Production of Monoclonal Antibodies That Detect Hodgkin's High Molecular Weight Transforming Growth Factor-β

By Samuel R. Newcom, Lisa H. Muth, and Ernest T. Parker

High molecular weight transforming growth factor-β (TGFβ) is a physiologically active TGF secreted by nodular sclerosing Reed-Sternberg cells. Five monoclonal murine antibodies were prepared that distinguished Hodgkin's TGFβ from platelet-derived TGFβ using an enzyme-linked immunosorbent assay, neutralization of biologic activity, and Western blotting. These monoclonal antibodies directed at unique antigenic determinants (epitopes) of Hodgkin's TGFβ will allow further characterization of the role of Hodgkin's TGFβ in Hodgkin's disease and related entities.

© 1990 by The American Society of Hematology.

HODGKIN'S-DERIVED transforming growth factor-β (TGFB) is a high molecular weight TGF secreted in a physiologically active form. Hodgkin's TGFβ mediates its actions through the ubiquitous TGFβ cell membrane receptor and crossreacts with anti-TGFβ antibodies. Unlike the TGFβ characterized to date, Hodgkin's TGFβ is almost, but not completely, inactivated by acidification (97%). Both acidification and reducing agents release the 25,000 d TGFβ molecule from Hodgkin's TGFβ. Endogenous and exogenous proteases destroy the activity of Hodgkin's TGFβ.

Sources other than Reed-Sternberg cells have been reported to secrete physiologically active TGFβ. Although less well characterized, these active TGFBs have been identified in the conditioned media of the MCF-7 human breast cancer cell line and in certain rat liver tumors. All of these physiologically active TGFBs appear to have potentially important biologic activity.

To determine if Hodgkin's TGFβ is composed of unique epitopes or merely aggregates of 25,000 d TGFβ molecules, and to prepare reagents for identifying Hodgkin's TGFβ and characterizing its role in human disease, we have prepared monoclonal antibodies (MoAbs) against Hodgkin's TGFβ. In this article, we show that Hodgkin's TGFβ has unique epitopes that distinguish Hodgkin's TGFβ from the chemically extracted 25,000 d forms, and that specific MoAbs can partially inhibit the biologic activity of this interesting growth factor.

MATERIALS AND METHODS

Growth factors. TGFβ1 was from R&D Systems, Inc (Minneapolis, MN). This is a 25,000 d human platelet TGFβ1 preparation that has been shown to be homogeneous by analysis on sodium dodecyl sulfate (SDS)-polyacrylamide gels and identical to other TGFβs in bioassay, receptor binding, and amino acid sequence. Hodgkin's TGFβ was prepared from serum-free medium conditioned by L-428 Hodgkin's cells, as previously described. This 300,000 d growth factor is 90% homogeneous on gels stained with silver. The purification steps and yields for a sample are listed in Table 1. A total of 48 L of serum-free conditioned medium were used in these experiments. Chromatography was done using 1.6 x 100 cm columns with adaptors (Pharmacia, Piscataway, NJ) packed with a crosslinked agarose gel (Sepharose-CL6B) equilibrated in a Tris-saline buffer (100 mmol/L Tris, 200 mmol/L NaCl, 2 mmol/L EDTA, pH 8.0). Samples were loaded into the buffer line using a three-way valve and a manually controlled peristaltic pump. All chromatography was done at 4°C. The sample was layered onto the gel bed and a flow-rate of 8 to 10 mL/h was used to collect 2.5-mL samples in a fraction collector (Gilsen, Middleton, WI). An ultraviolet flow cell and strip recorder were used to detect protein peaks and assure collection of all protein. Individual sample protein concentrations were estimated using an ultraviolet spectrophotometer (OD280).

TGFB activity was assessed for each fraction using AKR-2B cell colony formation in soft agar, as described below. Electroelution of gel slices was done as previously described.

