Detection of Urinary TGF-α by HPLC and Western Blot in Patients with Melanoma

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Transforming growth factor-alpha (TGF-α) secretion in the urine may reflect autonomous production by neoplasms. Previous measurements of TGF-α in the urine were limited by the large volumes of urine required for detection. Sensitive assays are required to detect TGF-α in small urine samples from individual patients with melanoma. An assay which detects ng quantities of immunoreactive TGF-α in 10 ml of urine using dialysis, reverse-phase high-performance liquid chromatography (HPLC), and Western blot of the HPLC fractions was used to study the urine from 6 melanoma patients and 6 normal human volunteers. No TGF-α was detectable in the first voided urine specimens from the normal volunteers. In contrast, TGF-α was detected in the urine specimens of two of three patients with primary melanomas and two of three patients with metastatic melanoma. The sensitivity and reliability of the assay were most affected by methods of urine collection, sample preparation, and storage. TGF-α was less often found in randomly voided urine samples from melanoma patients (1 of 3 detectable) as compared to first voided morning urine samples (3 of 3 detectable). This data indicates urinary TGF-α assay may be useful in the diagnosis and management of patients with melanoma.


Melanoma cells have been shown to produce growth factors both in vivo and in vitro [1–7]. TGF-α found in tumors shows heterogeneity in molecular weight (6–34 kD) [8], probably due to variable post-translational processing such as proteolysis or glycosylation of the 160–amino acid TGF-α precursor molecule [9]. Western blot assays utilizing anti-TGF-α antibodies make identification of these various forms of TGF-α possible in urinary samples from patients with carcinomas, if a sufficient concentration of TGF-α is excreted by the tumor. Previous methods of detection of urinary TGF-α in patients with melanoma and carcinoma required large volumes of urine [4,10], which are impractical for clinical use. To develop a more sensitive assay for TGF-α, we combined the techniques of HPLC and Western blot [11]. The use of these techniques allows: 1) the use of small aliquots of urine (minimum of 10 ml), a volume practical for clinical use; and 2) detection of possible multiple M, forms of TGF-α. This urinary assay initially detected immunoreactive TGF-α in the urine of a patient with a primary melanoma and the cutaneous paraneoplastic syndromes of acanthosis nigricans, the sign of Leser-Trelat, and eruptive acrochordons [11]. Because this patient’s constellation of paraneoplastic syndromes is rarely found in melanoma patients, it was possible that he also had uniquely high values of TGF-α in the urine. The urinary TGF-α assay therefore might not detect TGF-α in urine samples from patients with melanomas who did not have cutaneous paraneoplastic syndromes, making the assay less useful clinically. This study tested the urinary TGF-α assay we developed for its sensitivity and reliability in detecting TGF-α in urine samples from normal volunteers and patients with primary or metastatic melanoma who did not have cutaneous paraneoplastic syndromes.

MATERIALS AND METHODS

Patient Population

Controls: Six normal volunteers without malignancies or cutaneous disease were studied. These volunteers were sex and age matched controls for the melanoma patients studied (five males, one female; median age, 49).

Melanoma Patients: Three patients with primary melanomas and three patients with metastatic melanomas were also studied (Table I) (five males, one female; median age, 52). Specimens from patients with primary melanomas were obtained from patients seen in Pigmented Lesion Clinics at Vanderbilt University and Veterans’ Affairs Medical Centers (Nashville, TN) and the University of Oklahoma Medical Center (Oklahoma City). Specimens from patients with metastatic melanomas were obtained from hospitalized patients on the Veterans’ Affairs Medical Center Oncology Service, in addition to the Pigmented Lesion Clinics. None of these melanoma patients had any clinical evidence of a cutaneous paraneoplastic syndrome.

Specimen Collection First voided morning urine specimens (approximately 100 ml) were collected from three melanoma patients and all normal human volunteer control patients. Random spot urine specimens (approximately 100 ml) were obtained from

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Abbreviations:
DNS: dysplastic nevus syndrome
HPLC: high-performance liquid chromatography
SSM: superficial spreading melanoma
TFA: trifluoroacetic acid
TGF-α: transforming growth factor-alpha

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patients 2 and 3 just prior to excision of their primary melanomas, and from patient no. 6, who had a metastatic melanoma. 1% thymol in glacial acetic acid (1 ml/10 ml urine) was added to each specimen. Samples were immediately frozen at −70°C until processing for the HPLC column, then stored at −20°C until analysis.

