Human Epithelial Cancers Secrete Immunoglobulin G with Unidentified Specificity to Promote Growth and Survival of Tumor Cells

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ABSTRACT

Immunoglobulins (Igs) are found thus far only to be produced by differentiated B lymphocytes. By immunohistochemistry analysis, in situ hybridization, and laser capture microdissection-assisted single-cell PCR, we demonstrate that human cancers of epithelial origin, including carcinomas of breast, colon, liver, and lung, established epithelial cancer lines, as well as some normal lung tissues, also produce IgG in both cytoplasmic and secreted forms. Furthermore, blockade of tumor-derived IgG by either antisense DNA or antihuman IgG antibody increased programmed cell death and inhibited growth of cancer cells in vitro. More importantly, administration of antihuman IgG antibody also suppressed the growth of an IgG-secreting carcinoma line in immunodeficient nude mice. Our results support a role of tumor-derived IgG as a growth factor for epithelial cancers. Prevalent expression of IgG in human carcinomas and its growth-promoting functions may have important implications in growth regulation and targeted therapy of human cancers.

INTRODUCTION

Elevated level of serum IgG, IgA, or IgM antibodies is frequently observed in patients with cancers of epithelial origin, including carcinomas of breast, colon, and liver (1, 2). These tumor-reactive Iggs⁴ have been interpreted as humoral responses of host to cancer growth (3). In fact, by Serex method, it has been firmly established that these tumor-reactive antibodies are capable of binding to normal and tumor-associated antigens, including those of cell surface and intracellular proteins (3–5). However, monoclonal gammopathy was observed not only in patients with neoplastic proliferation of B cell and/or plasma cells but also in those with malignant tumor of epithelial origin (6–9). These observations suggest the possibility that the Ig is produced by the epithelial cancer cells. In this article, we present unexpected findings that IgG with unidentified specificity could be secreted directly from cancer cells, and these antibodies are involved in their survival and growth.

MATERIALS AND METHODS

Tissue Samples. Formalin-fixed, paraffin-embedded biopsy specimens of tumor and corresponding normal tissues were collected from Department of Pathology, Norman Bethune University of Medical Sciences and Department of Pathology, Taiping Road Hospital. Fresh biopsy samples were obtained from Department of Pathology, Beijing Railway Hospital. All procedures were performed with the approval of the Institutional Review Board and proper consent from patients (if any). For immunohistochemical staining, the biopsy tissues had been fixed immediately in 10% buffered formalin and, 24 h later, dehydrated in increasing concentrations of alcohol and embedded in paraffin. Sections of 5 μm were made. Fresh biopsy samples were used for the isolation of tumor cells.

Cell Lines. Human breast cancer cell line (MCF-7) and colon cancer cell lines (HT-29, LOVO) were purchased from Beijing Institute for Tumor Prevention and Treatment; human cervical cancer cell line (HeLa MR) was a gift of Dr. Shouping Ji (Academy of Military Medical Science); cervical cancer cell line (HeLa S3), lung cancer cell line (A549), ovarian cancer cell line (CaOV3), and B lymphocytic leukemia cell lines (Raji and Daudi) were supplied by Beijing University Center for Human Disease Genomics. The MCF-7, HT-29, LOVO, HeLa MR, HeLa S3, A549, and CaOV3 were cultured in DMEM with 10% FCS, whereas Raji and Daudi cell lines were cultured in RPMI 1640 10% FCS at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂.

Immunohistochemistry. The tissue sections were dewaxed, rehydrated through graded ethanol washes, placed in 10 mM citrate buffer (pH 6.0), and then heated twice in microwave oven for 5 min each. The slides were incubated with 0.3% hydrogen peroxide for 5 min, washed with PBS, and blocked in PBS plus 10% normal goat serum for 10 min. After excess blocking buffer was removed, the direct immunohistochemical staining was performed with goat antihuman IgG-HRP to detect IgG (1:100; Dako, Carpinteria, CA). Slides were incubated for 45 min with antibody at 37°C in a humidified chamber. After thorough washing, the bound antibody was detected using DAB; control sections were stained with goat antimouse IgG-HRP (1:100; Dako). Indirect immunohistochemical staining was performed to detect CD20 using mouse antihuman CD20 mAb (CD20cy, clone: L26, 1:200; Dako). To do so, the mAb was added to slides and incubated for 45 min with antibody at 37°C, after washing three times with PBS, the slides were incubated with goat antimouse IgG-HRP for 40 min at 37°C; after extensive washing, the bound antibody was detected using DAB.

