High Molecular Weight Transforming Growth Factor β Is Excreted in the Urine in Active Nodular Sclerosing Hodgkin’s Disease1

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ABSTRACT

To measure the in vivo secretion of high molecular weight (HMW) transforming growth factor (TGF)β by Reed-Sternberg cells from patients with nodular sclerosing Hodgkin’s disease, we studied the urinary samples from untreated patients. The urinary proteins did not promote the proliferation of NIH-3T3 cells in monolayer culture and contained similar amounts of total TGF activity when compared with normal controls. Urinary proteins from 24 different control and test urines were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Either of two primary antibodies were used for immunoblot detection: (a) affinity column purified polyclonal anti-TGFβ1 prepared against HMW-TGFβ or (b) monoclonal anti-HMW-TGFβ prepared against HMW-TGFβ secreted by cloned L-428 Reed-Sternberg cells. All patients with active nodular sclerosing Hodgkin’s disease had a detectable HMW-TGFβ (~300,000) which cross-reacted with both anti-TGFβ1 and anti-HMW-TGFβ. Purification demonstrated HMW-TGFβ which was active at physiological pH. Twelve control urine samples from healthy adults and 5 follow-up samples from the Hodgkin’s patients after successful treatment contained no detectable urinary HMW-TGFβ. The in vitro production of HMW-TGFβ by untreated nodular sclerosing Hodgkin’s disease supports the conclusion that this growth factor is secreted in large amounts by Reed-Sternberg cells or cells stimulated by Reed-Sternberg cells.

INTRODUCTION

Reed-Sternberg cells from patients with nodular sclerosing Hodgkin’s disease secrete HMW TGFβ (1, 2). The native molecular structure of TGFβ from cloned L-428 Reed-Sternberg cells has been shown to contain TGFβ1 and to mediate its activity through the high-affinity TGFβ1 receptor (3, 4). The unique high molecular weight configuration of this growth factor (~300,000) is stoichiometrically optimal at physiological pH and disrupted by acidification, proteases, or boiling (3).

Because a HMW-TGFβ is created when platelet TGFβ is bound to α2-macroglobulin in normal serum (5, 6), the addition of Reed-Sternberg cell-derived antigenic TGFβ is difficult to measure accurately. Platelet-free plasma preparations are also unpredictably contaminated with TGFβ1 (7). Urine represents a filtered plasma product that is not normally contaminated by platelet-release products and contains no TGFβ1.

Prior study of urine TGFs demonstrated their presence in the urine of patients with metastatic carcinoma (8, 9). The urine samples studied were extracted with acetic acid/ethanol, chromatographed, and tested for NRK fibroblast colony formation and epidermal growth factor receptor competition. Both patients and normal controls excreted a low molecular weight TGF activity (~6000) (8, 9). However, cancer patients had a significantly greater likelihood of demonstrating a second high molecular weight TGF peak (~30,000) (9). None of the patients’ urine samples contained the M, 300,000 molecule described in nodular sclerosing Hodgkin’s disease. These authors (8, 9) did not study the urine samples of any Hodgkin’s disease patients and that is the focus of the present investigation.

MATERIALS AND METHODS

Study Patients and Controls

Patients with untreated nodular sclerosing Hodgkin’s disease were selected for study. All patient information and specimens were provided to the investigators after full written consent had been obtained from each patient. The research plan and consent form were reviewed and approved by the institutional Human Investigations Committee. The 12 controls (6 adult females and 6 adult males) were young healthy adults in the same age range as the patients (15–40 years old). All medications were stopped for 48 h prior to study. None of the female patients or controls were known to be pregnant.

Urine Specimens

Urine collections (24 or 12 h) were obtained in refrigerated plastic containers (Nalgene, 500 ml) with 500 mg of sodium azide. The urine samples were identified by a laboratory code and numbered. Filtered specimens (0.2 μm) were either processed immediately or stored (~70°C). Urine specimens were exhaustively dialyzed in 30-liter containers (4°C) with constant stirring, using M, 3500 cut-off dialysis tubing (Spectrapor, Los Angeles, CA). The dialysis fluid was Ringer’s lactate (pH 7.4) changed each 24 h three times. The retained colloids were shell-frozen, lyophilized to dryness, and stored in desiccators at 4°C. Specimens yielded 3.7–34.07 mg of dry colloid/100–2400 ml. There was no difference in the amount of colloid/volume obtained from the Hodgkin’s disease patients and the controls.

