Effects of Monoclonal Anti-Transferrin Receptor Antibodies on in Vitro Growth of Human Solid Tumor Cells

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ABSTRACT

To determine whether anti-transferrin (TF) receptor monoclonal antibodies might be useful in treatment of human solid tumors, in vitro effects of immunoglobulin A (42/6) and immunoglobulin G (B3/25) anti-TF receptor antibodies on human solid tumor growth were examined. In colony and liquid cultures containing 10% serum, B3/25 did not inhibit growth of melanoma or ovarian carcinoma cell lines, 42/6 caused modest dose-dependent inhibition in colony cultures (maximum inhibition approximately 50%), and slowed growth of melanoma, ovarian carcinoma and epidermoid carcinoma cells in liquid culture. Inhibition was more pronounced in low (1%) serum, and was abrogated by 200 µg/ml iron-saturated Tf or 50 µM ferric nitrolate. All cells displayed high affinity Tf receptors (4–20 × 10³/cell). Cells grown in 1% serum and epidermoid carcinoma cells displayed more receptors, and susceptibility to 42/6 inhibition appeared related to higher receptor number. After culture with anti-TF receptor antibodies, solid tumor cells showed a 57–93% reduction in surface Tf-binding sites. Tf uptake by cells grown for 24 h in B3/25 was approximately 50% of control, but was reduced to <10% of control with 42/6. Immunofluorescence staining of melanoma and HL60 promyelocytic leukemia cells suggested greater heterogeneity of TF receptor display on melanoma than on leukemia cells. Previous studies showed 42/6 completely blocked blood cell Tf internalization and is a potent inhibitor of hematopoietic cell growth. In contrast, in solid tumor cells, inhibition of Tf uptake and growth inhibition are subtotal. Solid tumor resistance to 42/6 may be due in part to greater heterogeneity of TF receptor display by proliferating cells. However, responses to iron-saturated Tf and ferric nitrolate in the presence of 42/6 also differ in hemopoietic and solid tumor cells, suggesting possible differences in TF processing or iron growth requirements.

INTRODUCTION

Monoclonal antibodies to human and murine Tf receptors have recently been described (1–6) and can be used to detect Tf receptors on tissues and cultured cells (1–7). These antibodies are also powerful tools for investigating the roles of Tf, iron, and Tf receptors in normal and malignant cell growth (5, 8–11). An unusual IgA anti-Tf receptor antibody, 42/6 (3), profoundly inhibits growth of cultured, normal and malignant human hemopoietic cells (8–11). Rat IgM, anti-mouse Tf receptor antibodies also inhibit in vitro (8, 12) and in vivo (13) malignant murine blood cell growth. These studies suggest anti-Tf receptor antibodies may provide an innovative passive serotherapy for hematological tumors by both marshalling immune responses and inhibiting cell growth (8, 11–14).

Trowbridge and Domingo (2) also showed an anti-human Tf receptor antibody inhibited in vitro growth of a human melanoma xenograft. To determine whether anti-Tf antibodies might be used to treat nonhemopoietic tumors, we examined their in vitro antiproliferative effects on human carcinoma and melanoma cells. In contrast to studies using malignant hemopoietic cells (3, 10, 11, 14) in which 42/6 completely inhibited cell growth and Tf uptake, in solid tumor cells, inhibition of growth and Tf uptake was subtotal. In part, antibody resistance was attributed to heterogeneity of Tf receptor display by solid tumor cells. However, the ability of iron-saturated Tf to overcome solid tumor but not hemopoietic cell 42/6-induced growth inhibition suggests Tf processing or receptor function may differ in these cells.

MATERIALS AND METHODS

Cell Lines. Melanoma lines 242 and 354 were derived from human tumor xenografts (15). These lines contain only human tissue, express human melanoma and other surface antigens, and have karyotype abnormalities similar to other human melanomas. Ovarian carcinoma line 547 was also established from a xenograft. This line contains only human lactic dehydrogenase isoenzyme and chromosomes and has a near tetraploid karyotype (Karyotype analysis was kindly performed by Dr. O. W. Jones, University of California, San Francisco, CA.). Epidermoid carcinoma A431 was originally established from a patient with vulvar carcinoma (16). HL60 promyelocytic leukemia cells (17) were originally a gift from Dr. Stephen Collins (University of Washington, Seattle, WA) and were maintained in RPMI 1640 with 10% FBS (HyClone Laboratories, Logan, UT). Doubling times of these cell lines were: 242, 20–22 h; 354, 20–22 h; 547, 26–28 h; A431, 15–16 h; and HL60, 20–22 h.