Purification of Tumor Cells. Tumor tissue suspension was prepared from fresh biopsy cancer tissues of the breast, colon, liver, and lung. After passing through a 90-µm mesh and washing twice with Hanks solution, lymphocytes in the cell suspension were removed by gradient centrifugation in lymphocyte separation medium (Shanghai Chemical Reagent, Inc., Shanghai, China). The separated cancer cells were washed and resuspended in Hanks solution. To detect IgG in the isolated cancer cells, goat antihuman IgG-HRP (1:100) was used in direct immunocytochemical staining.

Extraction and Purification of Ig Molecules. Cancer cells were treated with lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH7.5), 25% sucrose, 0.8 µM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride] on ice for 20 min. The lysate was centrifuged for 25 min at 15,000 rpm/min at 4°C. The pellet was discarded, and the supernatant containing the cytosol protein was added to CM-Sepharose (Pharmacia, Peapack, NJ), and the bound IgG was eluted by different concentrations (0.5, 0.8, 1.0, and 1.5 M) of NaCl. Fractions collected were analyzed by SDS-PAGE, Western blot, or capillary electrophoresis. For simplicity, sometimes the Ig in the cytosol protein was not separated by CM-Sepharose but directly analyzed by SDS-PAGE and Western blot after protein concentration was determined according to A_{280} nm.

SDS-PAGE and Western Blot. The cytosol protein (crude protein at 40 µg/well; purified protein at 10 µg/well) was analyzed in 10% SDS-PAGE (under reducing and nonreducing conditions) and stained with Coomassie Brilliant Blue. For Western blot analysis, after electrophoresis, the separated proteins on the gel were transferred to polyvinylidene difluoride membrane. In

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3 The abbreviations used are: Ig, immunoglobulin; HPR, horseradish peroxidase; DAB, 3,3′-diaminobenzidine; mAAb, monoclonal antibody; CD83, immunoglobulin heavy chain complementary-determining region 3; RT-PCR, reverse transcription-PCR; LCM, laser capture microdissection; FACS, fluorescence-activated cell sorting; FR3, immunoglobulin heavy chain framework 3 region; RAG, recombinase-activating gene; ASODN, antisense oligodeoxynucleotide; RODN, random oligodeoxynucleotide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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direct staining, goat antihuman IgG-HRP (1:1000) was used. In indirect staining, mAbs against IgG heavy chain (1:1000; Sigma, St. Louis, MO), κ chain, and A chain (Dako, Glostrup, Denmark; 1:1000) were used as primary antibodies, and goat antihuman IgG-HRP (Dako; 1:1000) was used as secondary antibody.

In Situ Hybridization. Parafomaldehyde-fixed and paraffin-embedded tissue sections (5 μm) were deparaffinized, dehydrated, and incubated in 0.2 M HCl for 20 min. The sections were treated with protease K, fixed with paraformaldehyde, prehybridized at 42°C for 4 h, and hybridized overnight at 46°C with human IgG Fc cRNA as probe. After hybridization, the sections were washed in 2× SSC and 0.2× SSC (65°C) and then treated with RNase. The samples were incubated with antidigoxigenin antibody conjugated with alkaline phosphatase (dilution 1:500; Roche Diagnostics, Rotkreuz, Switzerland). 5-Bromo-4-chloro-3-indolyl phosphate and nitro-blue-tetrazolium (Sigma) were used for color reaction. In control experiments, the slides were incubated with RNase or with corresponding sense probes.

Northern Blot Analysis. Total RNA was extracted from normal lung and lung carcinoma tissues using Trizol Reagent (Life Technologies, Inc.) followed by electrophoresis. The RNA was transferred onto nylon membranes and cross-linked by UV irradiation. The membranes were prehybridized using hybridization solution (Life Technologies, Inc.) for 6 h at 42°C and hybridized with 32P-labeled IgG Fc cDNA probe for 16 h at 68°C. After extensive washing, positive signals were displayed on a phosphor screen.