Bioassays

NIH-3T3 and NRK-49F cells were a kind gift (George Todaro, M.D., Bethesda, MD). CCL-64 epithelial cells were obtained from the American Type Culture Collection (Rockville, MD). Early passage aliquots of cells were cryopreserved in liquid nitrogen for study. Thawed cells were maintained in IMDM supplemented with calf serum (10%) or fetal calf serum (10%), L-glutamine (2 mM), and penicillin-streptomycin (1%). All cell cultures were placed in 37°C incubators with 7.5% CO2 and maximum humidity. Cell viability and concentration were determined each passage using trypsin blue dye exclusion and hemocytometer cell counting. Cells were passed to maintain a concentration of 1 x 106 to 2 x 106 cells/ml.

Adherent 3T3 Cell Proliferation (FGF-like Activity). To determine fibroblast proliferation on plastic surfaces, 3T3 cells were removed from contact-inhibited cultures using trypsin (0.25%) and replated in 35-mm test dishes (10 cells/dish). All cultures, including controls, were incubated for 48 h. Test dishes received supplements (200 ng FGF every other day, 10 μg urine protein every other day, or 50% nodular sclerosing Hodgkin’s lymph node serum-free conditioned medium weekly). The initial cell count was confirmed by trypsinizing the adherent monolayer from 2 test dishes after 18 h and counting the resulting cell suspension using an automatic cell counter (Coulter,

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1 The abbreviations used are: HMW, high molecular weight; TGF, transforming growth factor; NRK, normal rat kidney; EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; FGF, fibroblast growth factor; HDU, Hodgkin’s disease urine specimen; CU, control urine specimen; IMDM, Iscove’s modified Dulbecco’s medium; MOPP, Mustargen, Oncovin, prednisone, and procarbazine; ABVD, Adriamycin, bleomycin, vinblastine, and dacarbazine.

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Simultaneously, the monolayer from a fifth dish was fixed in formalin from L-428 Reed-Sternberg cells (11) and from Ki-l-positive from each control and test condition were sacrificed for cell counts. Hialeah, FL). After 7 days, and daily thereafter, quadruplicate dishes were used for each test condition; after 24 h, the cells were at a stable rate of DNA synthesis (~30,000 cpm). A 12-point titration curve of native urine proteins was tested by dissolving in IMDM and followed by neutralization with 1 M sodium bicarbonate) to determine filtration (0.2 urn). Urine proteins were also acidified (1 M acetic acid in vitro activation (7)).

Soft Agar Colony Formation (Total TGF Activity). This assay was performed as previously described (1–3). Clone 49F NRK cells were plated in 1 ml 0.35% Noble’s agar (3 x 103 cells/35-mm scored dish). The cell-containing top agar layer was plated over a 1-ml 0.5% agar layer in complete medium. Test materials were added to the upper layer. Controls were complete medium and complete medium supplemented with EGF (6 ng/dish). Test dishes were treated with 100, 10, and 1 gM urine protein. All conditions were cultured in duplicate. Incubation was at 37°C with 100% humidity. A viable single-cell suspension was identified with an inverted microscope on day 1 using trypan blue dye exclusion. Colonies (>10 cells) were looked for weekly. All plates were scored weekly for 3 weeks. Results were expressed as the mean colonies achieved at 14 days ± SEM.

Epithelial Cell DNA Synthesis Inhibition (TGFβ Activity). This assay was performed as previously described (3). Mink lung epithelial cells (CCL-64) were plated into the microtiter wells of a flat-bottomed 96-well plate (104 cells/well in 1% fetal calf serum). Triplicate test wells were used for each test condition; after 24 h, the cells were at a stable rate of DNA synthesis (~30,000 cpm). A 12-point titration curve of TGFβ1 inhibitory activity (0–240 pm) [3H]Thymidine (0.5 μCi; New England Nuclear, Boston, MA) was added to each well for 16 h, and the cells were collected on glass filters, dried, and counted in a scintillation counter with Hydrofluor scintillation fluid (National Diagnostics, Mari- ville, NJ). Native urine proteins were tested by dissolving in IMDM and filtering (0.2 μm). Urine proteins were also acidified (1 M acetic acid followed by neutralization with 1 M sodium bicarbonate) to determine the role of in vitro activation (7).