Cell Culture Techniques. Tumor colony assays were performed as described elsewhere (15). Briefly, 1 ml underlayers contained McCoy's 5A medium (Grand Island Biological Co., Grand Island, NY) enriched with amino acids, 10% FBS, and 0.6% tryptic soy broth in 0.5% agarose (Seaplaque, Rockland, ME). The overlay contained 10⁴ cells in 1 ml CMRL medium (Grand Island) with 15% FBS, supplemental vitamin C, 2.5 µg/ml porcine insulin, relatively unsaturated human transferrin (Apotransferrin; Sigma Chemical Co., St. Louis, MO), catalase, hydrocortisone, and amino acids in 0.33% agarose. After 10–14 days at 37°C in 5% O₂ and 7.5% CO₂, aggregates of >75 µm were scored as colonies using an inverted microscope equipped with an eyepiece reticle. Cloning efficiency (colonies/cells plated) were 2–5%. To detect effects of anti-Tf receptor antibodies, various concentrations of B3/25 or 42/6 were added at initiation of culture.

Lines 242, 354, and 547 were established and maintained in RPMI medium supplemented with 10⁻⁴ M hydrocortisone, 5 µg/ml porcine insulin, 5 µg/ml relatively unsaturated human transferrin, 5 ng/ml selenium, and 1% FBS. For some studies, these cells were transferred into RPMI 1640 with 10% FBS, maintained for 2–3 weeks, and effects of anti-Tf receptor antibodies and Tf binding were restudied. A431 cells were maintained in RPMI 1640 with 5% NCS (HyClone) as described elsewhere (18). Cells were grown at 37°C in 5% O₂, 7.5% CO₂, and 87.5% N₂ using a Model 2200 incubator (Queue Systems, Parkersburg, WV) equipped for 3-gas injection. When confluent, cells were detached with 0.05% trypsin/0.2% EDTA, and replated at 2 × 10⁶/ml. For cell growth studies, duplicate cell counts were performed on indicated days using trypsin blue dye exclusion. B3/25 or 42/6 at a final concentration of 50 µg/ml were added at initiation of cultures, and cultures were not re-“fed” with antibody.

Antibodies. Monoclonal IgG anti-transferrin receptor antibody B3/25 (6), and IgA anti-Tf receptor 42/6 (3) were kindly provided by Dr. Ian Trowbridge, Salk Institute for Biological Studies, La Jolla, CA. Anti-Tf receptor antibody OKT9 was obtained from Coulter Diagnostics (Hialeah, FL).
Addition of Iron and Transferrin to Cultures. Sera were assayed for iron, and Tf concentrations were estimated by TIBC using a Ferro Chem Model 3050 serum iron/TIBC analyzer. Fetal bovine serum had a TIBC of 299 mg/dl, 72% saturated with iron. Newborn calf serum had a TIBC of 485 mg/dl, 17% iron saturated. FeNTA was prepared as previously described (11), and was added to cultures at final concentrations of 25–50 μM. Relatively unsaturated Tf (Apotransferrin) or fully iron-saturated Tf (Miles Laboratories, Naperville, IL) were added to cultures at concentrations of 5–200 μg/ml.

Detection of Surface Transferrin Receptors. Iron-saturated human Tf was 125I-labeled using chloramine T to a specific activity of approximately 1.5 X 10⁶ cpm/ng protein as previously described (11). Log phase melanoma cells were grown for 24–72 h and then incubated for 2 h in Tf-free medium. Cells were detached using 1 mM EDTA/phosphate-buffered saline, and incubated with various concentrations of labeled Tf for 90 min at 4°C in prewashed 96-well filter plates (V and P Scientific, San Diego, CA). The cells were incubated and washed in phosphate-buffered saline containing 0.2% bovine serum albumin and 0.2% sodium azide, and filters were counted in a gamma counter. Nonspecific binding was detected by incubation with 100-fold excess unlabeled Tf. Data were analyzed by Scatchard analysis (11) using regression lines determined by least squares analysis.

To detect Tf binding to 547 and A431 cells, cells cultured for 24–72 h were detached using trypsin/EDTA and plated into 16-mm multiwell dishes (Falcon Plastics, Cockeysville, MD). After 24 h in culture, cells were incubated in Tf-free medium and then with various concentrations of labeled Tf at 4°C, washed as described above, and solubilized with 0.1 N NaOH. The solubilized cells were then counted in a gamma counter.