Single-Cell RT-PCR. Frozen lung cancer samples were sectioned at 8 μm and mounted on slides, and slides containing frozen sections were quickly fixed in 70% ethanol for 5 min, stained with hematoxylin for 1 min, counterstained with eosin for 2 min, and then quickly rinsed in diethyl pyrocarbonate-fixed in 70% ethanol for 5 min, stained with hematoxylin for 1 min, counterstained with eosin for 2 min, and then quickly rinsed in diethyl pyrocarbonate-treated water. Slides were dried in a fume hood for 5 min and then was used to label using the PALM Microlaser Systems (PALM MikroLasertechnologie). The total RNA microdissected cancer cells was isolated following the standard RNeasy Mini Kit (Qiagen, Hilden, Germany) protocol.

Reverse transcription was carried out with SssI cDNA synthesis enzyme (Stratagene) and random hexamers (Pharmacia). The synthesized cDNA was amplified using the primers 5′-GAGGTTCGGTAGTGGACG-3′ and 5′-GACAGACGTAGCCTTG-3′, and the downstream primer was 5′-GAGGTTCGGTAGTGGACG-3′. PCR products were separated on 1.5% agarose gel by electrophoresis. The identification of the PCR product was achieved after annexin V and PI staining by flow cytometry.

Table 1 IgG expression in human cancers and normal tissues.

<table>
<thead>
<tr>
<th>Case</th>
<th>Positive/total (%)</th>
<th>Positive degree</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Malignant epithelial tumor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast cancer</td>
<td>10/10 (100%)</td>
<td>++</td>
</tr>
<tr>
<td>Liver cancer</td>
<td>14/14 (100%)</td>
<td>++</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>6/6 (100%)</td>
<td>++</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>8/12 (66.6%)</td>
<td>+</td>
</tr>
<tr>
<td>Total</td>
<td>38/42 (90%)</td>
<td>++</td>
</tr>
<tr>
<td><strong>Normal epithelial tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal breast tissue</td>
<td>0/10 (0)</td>
<td></td>
</tr>
<tr>
<td>Normal liver tissue</td>
<td>0/14 (0)</td>
<td></td>
</tr>
<tr>
<td>Normal colon tissue</td>
<td>0/6 (0)</td>
<td></td>
</tr>
<tr>
<td>Normal lung tissue</td>
<td>4/10 (40%)</td>
<td>+</td>
</tr>
<tr>
<td>Total</td>
<td>4/40 (10%)</td>
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</table>

- Percentage of IgG positive cancer tissues and normal tissues.
- Degree of positive IgG expression on tumor tissues and normal tissues is determined by immunohistochemical staining. ++, positive cell under 10%; +, positive cells 10–50%; ++, positive cells 50–70%; +++, positive cells 70–100%.

Results

IgG Expression in Human Cancer Tissues. We first used human IgG-specific monoclonal or polyclonal antibodies in immunohistochemistry to determine the expression of IgG in malignant epithelial tumors. In all evaluated cancer tissues, including those from breast cancer (n = 10), liver cancer (n = 14), colon cancer (n = 6), and lung cancer (n = 12), positive staining was detected in the cytoplasm of all cancer tissues. In contrast, IgG was not detected in similar numbers of normal tissues of breast, colon, liver, and lung, except 4 of 10 normal lung tissues, some cells in the basal layer of

Animal Studies. Cultured HeLa MR cells (5 × 10⁴) were s.c. inoculated into nude mice (BALB/c). When the tumor became palpable, polyclonal antibody of goat antihuman IgG [purified by protein A-Sepharose 4B column, a gift from PLA Logistic University (Changchun, China), with an antibody titer of 1:125 by double diffusion test] was injected 1.5 mg/tumor intratumorally for five times with a 3-day interval. The growth of the tumor was monitored every day. On day 21 after tumor cell inoculation, the tumors were excised and histologically examined. Nude mice with HeLa MR xenografts treated with normal goat IgG (PLA Logistic University, also purified by protein A-Sepharose 4B column), and PBS was used as controls.