Urine Specimen Preparation

Urine samples were dialyzed at 4°C against 1 M acetic acid for 24 h and lyophilized as per Ellis et al [11]. Alternative methods of urinary protein concentration tested included precipitation of control normal urine specimens containing a known amount of 125I TGF-α with quinine sulfate [12] and 10% trichloroacetic acid.

Each urine sample was reconstituted in one-tenth the original volume with 0.05% trifluoroacetic acid (TFA) (Fisher Scientific, Springfield, NJ), spun at 110,000 × g for 30 min, and the supernatant filtered through a 0.45 μm Nalgene filter. The filtrate pH was adjusted to 2.5 with NH₄OH.

Reverse Phase High-Performance Liquid Chromatography

A 1-ml sample of each processed urine specimen was applied to the SynChrom Synchropak RP-P C18 (250 × 4.1 mm) HPLC column and a linear 0–60% acetonitrile gradient elution in 0.05% TFA, done as per Ellis et al [11]. One-milliliter HPLC fractions were collected, pooled into five samples (A,B,C,D,E) [11], and lyophilized.

Polyacrylamide-Gel Electrophoresis

Lyophilized HPLC fractions (A,B,C,D,E) and standards were prepared for sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) and run as previously described [11,13]. Standards (M,) included human TGF-α (200 ng) (Bachem, Torrance, CA), and previously described control proteins [11].

Western Blots

In brief, proteins separated on the 16.5% resolving and 4% stacking SDS-PAGE gels were transferred to nitrocellulose paper for 18 h at 30 V [14]. After equilibration with blocking solution, the nitrocellulose sheet was incubated overnight at 4°C with anti-TGF-α antibody, diluted 1:100 in blocking solution [11]. The primary antibody was sheep anti-human TGF-α (Triton Biosciences, Alameda, CA), a polyclonal antibody raised against synthetic rat TGF-α and affinity purified, which cross-reacts with human TGF-α. Immunoreactivity was confirmed in each experiment by including known amounts of human TGF-α as an internal control. Western blots were then developed as previously described [11].

RESULTS

Immunoreactive TGF-α was detectable in urine samples from patients with melanomas that did not have any cutaneous paraneoplastic syndromes, but not from normal volunteers (Table II). Clinical characteristics of these patients are shown in Table I. The lesions were predominantly superficial spreading melanomas (five of six). The primary melanomas studied were of intermediate thickness (1.10–2.03 mm). Four of six (67%) of the melanoma patients in this series had DNS. This increased incidence of DNS was attributable to the fact that most patients were obtained from Pigmented Lesion Clinics, where the majority of the clinic patients have DNS.

Urinary TGF-α was detected in two of three patients with either primary or metastatic melanomas. The Western Blot of the urinary HPLC samples from Patient 5, who had metastatic melanoma, stained for immunoreactive TGF-α is shown in Fig 1. Immunoreactive bands are seen in HPLC fractions A, B, and C, which correspond to the TGF-α standard. Some variability was noted in the presence of immunoreactive TGF-α in the HPLC fractions of the melanoma patients. All patients’ specimens in which immunoreactive TGF-α was detected had 6,000 mw bands, corresponding to the TGF-α standard in the B and C fractions. Specimens from patients no. 2 and no. 5 also had similar bands in the A fraction. Molecular variability of TGF-α was detected in the specimen from Patient 1, where an approximately 23,000 M, immunoreactive TGF-α band was seen in the D fraction in addition to the 6,000 M, form in the B and C fractions.

Preliminary studies indicated that TGF-α levels varied in samples from the same patient and even in the same specimen. A major variable affecting the TGF-α assay was found to be storage conditions. Urine samples from melanoma patients stored at −70°C retained TGF-α immunoreactivity, but samples stored at −20°C lost all TGF-α immunoreactivity within 3 months. Timing of specimen collection was also found to be a factor affecting TGF-α detection. First voided morning urine samples of patients with melanoma all (three of three) demonstrated immunoreactive TGF-α, as opposed to only one of three randomly collected urine samples.