For immunofluorescence staining, melanoma cells were detached with 0.5% EDTA, incubated at 4°C with 10–20 μg/ml of anti-Tf receptor antibodies, and stained with fluoresceinated goat anti-mouse immunoglobulin (Tago, Inc., Burlingame, CA). Positive cells were enumerated on an Ortho 50H cytofluorograph. A mixture of mouse immunoglobulins served as a control. Simultaneous analysis of DNA content was performed as previously described (11) using propidium iodide. HL60 promyelocytic leukemia cells were stained in a similar manner. For comparisons of fluorescence intensity, cytofluorographic analysis was performed using a single gain setting.

Assessment of Antibody Effects on Transferrin Binding and Uptake. To assess effects of anti-Tf receptor antibodies on Tf binding, binding at 4°C was determined on 547 ovarian carcinoma cells grown for 24 h in media with 10% FBS or with FBS and 50 μg/ml B3/25 or 42/6. Previous studies using hemopoietic cells showed maximal antibody effects on Tf binding and uptake by this time (14). To assess Tf uptake, 547 cells were grown with or without antibodies for 24 h, incubated for 2 h at 4°C with 100 nm radiolabeled Tf in binding buffer, and then incubated for 30 min at 37°C. An aliquot of cells was solubilized to determine total cell-associated Tf. From a separate aliquot, cell surface bound Tf was eluted by a 10-min incubation with 0.5 M NaCl/0.2 M acetic acid (pH 2.5), the cells were solubilized, and non-surface bound Tf was determined by gamma counting (14). Binding studies using cells incubated at 4°C with 0.2% sodium azide showed that >98% of surface bound counts were removed by this procedure.

Statistical Methods. Correlation analysis was performed using the standard product-moment formula. When the same cell line was tested for effects of anti-Tf receptor antibodies under differing culture conditions (i.e., 1 or 5% serum), each set of inhibition curves and Tf binding were considered as separate events.

RESULTS

To compare effects of anti-Tf receptor antibodies on solid tumors to previous studies using hemopoietic cells (10), melanoma and ovarian carcinoma cells were grown in 10% FBS using a colony-forming assay. As shown in Fig. 1, antibody 42/6 had no effect on ovarian carcinoma colony growth, and caused minimal (approximately 50%) dose-dependent inhibition of melanoma colony growth. In parallel studies using HL60 cells grown in colony assay (10), 10 μg/ml 42/6 completely abrogated colony formation. Antibody B3/25 was an even less effective inhibitor of solid tumor colony growth (Fig. 1). Increasing the antibody concentrations to 100 μg/ml did not result in further growth inhibition (data not shown).

When liquid cultures containing 5–10% serum were used, 50 μg/ml B3/25 did not inhibit melanoma or ovarian carcinoma growth (data not shown), and antibody 42/6 showed little ability to inhibit solid tumor cell growth (Fig. 2). To allow for widely differing growth rates, average results of 2–3 experiments using each cell line are shown as percentage of control growth. In the presence of 50 μg/ml 42/6 and 10% FBS, melanoma and ovary carcinoma cell growth was inhibited only slightly (<20%), but A431 cells grown in 5% NCS showed more growth inhibition. Again, increasing the antibody concentration to 100 μg/ml did not increase growth inhibition (data not shown). Thus, in 5–10% serum, anti-Tf receptor monoclonal antibodies were weak inhibitors of non-hemopoietic tumor cell proliferation.

Since A431 cells were grown in NCS containing less saturated Tf, these results suggested serum concentration or Tf/iron content might affect 42/6-induced inhibition. To examine this possibility, cell lines 242, 354, and 547 were grown as previously described (15) in Tf-supplemented medium with 1% FBS and 50 μg/ml 42/6. Representative studies using melanomas 242 and 354 are shown in Fig. 3. Antibody-induced inhibition increased (45–60% of control at day 7) somewhat over that seen in 10% FBS, but cell growth was still observed. Line 547 ovarian cancer cells, which showed less than 10% inhibition in 10% FBS (Fig. 2), also showed increased inhibition (60% of
control on day 7) in 1% FBS. Addition of relatively unsaturated Tf at concentrations of 25–50 μg/ml failed to reverse 42/6-induced growth inhibition. However, 50 μg/ml fully iron-saturated Tf partially reversed the inhibition (Fig. 3). Further studies showed dose-dependent effects and full reversal of antibody inhibition at a concentration of 200 μg/ml iron-saturated Tf. In contrast, parallel studies with HL60 cells and 42/6 showed no effect of iron-saturated Tf at any concentration. FeNTA also completely reversed 42/6-induced inhibition of ovarian carcinoma cells grown with 1% fetal bovine serum, and A431 epidermoid carcinoma cells grown in 5% newborn bovine serum.