RESULTS

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IgG EXPRESSION IN EPITHELIAL TUMOR CELLS

A

Breast cancer
Normal breast tissue
Liver cancer
Normal liver tissue
Colorectal cancer
Normal colon tissue
Lung cancer
Normal lung tissue

C

59KD
23KD

B

Breast cancer cells
Liver cancer cells
Solvent control

D

Western blot
Non-reduce SDS-PAGE
Non-reducing
Reducing
150KDa
50KDa

E

Breast cancer
Normal breast tissue

F

Lung cancer
Normal lung tissue

G

Before LCM
After LCM
epithelium and cells close to cancer tissues. In addition, immunoreactivity did not correlate with the amount of B lymphocyte (CD20-positive cells) and plasma cell found in cancer tissues; furthermore, tumor-infiltrating plasma cells were generally negative or weakly positive reaction by antihuman IgG staining (Table 1, Fig. 1A).

To minimize contamination of infiltrating B cells in cancer tissues, we isolated tumor cells by gradient centrifugation from fresh biopsy tissues of breast cancer (n/H11005/4), colon cancer (n/H11005/4), liver cancer (n/H11005/4), and lung cancer (n/H11005/2). After removal of lymphocytes (including plasma cells) by gradient centrifugation, IgG in the separated breast, liver, and colon cancer cells were detected by immunocytochemical staining (Fig. 1B). IgG was distinctly detected in the cancer cells by Western blotting (Fig. 1C).

Protein extract of enriched cancer cells from a case of lung cancer was further purified by ion exchange chromatography on CM-Sepharose, and IgG in these tumor cells was confirmed in Western blot analysis using specific antibodies (Fig. 1D). Finally, NH2-terminal sequence analysis of the purified protein detected the 14 amino acid residues that were identical to those of the published IgG sequences (N-VQLVESGGGLVHPG).

We also examined the expression of Ig mRNA by in situ hybridization using the cRNA of IgG Fc segment as a probe. Human IgG gene transcripts were demonstrated in all 6 breast cancer samples (Fig. 1E), all 10 lung cancer samples, and a part of adjacent normal epithelial cells of the breast and lung. Furthermore, we also demonstrated IgG expression by Northern blot analysis using cDNA of IgG Fc segment in 5 lung cancer tissues and in 5 adjacent normal lung tissues. Finally, Ig γ chain mRNA was also detected in all 5 lung cancer tissues and 3 of 5 adjacent lung tissues (Fig. 1F).

Cancer cells were isolated by LCM technique from the same lung cancer sample (the protein purified from this sample was Ig by NH2-terminal sequence analysis as described above), and the IgG heavy chain (including V, D, J, and CH1 region) was amplified by single-cell RT-PCR. This result confirmed that Ig gene recombination, a hallmark of functional Ig, exists in the cancer cells. Moreover, three clonal sequences sequenced were completely identical (Fig. 1H), suggesting limited diversity of cancer-associated IgG.

IgG Expression in Long-Term Cultured Human Cancer Lines. To entirely eliminate contamination of B cells in cancer tissues, we examined the expression of IgG also in long-term cultured human cancer lines of epithelial origin. FACS analysis demonstrated IgG expression in breast cancer (MCF-7), colon cancer (HT-29, LOVO), liver cancer (BCL-7402), ovarian cancer (CaOV3), lung cancer (A549), cervical cancer (HeLaS3 and HeLa MR), and two B lymphoma cell lines (Raji and Daudi). IgG immunoreactivity located predominantly in cytoplasm (30–90%) than in plasma membrane (15–20%; Fig. 2A). We have used antihuman CD19 (pan B cells) mAb to distinguish cancer cell lines from B-cell lines.
CD19 was found on B-cell line (Raji) but not on cancer cell line A549 (Fig. 2B), HT-29, and HeLaS3 (data not shown). The culture supernatant of two cervical cancer cell lines was also positive for IgG as determined by ELISA, suggesting that these cancer lines secrete human IgG (Fig. 2C). Consistent with this finding, Western blot analysis of lysate of cancer cell lines demonstrated immune staining at \( M_r \) 150,000, \( M_r \) 50,000, and \( M_r \) 25,000, which correspond to the molecular sizes for IgG, in several cancer cell lines using monoclonal antibodies against human Ig \( \gamma \) chain and \( \kappa \) chain (Fig. 2D).