Polyclonal anti-TGFβ1 antibody. This antibody (from R&D Systems) was prepared in rabbits by injection of samples of highly purified (greater than 95%) TGFβ1 prepared from porcine platelets. Anti-serum was further purified on an affinity column containing crosslinked agarose columns with adaptors (Pharmacia, Piscataway, NJ) packed with a crosslinked agarose gel (Sepharose-CL6B) equilibrated in a Tris-saline buffer (100 mmol/L Tris, 200 mmol/L NaCl, 2 mmol/L EDTA, pH 8.0). Samples were loaded into the buffer line using a three-way valve and a manually controlled peristaltic pump. All chromatography was done at 4°C. The sample was layered onto the gel bed and a flow-rate of 8 to 10 mL/h was used to collect 2.5-mL samples in a fraction collector (Gilsen, Middleton, WI). An ultraviolet flow cell and strip recorder were used to detect protein peaks and assure collection of all protein. Individual sample protein concentrations were estimated using an ultraviolet spectrophotometer (OD280).

TGFB activity was assessed for each fraction using AKR-2B cell colony formation in soft agar, as described below. Electroelution of gel slices was done as previously described.

Monoclonal anti-Hodgkin's TGFβ antibodies. MoAbs were raised using a modification of previously published methods. These antibodies were prepared by injecting partially purified Hodgkin's TGFβ into 6- to 8-week-old female BALB/C mice. Pooled chromatography fractions with TGFβ activity containing 5 mg protein emulsified in 0.2 mL Freund's complete adjuvant were injected both intraperitoneally (IP) and subcutaneously (SQ) on day 1. A boost of 5 mg of protein emulsified in incomplete Freund's adjuvant was given on day 22. Immune mice were killed on day 29, and splenocytes were fused with exponentially growing BALB/C myeloma cells (SP2/0-Ag14). Before fusion, the myeloma cells were grown in Optimum medium supplemented with 4% fetal calf serum. The medium was supplemented 1 week before fusion with 15 μg/mL 8-azaguanine for 3 days. The cells were then passed twice at a 1:4 split ratio before fusion to assure exponential growth. Fusion partners were selected for growth using HAT selection medium (1 x 10^-4 mol/L hypoxanthine, 4 x 10^-7 mol/L aminopterin, and 1.6 x 10^-3 mol/L thymidine). Hybridomas cloned in 96-well microtiter plates were screened using an ELISA (see below) to determine if the supernatants contained anti-Hodgkin's TGFβ antibodies. Subclones of positive hybridomas were screened using both ELISA and biologic activity inhibition. Selected clones were
subcloned twice, retested, and stored in the gas phase of liquid nitrogen (−196°C; 8% dimethyl sulfoxide [DMSO] in fetal calf serum). Thawed viabilities were routinely greater than 80% by trypan blue dye exclusion. Serum-free Iscove’s modified Dulbecco’s medium (IMDM) was used to collect the secreted MoAb from thawed clones. The IgG from pooled medium was purified using Staph A gel chromatography (MAPS II; Bio-Rad, Richmond, CA), concentrated to the desired concentration of 10 to 100 μg/mL (Amicon Stirred Cell, Danvers, MA), dialyzed, filtered through a 0.2-μm filter, and stored at 4°C.

**ELISA.** The ELISA was done according to previously described methods. Briefly, TGFβ or Hodgkin’s TGFβ were coated on the wells of a polystyrene 96-well microtiter plate and fixed with formaldehyde (10%). Unreactive sites were blocked with 5% bovine serum albumin. The primary antibody (polyclonal or monoclonal) was detected using peroxidase-Vectastain ABC Kits (Vector Laboratories, Burlingame, CA). OD_{492} was determined in a microprocessor-controlled spectrophotometer.

**Immunoblotting.** Western blotting was done using a previously described procedure. Polyclonal anti-TGFβ is capable of detecting nanogram concentrations of TGFβ in this assay. Briefly, after electrophoresis into 4% to 30% polyacrylamide gradient gels, TGFβ and electroeluted, purified Hodgkin’s TGFβ were transferred to nitrocellulose paper in transfer buffer (20 mmol/L Trizma base, 150 mmol/L glycine, 20% methanol). The transfer was done overnight at 60 V. Transfer blots were either stained immediately or press-dried for later staining. Blocked papers (3% gelatin for 1 hour) were reacted for 3 hours in anti-TGFβ polyclonal or monoclonal antibody in 1% gelatin (20°C). The biotinylated second antibody was detected with an alkaline-phosphatase-conjugated avidin detected with nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolylphosphate-p-toluidine).