Because dialysis and lyophilization are time consuming techniques, alternative methods of urinary protein concentration by precipitation were tried, using 125I TGF-α as an internal control.

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**Table I.** Clinical Characteristics of Melanoma Patients

<table>
<thead>
<tr>
<th>PT. no.</th>
<th>Sex</th>
<th>Age</th>
<th>Primary Melanomas</th>
<th>Lesion thickness (mm)</th>
<th>Lesion location</th>
<th>Other factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>57</td>
<td>SSM</td>
<td>1.90</td>
<td>R. arm</td>
<td>DNS</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>39</td>
<td>SSM</td>
<td>2.03</td>
<td>L. lower back</td>
<td>DNS</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>33</td>
<td>SSM</td>
<td>1.10</td>
<td>R. upper back</td>
<td>DNS pregnant</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>52</td>
<td>SSM</td>
<td>0.76</td>
<td>R. scalp</td>
<td>DNS</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>70</td>
<td>Spindle cell</td>
<td>3.00</td>
<td>L. forearm</td>
<td>DNS</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>58</td>
<td>SSM in congenital nevus</td>
<td>3.90</td>
<td>R. lower back</td>
<td>DNS</td>
</tr>
</tbody>
</table>

Metastatic Melanomas

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<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age</th>
<th>Melanoma type</th>
<th>Lesion thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>M</td>
<td>52</td>
<td>SSM</td>
<td>0.76</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>70</td>
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<td>6</td>
<td>M</td>
<td>58</td>
<td>SSM in congenital nevus</td>
<td>3.90</td>
</tr>
</tbody>
</table>

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### Table II. Urinary TGF-α Western Blots

<table>
<thead>
<tr>
<th>Patient</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>1st void</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Random</td>
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<tr>
<td>3</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Random</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1st void</td>
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<td>5</td>
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<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Random</td>
</tr>
</tbody>
</table>

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* *: No reactivity.

+ 6000 MW band.

**: Approximately 23,000 MW band.
Quinine sulfate and trichloroacetic acid precipitation techniques were both unsuccessful. Although the TGF-α could be precipitated, it was insoluble in HPLC compatible solutions such as 0.05% TFA, 10% acetonitrile in 0.05% TFA, and 0.4 N HCl (data not shown).

### DISCUSSION

Our previous report of a patient with primary malignant melanoma [11] indicated that measurement of urinary TGF-α may be a sensitive assay for detecting the presence of melanomas. However, that patient was unique because he had cutaneous paraneoplastic syndromes that appeared to correspond to the presence of increased levels of TGF-α [11]. The present study detected urinary TGF-α in patients with primary (two of three) and metastatic (two of three) melanomas without associated cutaneous paraneoplastic syndromes. This data indicates that the described urinary TGF-α assay may indeed be useful as a screening and/or monitoring test for melanoma patients in general, and not limited to the subset of patients with cutaneous paraneoplastic syndromes. We also observed molecular variability in the immunoreactive TGF-α detected in the urine specimen of one patient. This is consistent with previous observations, where TGF-α showed heterogeneity in molecular weight (6-34 kD) [8], and, as previously reported [9], may be due to proteolysis or glycosylation of the TGF-α precursor molecule. Although there are not enough patients in this study to determine any significance of different M, of TGF-α, melanoma patient populations defined by differences in the M, of TGF-α fractions found in their urine specimens may be studied in the future with this assay to detect any differences in their clinical courses.

The present study indicates that urine specimens to be tested for TGF-α must be stored at -70°C and should be first morning voided specimens. Both specimens from patients (nos. 3 and 6) with undetectable levels of TGF-α were random spot urine specimens, and were tested with triplicate assays on each specimen. Because the primary melanoma of patient no. 3 was removed and patient no. 6 died, first morning voided urine specimens could not be obtained. First morning voided urine specimens should have higher protein concentrations, which may account for the apparent increased sensitivity of these specimens in the TGF-α assay. The Western blot immunooassay method may be a limiting factor in this assay's sensitivity. Using internal TGF-α standards, the lower limit of detection in this assay was 50 ng per sample (data not shown). Undetectable urinary levels of TGF-α may also occur if not all melanomas actively secrete TGF-α. Patient no. 3 was in the first trimester of pregnancy at the time her melanoma was removed. This assay did not detect TGF-α in her urine specimen, which was surprising because pregnant patients have been reported to have increased urinary levels of transforming growth factors [16].