Primary Antibodies

Anti-TGFβ1 is a polyclonal rabbit antibody (R & D Systems, Inc.) prepared by injection of highly purified TGFβ1 from porcine platelets. The antiserum is purified by Staph A chromatography and has been shown to cross-react with TGFβ1 in immunoblotting (3, 10). This antibody is nonneutralizing in bioassay and cross-reacts with TGFβ1 but not with acidic or basic FGF or EGF (10). Anti-Hodgkin’s TGFβ (T1A5) is a monoclonal IgG1 murine antibody prepared against HMW-TGFβ (11). This monoclonal antibody partially neutralizes Hodgkin’s TGFβ from L-428 Reed-Sternberg cells (11) and from Ki-l-positive lymphoma cells (12). Immunoblot and enzyme-linked immunoabsorbent assay indicate that T1A5 cross-reacts with a unique epitope on Hodgkin’s TGFβ distinct from TGFβ1 (11). Immunoblot Detection of Urinary TGFβ

Unreduced urinary colloids (1 mg/20 μL) were solubilized in 2% SDS electrophoresis buffer (0.01 M Tris-HCl-0.001 EDTA, pH 8.0). Electrophoresis was performed using 4–30% polyacrylamide gradient gels (Pharmacia, Piscataway, NJ) and a modification of the Laemmli method (13). Electrophoresed proteins were blotted onto nitrocellulose paper using a 24-h transfer (80 V) with a cooling coil (4°C). Complete transfer was documented by staining control and electrophoresed gels (0.1% Coomassie blue). Immunoblotting was performed according to the directions of the manufacturer (R & D Systems, Inc.). Immunoblots were blocked for 2 h using 1% bovine serum albumin in buffer (500 mM NaCl-20 mm Tris-HCl-0.05% Tween 20, pH 7.2). Primary antibodies were applied for 2 h at 20°C (rabbit polyclonal anti-TGFβ1 from R & D Systems:1:1000) or T1A5 murine monoclonal IgG1 anti-HMW-TGFβ (11) (10 µg/ml). Reactions of primary antibodies were detected using a 1:2000 dilution of a biotin-conjugated goat anti-mouse IgG purified by immunoaffinity chromatography (Pierce, Rockford, IL). Biotin was detected using alkaline phosphatase-conjugated avidin and enzymatic color development of nitroblue tetrazolium. Control blots were routinely performed to detect nonspecific reactions caused by bridging of the second antibody or endogenous enzymes.

Molecular Weight of TGFβ Activity from Hodgkin’s Urine. Urine proteins (HDU 3A, CU 13, CU 14) were solubilized in 2% SDS-electrophoresis sample buffer and electrophoresed into 7% polyacrylamide gels. Gels were sliced horizontally at 8-mm intervals, and protein from each slice was electrophoreted into dialysis tubing bags (M, 3500 cut-off). Each slice fraction was tested for TGFβ activity after equilibration in IMDM (PD-10 column; Pharmacia), concentration using an Amicon concentration chamber (YM2 membrane), and filtration through a 0.2-μm filter.

RESULTS

Study Patients and Controls. Each Hodgkin’s patient had a lymph node biopsy demonstrating nodular sclerosis. Initial

Table 1 Characteristics of nodular sclerosing Hodgkin’s disease patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stage</th>
<th>Treatment</th>
<th>Time of urine collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDU 1A</td>
<td>IIIB</td>
<td>Radiotherapy</td>
<td>Prior to treatment</td>
</tr>
<tr>
<td>HDU 2A</td>
<td>IIIB</td>
<td>MOPP × 6 cycles</td>
<td>Prior to treatment</td>
</tr>
<tr>
<td>HDU 3B</td>
<td>IVB</td>
<td>MOPP/ABVD × 12</td>
<td>6 mo of treatment, partial remission</td>
</tr>
<tr>
<td>HDU 3C</td>
<td>III</td>
<td>MOPP × 6 cycles</td>
<td>6 mo of treatment, clinical complete remission</td>
</tr>
<tr>
<td>HDU 3D</td>
<td>III</td>
<td>MOPP × 6 cycles</td>
<td>10 mo of treatment, clinical complete remission</td>
</tr>
<tr>
<td>HDU 4A</td>
<td>III</td>
<td>MOPP × 6 cycles</td>
<td>Prior to treatment</td>
</tr>
<tr>
<td>HDU 4B</td>
<td>III</td>
<td>MOPP × 6 cycles</td>
<td>6 mo of treatment, clinical complete remission</td>
</tr>
<tr>
<td>HDU 4C</td>
<td>III</td>
<td>MOPP × 6 cycles</td>
<td>3 mo of treatment, clinical complete remission</td>
</tr>
<tr>
<td>HDU 5A</td>
<td>III</td>
<td>MOPP × 6 cycles</td>
<td>Prior to treatment</td>
</tr>
<tr>
<td>HDU 5B</td>
<td>III</td>
<td>MOPP × 6 cycles</td>
<td>Clinical complete remission after 3 cycles of treatment</td>
</tr>
</tbody>
</table>