**Test cells.** AKR-2B cells (a gift from Harold Moses, Vanderbilt University, Nashville, TN) were used as test cells because of their lack of TGFβ receptors. AKR-2B cells were routinely cultured in IMDM supplemented with 10% calf serum, 2 mmol/L L-glutamine, 50 μg gentamicin/mL, and 2.5 μg Fungizone/mL. Cell stocks were passaged every 7 to 10 days and maintained in 7.5% CO₂ incubators at 37°C with full humidity. Fibroblasts were used for bioassay in passage 1 to 6 and then discarded. Early passage cells were cryopreserved in 8% DMSO in fetal calf serum at −196°C. Thawed viabilities were routinely greater than 80% by trypan blue dye exclusion.

**TGFβ bioassay.** The bioassay for TGFβ depends on the ability of TGFβ to induce anchorage-dependent growth of AKR-2B fibroblasts in soft agar. The agar assay was done as previously described. Briefly, scored 35-mm dishes were coated with 1 mL of 0.5% agar in complete medium. AKR-2B cells (3 × 10^5) in 0.35% agar were plated over the semisolid 0.5% layer. Control cultures were in medium, 10 to 12 concentrations of TGFβ1 (a titration curve from 0 to 800 pmol/L), or test concentrations of anti-TGFβ antibody. Test dishes contained Hodgkin’s TGFβ. At least three concentrations of anti-Hodgkin’s TGFβ were tested to determine the ability to neutralize Hodgkin’s or platelet TGFβ. All cultures were in duplicate. Using an inverted microscope with phase optics, a single cell suspension was identified on day 1, and colonies (greater than three cells) were counted on day 10. The titration curve of TGFβ1 was constructed and used to estimate the concentration of Hodgkin’s TGFβ. Background colony formation in antibody alone did not occur.

## RESULTS

**ELISA.** Spectrophotometric OD_{492} readings at least twice background were discovered for five distinct hybridoma fusions and were retested using electroeluted Hodgkin’s TGFβ and confirmed after cloning and subcloning twice. These anti-Hodgkin’s TGFβ murine MoAbs failed to crossreact with human platelet TGFβ1 in the ELISA. Polyclonal rabbit anti-TGFβ served as a positive control against both platelet and Hodgkin’s TGFβ and routinely gave OD_{492} greater than 1.000. (See Table 2 for the OD_{492} readings.)

**Western blotting.** Polyclonal anti-TGFβ against platelet TGFβ crossreacted equally well with TGFβ from Reed-Sternberg cells both before (Fig 1) and after treatment of Hodgkin’s TGFβ with SDS-2-ME. None of the MoAbs (C-10, 2H9, T1A5, M2A6, and M2B1) crossreacted with TGFβ1. Figure 1 shows the results with polyclonal antibody TGFβ1 and the five monoclonal anti-Hodgkin’s TGFβ antibodies.

**Inhibition of the biologic activity of Hodgkin’s TGFβ.** Adding polyclonal anti-TGFβ to the agar assay had no effect on TGFβ1 or Hodgkin’s TGFβ biologic activity. The monoclonal anti-Hodgkin’s TGFβ MoAbs were capable of significant inhibition of Hodgkin’s but not platelet TGFβ1 (Table 1). A titration curve could be established with each antibody, and maximum activity was present at 1 to 12 μg/35-mm dish. Using 2H9, the stimulatory effect of TGFβ on the L-428 Reed-Sternberg cell line could be blocked. In addition, the stimulatory effect of serum on L-428 Reed-Sternberg cells could be partially blocked by the anti-Hodgkin’s TGFβ MoAbs.

### Table 1. Hodgkin’s TGFβ Purification

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Protein (mg)</th>
<th>TGFβ (ng)</th>
<th>Yield (%)</th>
<th>Specific Activity</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditioned</td>
<td>6,625</td>
<td>30.72</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentrate</td>
<td>5</td>
<td>30.00</td>
<td>22.5</td>
<td>100</td>
<td>0.75</td>
<td>1.00</td>
</tr>
<tr>
<td>Chromatography</td>
<td>10</td>
<td>4.36</td>
<td>20</td>
<td>89</td>
<td>4.59</td>
<td>6.12</td>
</tr>
<tr>
<td>Electroelution</td>
<td>6</td>
<td>0.18</td>
<td>15</td>
<td>67</td>
<td>833.00</td>
<td>1,111.00</td>
</tr>
</tbody>
</table>

