Distinct High-Performance Liquid Chromatography Pattern of Transforming Growth Factor Activity in Urine of Cancer Patients as Compared With That of Normal Individuals


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ABSTRACT

Reverse-phase high-performance liquid chromatography (HPLC) performed on urine from cancer patients and normal controls revealed the presence of seven chromatographically distinct peaks of transforming growth factor (TGF) activity, as measured by colony formation of normal rat kidney cells in soft agar. Comparison of urines from normal donors and cancer patients showed no differences in EGF (epidermal growth factor)-dependent β-TGF-like activity but did reveal distinct patterns of EGF-related, EGF-independent α-TGF-like activity. All urine samples contained at least two chromatographically distinguishable forms of EGF-dependent TGF activity, eluting from HPLC as broad peaks with 30 and 43% acetonitrile. The remaining five TGFs eluted as sharp peaks with 32, 34, 35, 37, and 38% acetonitrile, demonstrated EGF-competing activity, and thus were functionally related to EGF. Two of the five EGF-related TGFs were consistently elevated only in the urine of cancer patients and eluted with 32% (TGF_A) and 37% (TGF_B) acetonitrile. Two of the other EGF-related TGFs, eluting with 34% (TGF_C) and 35% (TGF_D) acetonitrile, were commonly found in both normals and cancer patients. The fifth EGF-related TGF, TGF_E, eluting with 38% acetonitrile, was found only in normal donor specimens. TGF_A corresponded to the unique M, 30,000 TGF activity previously identified only in the urine of cancer patients.

These observations demonstrate that cancer patients produce high levels of EGF-related TGF activities which can be readily distinguished, using reverse-phase HPLC, from EGF-related TGFs produced by normal individuals. Using a solid-phase competitive radioreceptor binding assay for EGF, we demonstrated that quantitation of EGF-competing activity is as sensitive and effective as the soft-agar colony formation assay for distinguishing HPLC profiles of urinary TGF from cancer patients versus that from normal individuals.

INTRODUCTION

TGFs are peptides which reversibly promote anchorage-independent growth and colony formation in semisolid medium of nontransformed cells (6, 21). Two functional classes of TGFs have been described (18, 20): EGF-related α-TGFs, which compete with EGF for binding to EGF receptors (6, 18, 28) and which can promote growth of normal fibroblasts in soft agar in the absence of EGF; and β-TGFs, which do not bind to EGF receptors and must act in synergy with either EGF or α-TGFs to promote anchorage-independent growth (1, 3, 20). Like EGF, α-TGFs will catalyze phosphorylation of tyrosine moieties in the EGF receptor (17, 28).

TGF activity has been identified in tissue culture supernatants of virally and chemically transformed rodent cells (6, 15, 16) and from human tumor cell lines (13, 22, 26). In addition, acid extracts of both neoplastic and normal tissues have been shown to contain TGF activity (3, 19, 24). Since TGFs are acid stable, low-molecular-weight peptides, urine has been examined for the presence of TGF activity. Sherwin et al. (23) have recently reported elevated levels of M, 30,000 to 35,000 TGF activity in the urine of most cancer patients, whereas this molecular weight form was detected only infrequently, and at low levels, in normal controls. Normal controls as well as cancer patients express a M, 6,000 to 8,000 TGF.

In this report, we further characterize by using HPLC the EGF-competing activity and soft-agar growth-promoting activity found in acidified, concentrated human urine extracts. Reverse-phase HPLC revealed the existence of at least 5 species of EGF-related α-TGF-like activities and a smaller number of non-EGF related β-TGF-like activities when cancer patients and normal controls were examined, whereas gel filtration earlier had revealed only 2 different urinary TGFs (23, 27). In addition, we noted distinct differences between normal donors and cancer patients when compared with regard to the relative levels of the EGF-related TGF activities, one of which was found to correlate with the high M, 30,000 TGF observed previously (23, 27) in the urine of cancer patients. Furthermore, we observed that the M, 30,000 tumor-associated TGF gradually underwent conversion to a M, 6,000 TGF.