The detection of urinary TGF-α excretion in excess of 50 ng in patients with small primary melanomas indicates that small lesions may produce relatively large amounts of TGF-α. Urinary TGF-α measurement may thus prove useful in detecting metastatic malignant melanomas in patients at high risk for recurrence, and for monitoring responses of metastatic melanomas to therapy. Urinary TGF-α measurements may complement other assays such as S-cysteinyldopa [15], particularly in amelanotic melanomas, which may not secrete S-cysteinyldopa.

The mRNA for TGF-α has been detected in other tumors [2], as well as hyperproliferative cutaneous disorders such as psoriasis [17]. Urinary secretion of detectable levels of TGF-α may also be a feature of these conditions, but it is not found in normal volunteers without obvious skin diseases. The mRNA for TGF-α has also been detected in normal keratinocytes, but not melanocytes in culture [18]. Whether induction of mRNA for TGF-α is a primary or secondary event in the malignant transformation of melanocytes is clearly of interest. The urinary assay for TGF-α described above may thus be useful in studying the pathogenesis, diagnosis, and therapy of patients with melanomas.

### REFERENCES

14. Burnette WN: "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to immobi-

COURSE ANNOUNCEMENTS

The course “Selective Delivery of Therapeutic Polypeptides and Proteins” will be given September 24–26, 1990 in Montreux, Switzerland. The ability to control the biologic dispersion of conventional and polypeptide and protein drugs is one of the key features of modern drug design and development. This short course provides valuable insight into the rapidly emerging field of selective delivery and targeting of polypeptides and proteins intended for therapeutic use. The course will examine the rationale for selective drug delivery and targeting, the biologic opportunities for site-specific delivery, and examples such as macromolecular carriers and hybrid protein delivery systems, and the delivery of genetic material using retroviral vectors. Currently available systems will be critically reviewed and pharmaceutical development, clinical usage and regulatory issues pertaining to site-specific delivery will be addressed. Opportunity will be given for detailed tutorial discussion. Course leaders: Professor Eric Tomlinson and Professor Christopher Marriott.

The course “Gene Expression Under the Microscope: Recent Advances in In Situ Hybridisation” will be given September 26 and 27, 1990, Royal Society of Medicine, London, United Kingdom. This course of lectures and practical demonstrations has been designed to illustrate the principles, practical problems, and applications of in situ hybridization. Topics will include riboprobes, oligonucleotide and DNA probes, probe construction; tissue collection and preparation; optimising prehybridization and hybridization conditions; choice of radiolabeled or biotinylated probes; visualization using autoradiography or chromogenic methods; combination with immunocytochemistry to provide a “functional morphology;” quantification of results using computer-assisted image analysis; and applications to the study of neoplasia and endocrine pituitary manipulations. Course leader: Professor J.M. Polak.

The course “Image Analysis in Microscopy: The Unbiased Picture” will be given September 28, 1990, Royal Society of Medicine, London, United Kingdom. This course will provide an introduction to the use of computer-assisted image analysis in microscopy. Lectures will cover the principles of image analysis and its applications including measurement of area, perimeter, cell numbers, etc.; optical density measurement; thresholding techniques; image transformations; silver grain counting, particularly with respect to in situ hybridization; 3-D reconstructions and confocal microscopy. Many of these techniques will be demonstrated with the aid of a live video display and in addition, many of the leading companies in the field will be represented in a trade exhibition run in parallel with the course. Course leader: Professor J.M. Polak.

For details on all courses, please contact Fiona Morgan, IBC Technical Services Ltd., Bath House, 56 Holborn Viaduct, London EC1A 2EX. Tel: 01-236 4080. Tlx: 888870 IBC G. Fax: 01-489 0849.