, tumor cells were plated in hybridoma cell supernatants, normal medium, or medium from SPM4–0 cells. Plates were incubated for 30–60 min at 37°C and washed carefully with PBS. Remaining cells were fixed with 3% paraformaldehyde. An inverted microscope was used to count the attached cells, and inhibition of attachment was evaluated by comparison with controls.

Enrichment of Intermediate Filament Proteins. Cytoskeletal proteins were prepared as previously described (26) in the presence of 1 mM phenylmethylsulfonyl fluoride. This preparation represents the cellular material that is insoluble in solutions containing 1% Triton X-100 and high ionic strength (1.5 M KCl).

The identification of cytokeratin subvariants in two-dimensionally separated SDS-polyacrylamide gels was performed according to the methods given in Ref. 27.

Electrophoretic Procedures. SDS-polyacrylamide (10%) gel electrophoresis was performed as described by Laemmli (28).

Two-dimensional electrophoretic separations were carried out as isoelectric focusing in the first dimension and SDS-polyacrylamide gel electrophoresis in the second dimension (29). SDS-denatured samples were applied for isoelectric focusing as described by Comings and Peters (30).

Western Blotting. Electrophoretic transfer of separated components from polyacrylamide gels to 0.45-μm nitrocellulose membranes (Schleicher & Schüll, Dassel, FRG) was performed in a semidy immunoblot apparatus (LKB, Freiburg, FRG) according to the manufacturer's descriptions.

Thereafter, the dried membrane was treated with PBS containing 10% fetal calf serum and 0.5% Tween 20 (buffer A) for 1 h at room temperature followed by a 2-h incubation with hybridoma cell supernatant. After 5 washes with PBS-0.05% Tween 20 (washing buffer), peroxidase-conjugated rabbit anti-human IgM (Dako, Hamburg, Federal Republic of Germany) was added to buffer A at a dilution of 1:1000. After a 2-h incubation, the nitrocellulose membrane was washed 4 times with washing buffer and once with Tris-buffered saline. Color was developed in a freshly prepared mixture of 20 ml Tris-buffered saline, 4 ml 4-chloro-1-naphthol (3 mg/ml in methanol), and 5 μl hydrogen peroxide.

Protein staining on nitrocellulose membranes was performed with a solution of 0.1% Pelikan India ink type 17 (Pelikan, Hannover, FRG) and 0.5% Tween-20 in PBS and subsequent washing in washing buffer (31).

Competitive Immunoadsorption Using Anti-TPA Antibodies. The competition of anti-TPA antibodies with humAb 56/16 was studied on a Western blot prepared with a whole cellular extract of 23132 stomach carcinoma cells. After the initial treatment with buffer A the nitrocellulose membrane was incubated with a preparation of affinity-purified antibodies from a rabbit anti-TPA:Bi serum (20) (Sanget Medical, Bromma, Sweden) for 2 h at room temperature. Thereafter, the blot was washed 3 times with washing buffer, and binding of humAb 56/16 was investigated as described. In control experiments we detected strong binding of the rabbit antibodies to polypeptides in the molecular weight range of 40,000–53,000.

RESULTS

Generation and Initial Screening of mAb 56/16. The mAb 56/16 (IgM, lambda) was produced by fusion of spleen and lymph node lymphocytes from a patient with a gastric signet-ring carcinoma with the heteromyeloma cell SPM4–0. In the attempt to isolate cell surface-reactive antibodies, mAb 56/16 was preselected for its ability to inhibit the adhesion of primary tumor cells to cell culture dishes.

Immunohistochemical and Immunocytochemical Reactivity of mAb 56/16. Indirect immunoperoxidase-staining assays with fresh cryostat sections of autologous and allogeneic tumors reveal the presence of structures reactive with mAb 56/16 on signet-ring cells (Fig. 1, a and b).

In order to obtain further information concerning the subcellular distribution of the 56/16 antigen(s), fluorescence experiments were performed. On 23132 stomach carcinoma cells, which show a strong tendency to grow in tightly associated cell clusters, mAb 56/16 identifies components that are located on or near the plasma membrane, preferentially on the periphery of larger cell groups (Fig. 2, a).