MATERIALS AND METHODS

Urine Preparation. Morning void urines were collected from patients with a variety of disseminated cancers and stored at 4°C. The patients ranged in age from 18 to 63 years, had received no anticancer therapy for a period of at least 3 weeks, and had normal renal function, as judged by serum creatinine and the absence of proteinuria. Urines were also collected from normal laboratory workers ranging in age from 22 to 49 years and also consisted of morning voids. Normal female donors in this group were nonpregnant. Urine specimens were acidified with glacial acetic acid to a final concentration of 5 M, clarified by centrifugation at 20,000 × g for 30 min, and then filtered through a 0.45-μm filter (Nalge Corp., Rochester, NY). Between 20 and 50 ml of acidified urine, containing 10 mg of protein, were concentrated to less than 1 ml by ultrafiltration on an Amicon XM-50 membrane (Amicon Corp., Danvers, MA) (M, 50,000 cutoff). The concentrate was dialyzed against 1 M acetic acid in the ultrafiltration cell. This procedure generally yielded 200 to 500 μg of protein in the retained portion and contained almost all of the EGF-
competing activity and soft-agar growth-promoting activity when compared to dilution curves for the concentrated urine and the Amicon filtrate. The retained portion was then applied to a reverse-phase HPLC column.

HPLC. A Waters Associates (Milford, MA) C18 µBondapak reverse-phase column (3.9 x 300 mm) was used on an Altex Model 324 HPLC (Beckman Instruments, Palo Alto, CA). Gradient elution over a period of 2 hr at a flow rate of 1 ml/min at room temperature, from 0 to 60% acetonitrile (Burdick and Jackson Laboratories, Inc., Hoffman-LaRoche, Inc.) in 0.05% trifluoroacetic acid (Pierce Chemical Co.), adjusted to pH 2.5 with ammonium hydroxide, was used to develop the chromatogram after an initial isocratic elution for 5 min with 0.05% trifluoroacetic acid. One-ml fractions were collected. Duplicate aliquots (100 µl) were taken for EGF competition assays, and 800-µl aliquots were used for soft-agar colony assays, as described below.

Soft-Agar Growth Assay. Column fractions were tested singly for the presence of factors capable of stimulating nontransformed NRK (clone 49F) fibroblasts, taken at passages 17 to 19, to grow in colonies in soft agar as described previously (5, 6). A single-cell suspension of approximately 10⁶ NRK cells was mixed with the lyophilized sample to be tested and seeded in 2.0 ml of Dulbecco’s modified Eagle’s medium containing 10% calf serum (Grand Island Biological Co., Grand Island, NY) plus 0.3% agar (Difco Laboratories, Detroit, MI) into 60-mm Costar Petri dishes containing a base layer of the same medium plus 0.5% agar. Plates were refed with 2.0 ml of the same medium plus 0.3% agar at 7 days. Colonies which typically contained 8 to 20 cells (23) were scored in 4 random low-power fields at both 7 and 14 days. Two independent readings were performed for each assay. NRK cells do not grow as colonies in 0.3% agar except in the presence of soft-agar growth-promoting factors, nor do they form colonies in the presence of EGF at concentrations as high as 10 ng/ml (6, 23).

Solid-Phase EGF Competition Assay. To quantify EGF competition in the HPLC eluates, a rapid solid-phase competitive binding assay suited for screening a large number of samples was developed and is discussed in detail elsewhere (12). In brief, receptor-rich A-431 (7) cell membrane fragments in 100 µl Dulbecco’s phosphate-buffered saline, pH 7.2, were dried overnight at 37° onto 96-well polyvinyl chloride plates (Dynatech Laboratories, Inc., Arlington, VA). 2.5 µg membrane protein per well. Receptor grade mouse EGF (Collaborative Research, Inc., Waltham, MA), was further purified to homogeneity over HPLC as described (2, 12, 14), radiolabeled with ¹²⁵I (Amersham/Seale Corp., Chicago, IL) as described (8), and used as the ¹²⁵I-labeled binding component. HPLC-purified unlabeled mouse EGF was also used for deriving standard inhibition curves. Binding was initiated by the addition of 100 µl binding buffer (6) (Dulbecco’s minimum essential medium containing bovine serum albumin (1 mg/ml) and 50 mM 2-[bis(2-hydroxyethyl)aminio]ethanesulfonic acid adjusted to pH 6.8), containing ¹²⁵I-labeled EGF (4 ng/ml) with or without an aliquot of the sample to be tested. After a 1-hr incubation at 23°, the contents of the wells were aspirated, and the wells were washed 4 to 5 times with binding buffer (150 µl). Finally, the wells were cut out of the plate with a hot wire, deposited into test tubes, and the amount of ¹²⁵I-labeled EGF specifically bound was determined. EGF-competiting activity was quantitated as ng equivalents of EGF activity by comparison to a standard inhibition curve.