Fig. 1. Effect on NIH/3T3 cells of urine HMW-TGFβ from 4 patients with active nodular sclerosing Hodgkin’s disease. All media were renewed weekly. HDU and FGF were added every other day (200 ng FGF/35-mm dish and 10 μg HDU protein/35-mm dish). Serum-free conditioned medium from a Hodgkin’s lymph node cell suspension was added at 50% concentration once weekly. All cell counts were in quadruplicate. FGF and Hodgkin’s conditioned medium (CM) produced a doubling of the cell count. HDU proteins did not change the proliferation of 3T3 cells.
urine specimens were collected prior to therapy. Follow-up specimens were collected at the patient's home using containers provided by the investigators. Similarly, control samples were obtained from 12 healthy volunteers (100–500 ml). The clinical status of Hodgkin's disease existing at the time each of the 12 HDUs were collected is shown in Table 1.

**Fibroblast Monolayer Proliferation.** The absence of FGF-like biological activity in HDU is shown in Fig. 1. HDUs did not promote 3T3 cell proliferation. Data from 4 different experiments are pooled and show no statistically significant difference between the growth in complete medium and that supplemented with HDU proteins. A doubling of cell number was measured at day 8 and day 10 when 3T3 cells were treated with either FGF (200 ng/ml every other day) or 50% nodular sclerosing Hodgkin's disease serum-free conditioned medium.

**Fibroblast Colony Formation in Soft Agar.** Colony formation was induced by all HDUs studied (range, 16–63 colonies/35-mm dish). Similar to previous reports (8, 9), there was no statistically significant difference between the TGF activity of control urine samples from healthy subjects (69.78 ± 35.4 colonies/35-mm dish) versus samples from Hodgkin's patients (44.8 ± 22.9, P < 0.1). The data are summarized in Table 2.

**Immunoblot Detection of Hodgkin's Urinary HMW-TGFβ.** HDU 1 was not tested by immunoblotting because of lack of material. Fig. 2 demonstrates the HMW-TGFβ detected in samples from the untreated HDU 2 and HDU 5 patients. After 6 months of MOPP chemotherapy (14), both patients had achieved a complete clinical remission. Follow-up specimens demonstrated clearing of the HMW-TGFβ.

Fig. 3 demonstrates the serial measurements for HDU 3. This 15-year-old female had a large tumor burden with mediastinal, liver, and retroperitoneal lymph node involvement. A year of alternating MOPP/ABVD chemotherapy (15) was given. After 6 and 8 months of therapy, restaging indicated a persistently elevated erythrocyte sedimentation rate (60 mm/h). Residual liver disease was found in liver biopsy tissue at 6 months. The HMW-TGFβ remained detectable at the same time. In the last urine specimen obtained prior to completion of therapy (10 months), the HMW-TGFβ had cleared. Results of an abdominal computed tomographic scan, liver function tests, and erythrocyte sedimentation rate had also returned to normal. A follow-up liver biopsy was refused by the patient.