V-D-J recombination of Ig heavy chain was analyzed in these epithelial tumor lines. The third CDR (CDR3) of IgG heavy chain gene was amplified by RT-PCR using a set of primers. The CDR3 segments, which include the V-D-J sequence and complementary to 3'-end of J region (bottom); the primers for nest-PCR amplification were segments of FR3 and J region. The identification of the PCR product was confirmed by 2.5% agarose gel electrophoresis and DNA sequencing. F. RAG-1 and RAG-2 mRNA were also found in cancer cell lines by RT-nest-PCR. The fact that the amplified segments of RAG-1 and RAG-2 are on one exon of RAG-1 and RAG-2, respectively, excluded the effect of genomic DNA. The total RNA was treated with DNase before amplification. As a negative control, treated total RNA was also used as the template. 1R, A549 RNA as template; 1C, A549 cDNA as template; 2R, HT-29 RNA as template; 2C, HT-29 cDNA as template; 3R, HeLa MR RNA as template; 3C, HeLa MR cDNA as template.
CDR3 in HeLa S3 (GenBank No. AY05372) was also amplified, although the primer sequences used for FR3 and VLJH were not completely complementary.

The RAG-1 and RAG-2 are required for the formation of VDJ recombination. We next examined whether or not human cancers actively expressed these genes. Interestingly, RAG-1 and RAG-2 gene transcription in these cancer cell lines were detected by RT-PCR (Fig. 2F), using the primers as described previously (10). Moreover, both the RAG-1 and RAG-2 segments from cancer cells were identical to the published sequences from B lymphocytes. Our results thus suggest that human epithelial cancer cells have ability to process VDJ recombination.

ASODN and Antihuman Ig Increases Apoptosis and Inhibits Growth of Cancer Cells. To determine the functions of cancer-derived Ig, we first introduced a 15-mer S-oligos of ASODN into A549, HT-29, HeLa MR, and HeLa S3 cells to block the expression of human IgG. RODN was used for comparison. The ASODN was complementary to FR3 of the IgV heavy chain derived from A549, HT-29, and HeLa MR but not completely complementary to HeLa S3. FACS analysis showed significant inhibition of IgG expression in A549 (inhibition rate: 50–70%), HT-29 (inhibition rate: 70–80%), and HeLa MR (inhibition rate: 30–50%) by ASODN. The inhibition of IgG expression induced by RODN in A549 and HT-29 was likely attributable to nonspecific effect, but it was significantly weaker than
LCM-assisted single-cell RT-PCR essentially ruled out this possibility. Our results support that ASODN is selective for the inhibition of Ig gene expression in tumor cells.

We next examined the effect of ASODN on survival of tumor cells. After ASODN was introduced into A549, HT-29, and HeLa MR cells accompanied with an inhibition of IgG expression, apoptosis of tumor cells increased significantly (Fig. 3B). In contrast, no apoptosis occurred in HeLaS3 cells. Our results suggest that tumor IgG expression is required, at least, in part, for survival of tumor cells. The effects of tumor IgG on the growth and survival of tumor cells were additionally confirmed by a goat antibody specific for human IgG. Inclusion of this antibody-induced cell apoptosis and growth inhibition on A549, HT-29, and HeLa MR cells, whereas goat-derived normal IgG and PBS had not effect (Fig. 3C). Accordingly, the antihuman IgG antibody significantly inhibited proliferation of these cancer cell lines in a MTT assay (Fig. 3D). Goat-derived normal IgG or PBS did not induce cancer cell death and growth inhibition.