### Table 2. ELISA and Biologic Inhibition By Monoclonal Anti-Hodgkin’s TGFβ

<table>
<thead>
<tr>
<th>Antibody</th>
<th>ELISA Against TGFβ1 (OD_{492})</th>
<th>ELISA Against Hodgkin’s TGFβ (OD_{492})</th>
<th>Inhibition of TGFβ1 (%)</th>
<th>Inhibition of Hodgkin’s TGFβ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-10</td>
<td>.146</td>
<td>.475</td>
<td>0</td>
<td>29</td>
</tr>
<tr>
<td>2H9</td>
<td>.010</td>
<td>.296</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>T1A5</td>
<td>.062</td>
<td>1.078</td>
<td>0</td>
<td>73</td>
</tr>
<tr>
<td>M2A6</td>
<td>.001</td>
<td>1.276</td>
<td>0</td>
<td>89</td>
</tr>
<tr>
<td>M2B1</td>
<td>.122</td>
<td>1.185</td>
<td>0</td>
<td>47</td>
</tr>
</tbody>
</table>

*Agar colony assay using AKR-2B cells.*
Fig 1. Immunoblotting of 25,000 d TGFβ extracted from platelets (A, lane 1) and physiologically active high molecular weight Hodgkin’s TGFβ (A, lane 2) stained with polyclonal anti-TGFβ1. MoAbs directed at Hodgkin’s TGFβ identify specific epitopes not present on the 25,000 d molecule; (B) lane 2, C-10; (C) lane 2, 2H9; (D) lane 1, T1A5; (E) lane 1, M2A6; (F) lane 1, M2B1. The second antibody was detected with an alkaline-phosphatase-conjugated avidin detected with nitroblue tetrazolium and BCIP.

antibodies: C-10 (34% reduction in activity), 2H9 (79%), T1A5 (73%), and M2A6 (29%).

DISCUSSION

To determine if Hodgkin’s TGFβ has unique epitopes when compared with platelet-derived TGFβ, and to aid in the identification of Hodgkin’s TGFβ, we have produced several murine MoAbs that crossreact with Hodgkin’s high molecular weight TGFβ. These monoclonal antibodies do not crossreact with the 25,000 d TGFβ derived from platelets, a TGFβ identical to TGFβ extracted from many sources including neoplasms, placenta, and kidney.

We have previously shown that the Hodgkin’s high molecular weight molecule contains the 25,000 d TGFβ structure that can be extracted by acidification or reduction of the molecule with SDS-2-mercaptoethanol. However, significant loss of biologic activity occurs with these extraction procedures (97% to 98%), suggesting that Hodgkin’s TGFβ may represent the biologically active form of TGFβ, which is normally stored in a poorly characterized inactive form. Previous extraction experiments may have artificially removed a 25,000 d portion from these inactive storage macromolecules. This 25,000 d TGFβ has biologic activity as a result of this harsh extraction procedure, but, possibly, the true biologically active molecule is similar to the Hodgkin’s TGFβ. The presence of unique epitopes on the Hodgkin’s TGFβ suggests that unique gene expression is responsible.

The possibility that TGFβ is sometimes secreted in a physiologically active form in situations other than Hodgkin’s disease is confirmed by the reports of active TGFβ secreted by MCF-7 breast cancer cells and certain rat liver tumors. The nature of these physiologically active TGFβs has not yet been determined.

Further analysis of Hodgkin’s TGFβ using the monoclonal antibodies described here may answer several questions: Do Hodgkin’s patients have circulating levels of detectable high molecular weight TGFβ? If detectable, does the level of Hodgkin’s TGFβ fall with effective therapy? Is the production of high molecular weight TGFβ unique to Hodgkin’s disease or can this molecule be detected in other clinical syndromes? Do normal activated lymphocytes, macrophages, and dendritic cells, which have been considered possible progenitor cells for the Reed-Sternberg cell, secrete high molecular weight TGFβ? The monoclonal antibodies described in this report will allow these questions to be investigated.

REFERENCES

from virally and chemically transformed cells by acid-ethanol extraction. Proc Natl Acad Sci USA 77:3494, 1980


Production of monoclonal antibodies that detect Hodgkin's high molecular weight transforming growth factor-beta

SR Newcom, LH Muth and ET Parker