The presence of Tf receptors on the solid tumor lines was confirmed by radioligand binding and immunofluorescence staining. As shown in Table 1, whether cultured in 1% or 10% FBS, all lines expressed high affinity surface Tf receptors. Scatchard analyses showed a single class of receptors, and affinities were similar to those reported previously for human Tf receptors (11, 14). However, average receptor numbers/cell of 3- to 5-fold higher on these cells than on myeloid leukemia cells studied in our laboratory (11). Cells cultured in 5–10% serum displayed fewer receptors than the same cells grown in Tf-supplemented medium with 1% FBS (Table 1), and A431 cells grown in NCS showed 3-fold greater receptor number than melanoma cells. Thus, the ability of 42/6 to inhibit cell growth appeared related to receptor number. Correlation analysis of percentage of inhibition on day 5 in liquid culture and surface Tf receptor number showed a significant relationship (r = +0.82, P < 0.01). A431 cells displayed the greatest sensitivity to 42/6 and the highest receptor numbers. Melanoma cells were intermediate in receptor number and sensitivity, while 547 cells were the least sensitive and had the lowest receptor numbers.

To assess effects of anti-Tf receptor antibodies on solid tumor cell Tf binding, ovarian carcinoma cells were grown for 24 h in 10% FBS or FBS with 50 μg/ml B3/25 or 42/6, and Tf binding was assessed at 4°C. As shown in Table 2, culture with B3/25 resulted in an approximately 60% decrease in surface Tf binding. In contrast, after culture with 42/6, Tf binding was reduced to <10% of control.

To assess antibody effects on solid tumor Tf uptake, cells were grown with antibodies for 24 h, incubated with a saturating concentration of Tf at 4°C, and warmed to 37°C (Table 2). Acid elution studies showed that control cells took up 83% of bound Tf. Cells previously grown with B3/25 showed reduced total cell-associated and internalized Tf, but the relative percentage of bound Tf taken up was identical to control (83%). Cells grown with 42/6 showed more reduced, but readily detectable, levels of both cell-associated Tf and Tf uptake. Thus, culture with either anti-Tf receptor antibody resulted in reduced surface Tf binding and uptake, but these effects were much more pronounced with 42/6.

In previous studies, 42/6 also failed to inhibit growth of cells from patients with acute nonlymphocytic leukemia (10, 11). Based on simultaneous staining for Tf receptors and DNA content, and on studies using differentiating HL60 cells, this was attributed to heterogenous Tf receptor display by proliferating cells (11). To compare relative Tf receptor display by growing hemopoietic and solid tumor cells, melanoma and HL60 leukemia cells were stained using immunofluorescence with antibodies B3/25, 42/6, and OKT9. Results with all 3 antibodies were identical, and a representative study using 42/6 is shown in Fig. 4. In multiple studies, 18–82% (mean, 44%) of melanoma cells were positive for Tf receptors, but there was no evidence for discrete receptor-positive and-negative tumor cell populations. In contrast, HL60 cells were 60–95% (mean, 83%) positive. Despite high average numbers of receptors per cell (Table 1), when 242, 354, and HL60 promyelocytic leukemia cells grown for 72 h were stained with 42/6 and analyzed in parallel, percentage of positive cells and median intensities of fluorescence were higher on HL60 than on melanoma cells (Fig. 4A). This suggests greater heterogeneity in receptor display by melanoma cells. When dividing cells in S-G2-M phases of the cell cycle were analyzed separately, 53–85% of melanoma cells were positive for Tf receptors, but there was greater heterogeneity of Tf receptor display by dividing melanoma cells.