The effect of antihuman IgG in the growth of HeLa MR tumor in nude mice was evaluated. Inoculation s.c. of HeLa MR led to progressive growth of tumor. Tumor-bearing mice on day 7 were treated intratumorally on a 3-day interval using antihuman IgG. Tumor growth was significantly retarded as compared with those treated with normal goat IgG and PBS (Fig. 4A). Histological examination demonstrated extensive necrosis and apoptosis in the mice treated with antihuman IgG but not control goat IgG or PBS (Fig. 4B). Some of the anti-IgG antibody-treated tumors became liquefied (data not shown). Our results thus indicate that blockade of tumor-derived IgG inhibits growth of tumor and implicates a new method for cancer immunotherapy.

DISCUSSION

In this article, we describe an unexpected finding that human cancers of epithelial origin produce IgG. Moreover, antisense oligos, which block the expression of IgG, also inhibit the growth of tumor cells in vitro. Administration of anti-IgG antibody induced regression of xenografted human tumors that secrete IgG. Our results reveal a novel mechanism for growth of cancer cells and implicate a new target for the manipulation of progressive tumor growth. Several methods were used to verify the expression of IgG from cancer cells. Initial immunohistochemical studies showed IgG immunoreactivity in many tissues derived from a variety of human cancer samples. In addition, tumor cells were isolated from cancer biopsy tissues, and additional analysis was performed. Western blot and NH2-terminal sequence analysis confirmed Ig molecular expression in the isolated cancer cells. Although it is possible that IgA and IgG are transferred into epithelial cells via poly-IgA/G receptor on plasma membrane (11), our further analysis by in situ hybridization and LCM-assisted single-cell RT-PCR essentially ruled out this possibility. Additional studies also detected IgG in many established tumor cell lines by FACS and Western blot analysis; furthermore, the FCS (5%) was used for culture of all cell lines and used in ELISA experiments to detect IgG in cancer cell supernatants. We did not observe significant increase in IgG level unless supernatants from cancer cell cultures were added. We also found that culture of tumor cells in serum-free medium (Chinese Hamster Ovary Cell Culture Media; Life Technologies, Inc.) up to 6 days did not eliminate the detection of IgG as shown by immunocytochemistry staining (12). Interestingly, we found that IgM was also expressed in these tumor cell lines, albeit in much lower level than IgG (data not shown). We have also found that Ig gene V-D-J rearrangement in conjunction with expression of RAG-1 and RAG-2 at mRNA level. Our results thus demonstrate the expression of IgG in human cancers of epithelial origin.

At this time, precise antigenic specificity of the cancer-derived Ig is unknown. High serum levels of autoantibodies were frequently found in patients with cancer (13, 14), and these autoantibodies often recognize intracellular proteins. There is yet no evidence that these autoantibodies are secreted by cancer cells. We found, however, that lung and breast cancer-derived IgG was capable of binding to smooth and striated muscles (data not shown). Moreover, the Ig V-D-J sequence of HT-29 was partially homologous with that of an autoantibody (GenBank no. Y17928). As the sequence of HeLa MR was identical to that of HT-29, it suggests that IgG from the two different cancer cell lines may have autoantibodies reacting to an identical antigen.

Tumor-derived IgG was found to involve, at least, in part, in the survival and growth of epithelial tumor cells because antihuman IgG and ASODN of Ig-induced apoptosis and growth inhibition of cancer cells in vitro and in vivo. It is likely that the cancer-derived Ig stimulates cell growth in an autocrine/paracrine fashion. As a matter of fact, Ig expression was not limited in malignant epithelial cells. Normal proliferating but not resting epithelial cells were also shown to express Ig, as seen in hyperplastic mammary glands, proliferating hepatocytes in cirrhotic liver (data not shown) and epithelium close to cancer tissue. Our results suggest that tumor-derived Ig contributes in cancer initiation in the precancerous stage when the epithelial cells are actively proliferating. Development of methods aiming at selective blockade of tumor-derived IgG thus may constitute a new approach for cancer therapy and prevention.

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Fig. 4. Growth inhibition of HeLa MR in nude mice treated with antihuman IgG. A, after tumor formation, antihuman IgG was injected intratumorally once every 3 days in 13 mice. Injection of normal goat IgG (n = 8) and PBS (n = 13) served as controls. B, on day 21 after treatment, the mice were sacrificed, and the tumors observed histologically (H&E staining).
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