Biochemical Characterization of the mAb 56/16-Binding Antigen. Western blot analysis was performed to study the distribution of the mAb 56/16-binding antigen in various tumor and normal tissues and in established cell lines. In these experiments, mAb 56/16 identifies various components in the molecular weight range of 38,000–45,000 (Fig. 3; Table 1).

A rather faintly stained band, M, 38,000, is observed in several normal tissues and tumors of the digestive tract. In some cases an additional more intense and sharp band appears at M, 45,000 (Fig. 3B and Table 1). High amounts of this component can be identified in extracts of pancreas or the submandibular gland (Fig. 3, D and E). This finding is further confirmed by immunohistochemical staining of pancreatic and submandibular gland tissue (Fig. 1, c–e). An M, 45,000 band is also observed in extracts of the stomach cancer cell line 23132. But the amount of the antigen in the extract prepared with the use of a nonionic detergent is low compared to the strong reaction with a whole-cell lysate of these cells (Fig. 3, G and H).

Therefore, we concluded that a part of mAb 56/16-reactive antigens had not been rendered soluble during the extraction step. This finding is reminiscent of the known insolubility of certain proteins of the cytoskeleton in buffers containing nonionic detergents. Hence, we made cytoskeletal preparations of 23132 cells according to a known procedure, which includes extraction with buffers containing Triton X-100 (1%) and high salt concentrations (1.5 M KCl) (26).

mAb 56/16-binding components (M, 45,000) are highly enriched in these preparations (Fig. 3D). Two-dimensional immunoblot analysis reveals a selective reaction of the antibody with minor polypeptides of the same molecular weight as cytokeratin 18 (45,000) but with more acidic isoelectric pH values (Fig. 4, A and B). The major variants of cytokeratin 8 and 18 are not stained.

Comparison of the Reactivity Pattern of Human mAb 56/16 with Antibodies Reactive with Cytokeratin 8 and the Tumor Marker TPA. mAb 35/H11, a mouse mAb reported to be specific for cytokeratin 8 (26), was chosen for a comparative analysis of its reactivity pattern with that of mAb 56/16. Immunohistochemical (Fig. 1F), immunocytochemical (Fig. 2B), and immunoblotting (Fig. 5) procedures reveal overlapping specificities of both mAbs. In two-dimensional immunoblot analysis with cytoskeletal preparations of 23132 cells, mAb 35/H11 identifies the major variant of cytokeratin 8 (52,500)
Fig. 1. Immunoperoxidase staining of humAb 56/16 (a, c, and e) and mouse mAb 358H11 (f) on cryostat sections of a gastric signet-ring cell carcinoma (a and b), normal submandibular gland (c and d), and normal pancreas (e and f). b and d, controls without a first antibody. a-f, × 370.
Fig. 2. Immunofluorescence staining of 23132 stomach carcinoma cells with humAb 56/16 (A) and mouse mAb 35/BH11 (B). (Under the conditions used, unspecific staining with the fluorescein isothiocyanate-conjugated second antibody alone was not detectable.) × 925.

Table 1: Identification of humAb 56/16-binding components in extracts of human cells and tissues by immunoblotting.