**Table 1. Transferrin binding by solid tumor lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>1% FCS</th>
<th>5–10% serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sites/cell (× 10^3)</td>
<td>Kd (m × 10^-4)</td>
</tr>
<tr>
<td>Melanomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>242</td>
<td>12.3</td>
<td>4.5</td>
</tr>
<tr>
<td>354</td>
<td>14.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Ovarian carcinoma 547</td>
<td>6.5</td>
<td>3.7</td>
</tr>
<tr>
<td>Epidermoid carcinoma A431</td>
<td>NT*</td>
<td>NT</td>
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</tbody>
</table>

* NT, not tested.
DISCUSSION

Previous studies using human and murine cells showed that multivalent IgA and IgM antibodies were potent inhibitors of in vitro blood cell growth (3, 8–10). Detailed studies showed that these antibodies undergo surface cross-linking (8, 14) and completely inhibit Tf uptake (14). Tf deprivation leads to iron deficiency and growth inhibition (8, 14, 19). IgG antibodies are much less effective, but, if chemically cross-linked on the cell surface, can partially mimic effects of IgA or IgM molecules (8, 14). On the basis of these studies, and on in vivo studies using a murine leukemia model (13), we suggested anti-Tf receptor antibodies might be effective in passive serotherapy of Tf receptor-positive, hematological malignancies.

In the present studies, we assessed effects of the same anti-Tf receptor antibodies on solid tumor cell growth. The general trend of the results is similar, i.e., B3/25, an IgG antibody, was much less effective than IgA 42/6. Similar to other studies using solid tumor and blood cells, after culture with B3/25, surface Tf-binding sites were reduced (14, 20). B3/25 does not compete for Tf-binding sites (14), and previous studies showed reduced Tf binding was due to accelerated receptor catabolism and decreased surface receptor display (8, 14, 20). After culture with 42/6, solid tumor cell Tf binding was profoundly reduced. We showed previously that, under these conditions, 42/6 blocks Tf-Tf receptor binding (10, 14), and that marked reductions in Tf binding were due to direct inhibition as well as to reduced surface binding sites (14).

Despite similarities in effects on blood and solid tumor cells, 42/6 was a relatively ineffective inhibitor of solid tumor cell growth. This is most likely due to inability of the antibody to prevent completely Tf internalization (Table 2). In previous studies using blood cells, Tf binding was not only markedly reduced after culture with 42/6, but no acid-stable, internalized Tf was observed (14). In contrast, using the same techniques and 547 ovarian carcinoma cells, culture with 42/6 markedly reduced Tf binding, but Tf uptake was still detected. These results are consistent with dose-response studies using deferoxamine to create iron deficiency (21). Low doses of deferoxamine slow cell growth, but higher doses cause complete growth arrest. The latter effect was attributed to inhibition of the iron-containing enzyme, ribonucleotide reductase (19, 21). Thus, 42/6 completely blocks Tf uptake in blood cells and abrogates their growth, but inhibition of Tf uptake and growth are subtotal in solid tumors.

Several observations suggest the relative ineffectiveness of 42/6 against solid tumors may relate to cell surface Tf receptor number or density. Using culture conditions in which Tf receptor number was reduced (10% FBS), the antibody was less effective. Further, using 3 different anti-Tf receptor antibodies, there was greater apparent heterogeneity of melanoma than HL60 leukemia cell staining. This difference persisted even when cells synthesizing DNA were analyzed separately. Previously, we showed identical findings in acute leukemia cells resistant to 42/6 (11), suggesting that reduced Tf receptor display leads to 42/6 resistance. The reduction in Tf receptor density need not be profound. Murine lymphoma cells became completely resistant to IgM anti-Tf receptor antibodies after mutation of only a single Tf receptor allele, and a 50% reduction in antibody-binding sites (12).

However, in the present studies, several observations suggest that other factors may contribute to solid tumor resistance to 42/6. Solid tumor, but not HL60, growth inhibition was reversed by iron-saturated Tf. Further, FeNTA reversed 42/6 inhibition of some blood cells, in others, it was ineffective (10). In contrast, all solid tumor cells tested obtained sufficient iron from FeNTA to overcome 42/6 inhibition. Although melanoma cells express a cell surface molecule with extensive homology to Tf (M, 97,000 or melanotransferrin) (22), the function of this protein is unclear. Further, FeNTA reversed inhibition of ovarian and epidermoid carcinoma cells which do not express this protein. These findings suggest that differences may exist in solid tumor and hemopoietic cell Tf and iron utilization or processing. Iron deprivation may also have varying effects on gene expression by blood and solid tumor cells, accounting, at least in part, for differing effects on cell growth.

The present preclinical studies suggest that anti-Tf receptor antibodies will be most active against hemopoietic cells with high levels of surface Tf receptors, such as thymic acute lymphoblastic leukemia cells (1, 6). Consistent with this idea, recent studies using a murine, thymic leukemia model showed that treatment with an anti-Tf receptor antibody caused tumor regressions and increased life span (13).

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