Fig. 4 demonstrates the positive urine obtained from patient HDU 4 prior to treatment and the negative urine after complete remission had been obtained. After 6 months of therapy, a faint TGFβ band persisted. A follow-up urine sample 3 months later revealed complete clearing without further therapy. These

Table 2. TGF activity for NRK fibroblasts in soft agar

<table>
<thead>
<tr>
<th>HDU sample no.</th>
<th>Colonies/35-mm dish</th>
<th>CU sample no.</th>
<th>Colonies/35-mm dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium only 0</td>
<td></td>
<td>EGF (6 ng/dish)</td>
<td>269 ± 5</td>
</tr>
<tr>
<td>1</td>
<td>41 ± 4</td>
<td>2</td>
<td>67 ± 7</td>
</tr>
<tr>
<td>2</td>
<td>63 ± 5</td>
<td>2</td>
<td>67 ± 7</td>
</tr>
<tr>
<td>3</td>
<td>72 ± 10</td>
<td>10</td>
<td>52 ± 6</td>
</tr>
<tr>
<td>4</td>
<td>32 ± 20</td>
<td>11</td>
<td>82.5 ± 15</td>
</tr>
<tr>
<td>5</td>
<td>16 ± 2</td>
<td>12</td>
<td>78 ± 22</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>43.5 ± 26</td>
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<td></td>
<td></td>
<td></td>
<td>124 ± 33</td>
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<td></td>
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<td>109 ± 19</td>
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<td>26 ± 12</td>
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<tr>
<td>Mean ± SEM</td>
<td>44.8 ± 22.9</td>
<td>69.78 ± 35.4</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2.** Immunoblot of HDU 2 and HDU 5. Electrophoresed native proteins were detected with monoclonal anti-Hodgkin's HMW-TGFβ (T1A5). Lane 1, HDU 2A, prior to treatment; lane 2, HDU 2B, after induction of complete clinical remission; lane 3, HDU 5B, after induction of complete clinical remission; lane 4, HDU 5A, prior to treatment. There is complete clearing of Hodgkin's HMW-TGFβ in both patients after successful treatment of their Hodgkin's disease with MOPP chemotherapy. Partial reduction of HMW-TGFβ yields an M, 90,000 molecule that is seen in lane 1.

**Fig. 3.** Immunoblot of HDU 3. Electrophoresed native proteins were detected with monoclonal anti-Hodgkin's HMW-TGFβ (T1A5). Lane 1, HDU 3D, after 10 months of chemotherapy and in complete clinical remission; lane 2, HDU 3C, after 8 months of treatment; lane 3, HDU 3B, after 6 months of treatment and pathological evidence of persistent disease; lane 4, HDU 3A, prior to treatment; lane 5, blank; lane 6, control urine. The patient had detectable Hodgkin's TGFβ prior to treatment and failed to clear completely after 6 and 8 months of treatment with alternating MOPP/ABVD. This finding correlated with clinical evidence of persistent Hodgkin's disease. By 10 months of treatment, there was complete clearing of TGFβ and complete remission of measurable Hodgkin's disease.
purification is the inhibitory activity of the native HMW-TGFβ measurable using the CCL-64 assay.

DISCUSSION

TGFβ is a member of a family of multifunctional polypeptides that induce anchorage-independent growth of nontransformed fibroblasts (7, 16, 17) and inhibit the proliferation of many cells, including epithelial cells (18, 19) and lymphocytes (20, 21). It has recently been established that there are at least three TGFβ mammalian proteins. Each TGFβ is encoded by a different gene (22).

TGFβ is important in limiting the expansion of nonmalignant activated lymphocytes (20, 21). TGFβ suppresses DNA
acrylamide gels were sliced at 8-mm intervals, and the protein from each slice was electroeluted for TGFB bioassay. The activity peak was measured at Mr 350,000+3,999. A 12-point TGFB titration control curve from 0-240 pM was conducted (e.g., 240 pm TGFB, gave cpm of 155 ± 24 or 99.2% DNA synthesis suppression).

DNA synthesis suppression (%) = \frac{Test cpm - Control cpm}{Control cpm} \times 100.

The identification of HMW-TGFβ in active nodular sclerosing HDU and the demonstration of clearing after treatment suggests that the source is the Reed-Sternberg cell. Confirmation of this conclusion would require specific in vivo labeling of Reed-Sternberg cell-derived HMW-TGFβ, a procedure not yet available.

The data presented here show that HMW-TGFβ, identical with that purified from cloned Reed-Sternberg cells, is secreted in the urine of patients with untreated nodular sclerosing Hodgkin’s disease and disappears with successful treatment. Although immune complex disease or reactive cells could be sources of this TGFB, the high molecular weight and antigenicity indicate that the likely source is the Reed-Sternberg cell.


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