<table>
<thead>
<tr>
<th>Tissue/cell line</th>
<th>Reactivity with humAb 56/16</th>
<th>M, (in thousands) of the recognized components</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach adenocarcinoma</td>
<td>14/16*</td>
<td>38 (14), 45 (2)</td>
</tr>
<tr>
<td>Stomach adenocarcinoma after a passage in the nude mouse</td>
<td>1/1*</td>
<td>45</td>
</tr>
<tr>
<td>Stomach lymphoma</td>
<td>0/1</td>
<td>38</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>1/1</td>
<td>45</td>
</tr>
<tr>
<td>Thymoma</td>
<td>0/2</td>
<td>45</td>
</tr>
<tr>
<td>Squamous cell carcinoma of the lung</td>
<td>0/2</td>
<td>45</td>
</tr>
<tr>
<td><strong>Normal tissues</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach mucosa</td>
<td>8/9</td>
<td>38 (8), 45 (5)</td>
</tr>
<tr>
<td>Colon mucosa</td>
<td>1/1</td>
<td>45</td>
</tr>
<tr>
<td>Duodenal mucosa</td>
<td>1/1</td>
<td>38</td>
</tr>
<tr>
<td>Esophagus</td>
<td>1/1</td>
<td>38</td>
</tr>
<tr>
<td>Pancreas</td>
<td>4/5</td>
<td>45</td>
</tr>
<tr>
<td>Submandibular gland</td>
<td>1/1</td>
<td>45</td>
</tr>
<tr>
<td>Parotis gland</td>
<td>1/1</td>
<td>45</td>
</tr>
<tr>
<td><strong>Cell lines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCECM (melanoma)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>59/8-2 (heteromyeloma)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>U937 (leukemia)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mol4 (leukemia)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>T671 (rhabdomyosarkoma)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>23132 (stomach carcinoma)</td>
<td>+</td>
<td>45</td>
</tr>
<tr>
<td>200 (stomach carcinoma)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HT29 (colon carcinoma)</td>
<td>+</td>
<td>45</td>
</tr>
<tr>
<td>A431 (vulva carcinoma)</td>
<td>+</td>
<td>45</td>
</tr>
</tbody>
</table>

* Number of reactive specimens/total number of specimens.

** DISCUSSION **

The human monoclonal antibody 56/16 was preselected for its ability to inhibit the adhesion of tumor cells in vitro. The activity in this functional binding assay together with the immunofluorescence staining pattern of 23132 cells (Fig. 2A) indicated an interaction of the antibody with the cell surface of stomach carcinoma cells. Furthermore, mAb 56/16 showed an exceptionally intense staining on immunoblots from 23132...
extracts, which is a rather uncommon feature in our experience with humAbs from tumor patients.

These early observations encouraged us to undertake a broader and more detailed screening of the reactivity pattern of the antibody. The data obtained from Western blot analysis of extracts from different normal and tumor tissues identify the mAb 56/16-binding antigens as Mr 38,000 and 45,000 polypeptides present in a variety of epithelial tissues of the gastrointestinal tract. An enrichment especially of the Mr 45,000 antigens is demonstrated in extracts of pancreas and submandibular and parotid glands.

These Mr 45,000 antigens were subsequently shown to be degradative products of the cytokeratin 8 molecule. Interestingly, the undegraded cytokeratin 8 variant is unreactive with mAb 56/16. TPA, one of the first serum tumor markers (22), has recently been immunologically related to soluble proteolytic.

Fig. 4. Immunoblot with mAb 56/16 (A) and mouse mAb 355H11 (C) performed on cytoskeletal preparations on 23132 stomach carcinoma cells two-dimensionally separated by isoelectric focusing (IEF) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). B, the same blot as in A, additionally stained with India ink; D, Coomassie-stained gel [actin (A); bovine serum albumin (B) added as internal standard; B, cytokeratin 8 (main variant), Mr = 52,500, isoelectric point (i.P.) = 6.1; 18, cytokeratin 18 (main variant), Mr = 45,000, ioelectric point = 5.7].

Fig. 5. Comparison of the reactivity patterns of humAb 56/16 (x) and mouse mAb 355H11 (y) as revealed by immunoblotting on extracts of pancreas (A), the submandibular gland (B), a tumor of 23132 stomach carcinoma cells in the nude mouse (C), and A431 vulva carcinoma cells (D), as well as on a cytoskeletal preparation of 23132 stomach carcinoma cells (E).
fragments of cytokeratins 8, 18, and 19 (20). Sequence analysis of human cytokeratin 8 revealed identity with reported amino acid sequences of fragments of human TPA (21). It consists of 38,000-44,000 polypeptides and its serum levels are often used to monitor tumor progression in carcinoma patients. For gastric cancers, Kanauchi et al. (32) reported particularly high serum TPA levels in well-differentiated and signet-ring cell carcinomas, which clearly correlate with the tumor stage. HumAb 56/16 might, therefore, be the result of an immune response against TPA-related polypeptides and identifies an epitope that has become immunogenic after proteolysis of cytokeratin 8. The presence of such cytokeratin 8 fragments in TPA preparations, as well as the relation of humAb 56/16 to TPA, is further confirmed by the competition between mAb 56/16 and polyclonal anti-TPA:B1 antibodies in detecting the M, 45,000 antigens on an immunoblot. The mAb 56/16 binding, M, 45,000 polypeptides in cell and tissue extracts represent soluble fragments of keratin 8. They can be regarded as indicators for intracellular turnover rates of cytoskeletal components. This turnover is expected to be high in cells with secretory activity, e.g., pancreas or in mucus-producing tumor cells, such as signet-ring cells or the 23132 cell line (33). In these cells, the transport of secretory vesicles is based on a permanent degradation and rebuilding of the cytoskeleton. Transformed secretory cells and tissues are thus a predominant potential source of TPA-like immunogens which may be released into the circulation either by a secretory process (23, 24) or during necrosis of tumor cells (25).

The molecular nature of the mAb 56/16-binding M, 38,000 antigen detected in tissues and tumors of the digestive tract has not been unequivocally clarified. It might be a proteolytic fragment of the M, 45,000 antigen that has lost the mAb 35/H11-relevant epitope. But, in extracts of 23132 and HT29 cells that express mainly keratins 8 and 18, we never observed a M, 38,000 component reactive with mAb 56/16. Using immunofluorescence or erythrocyte-rosetting tests (34) on viable cells, we were not able to clearly demonstrate the presence of mAb 56/16-binding structures on the surface of tumor cells. Therefore, a possible interaction of the antibody with the cell surface (as indicated by its reactivity in the attachment inhibition assay) can so far not be confirmed by further experimental data.

In conclusion, the detailed biochemical analysis of the humAb 56/16 reactivity pattern reveals a highly specific binding to an epitope on the cytokeratin 8 molecule that is rendered accessible solely after its proteolytic degradation. First, the specificity of this interaction gives strong evidence that mAb 56/16 is the result of an autoimmune reaction in the patient induced by a tumor-associated presentation of TPA-related epitopes to the immune system. Furthermore, it excludes mAb 56/16 from the common group of low-affinity and polycpecific antibodies (16) with cross-reactivity for cytoskeletal components. They have been observed by various authors (12, 13) and are preferentially detected by immunostaining procedures. Usually, these mAbs show only very weak or no reactivity in Western blotting, and this reactivity cannot be of great specificity. In contrast, Cote et al. (12) and Skaletsky et al. (5) reported the isolation of humAbs from axillary lymph nodes of patients with breast cancer that identify M, 45,000 cytokeratin components in Western blots of breast cancer cells. Although they did not define the exact nature of the recognized antigen, these antibodies might have a similar origin as mAb 56/16.

These findings indicate an association of the development of tumors with certain autoimmune phenomena. Because of the presence of the responsible antigens in the serum, the evoked autoimmune reactions cannot be expected to have a negative influence on tumor growth. On the contrary, these mechanisms have something in common with the known process of antigen shedding by tumor cells that is supposed to function as a defense mechanism of the tumor against destruction by the immune system.

ACKNOWLEDGMENTS

We thank Dr. T. Papadopoulos and Dr. M. G. Braun for helpful discussion and I. Weglein and J. Klug for excellent technical assistance. We are also grateful for the help of Dr. J. Müller and Dr. T. Kirchner for preparing the stomach carcinoma specimens and for the help of Dr. A. Marx for providing the extracts from thymomas.

REFERENCES

19. Vollmers, H. P., Imhof, B., Wieland, I., Hiesel, A., and Birchmeier, W. Monoclonal antibodies NORM-1 and NORM-2 induce normal behav...


Human Monoclonal Antibody against a Tissue Polypeptide Antigen-related Protein from a Patient with a Signet-Ring Cell Carcinoma of the Stomach


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/16/5192

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.