Other Growth Factors. Sarcoma growth factor from Moloney sarcoma virus-transformed 3T3 cells (clone 3B11) was prepared and partially purified according to the methods of DeLarco and Todaro (8). Chemically homogeneous, recombinant HEGF was a generous gift from Chiron Research Laboratories (Emeryville, CA).

RESULTS

Previous studies revealed that urine from cancer patients contained a unique soft-agar colony-promoting activity that had a molecular weight of approximately 30,000 (23, 27). In addition, elevated levels of a M, 6,000 to 8,000 fraction with colony-forming activity that is also present in normal control urine were detected. In order to further characterize such urinary TGF activity and permit comparisons to TGFs secreted by human tumor cell lines in vitro (13, 22, 26), acid extracts of human urine from normal controls and patients with disseminated neoplastic disease were examined by reverse-phase HPLC. Representative HPLC profiles of acidified urine extracts, from a normal 38-year-old male control and a 50-year-old male patient with renal cell carcinoma, are shown in Chart 1. Four peaks of soft-agar growth-promoting activity (Chart 1, top) which coeluted with EGF-competiting activity (Chart 1, bottom) were seen in the cancer patient, whereas the normal control contained only 3 such peaks. There were noteworthy quantitative and qualitative differences between the normal control and the cancer patient. The normal control showed very little, if any, of the TGF activity (TGF₄) which...
eluted at 32% MeCN, or of the activity (TGF₀) which eluted at 37% MeCN, whereas these activities were observed at high levels in the cancer patient urine. Furthermore, the activity designated TGFₑ (38% MeCN) in the control urine appeared to be below the limits of detection in the patient urine. The levels of TGFₐ and TGFₑ had similar ranges in the urine of patients and normal controls. The observation that all of the above soft-agar colony-promoting activities were associated with EGF-competing activity and formed small-to-moderate-sized colonies in the absence of EGF conforms to the definition of α-TGFs, as previously described (18, 20, 26).

The foregoing suggested a potential difference between TGF activity profiles of cancer patients and normal individuals with respect to the relative amounts of TGFₐ, TGF₀, and TGFₑ. To determine whether such differences were consistent, we performed similar HPLC analyses on a series of urines from cancer patients and normal controls. Table 1 compares levels of colony-forming and EGF-competing activity for the above-mentioned TGFs in urine from 2 pairs of age- and sex-matched normal donors and cancer patients. Soft-agar growth-promoting and EGF-competing activity were concomitantly elevated for TGFₐ and TGF₀ in cancer patients but depressed in normal controls, whereas the converse was observed for TGFₑ. It may also be noteworthy that although there were similar levels of EGF-competing activity for TGFₑ from normal male and female controls, the levels of soft-agar growth-promoting activities showed a wider distribution. Also, the male urines demonstrated more TGFₑ than did female urines when tested by the radioreceptor assay but less TGFₑ when tested in the soft-agar assay. This may be due in part to additional factors which are present in female urine, as observed by Twardzik et al. (27), which could contribute to the colony-forming activity present in those fractions.

Because there are often large variations in the absolute level of growth-promoting activity in individual urines, and because the soft-agar bioassay often demonstrates day-to-day variation, comparisons between specimens were facilitated by determining the TGFₐ/TGFₑ ratio for the number of soft agar colonies and for ng equivalents of EGF-competing activity recovered from reverse-phase HPLC (Chart 2). The TGFₐ/TGFₑ ratio was <0.5 in all normal controls and >0.5 in all cancer patients for both soft-agar colony promotion and EGF competition.

In order to determine the relationship of the above EGF-related TGFs to the tumor-associated M, 30,000 TGF described previously (23), high-molecular-weight TGF (M, 30,000) was isolated by gel filtration from the urine of a patient with breast carcinoma and then applied to reverse-phase HPLC. Chart 3 demonstrates that the M, 30,000 TGF activity eluted with the same retention as TGFₐ (32% MeCN). Since normal control urine had been shown previously by gel filtration to contain little or no M, 30,000 TGF (23) and also lacked significant levels of TGF₀ activity by reverse-phase HPLC, we tentatively concluded that TGFₐ is probably equivalent to high-molecular-weight (approximately, M, 30,000) tumor-associated growth factor in urine. However, in order to further correlate the M, 30,000 TGF with TGFₐ, the latter was isolated from reverse-phase HPLC and was promptly rechromatographed over Bio-Gel P-30. The result of that experiment, shown in Chart 4, reveals the presence of 2 molecular weight species, one of approximately M, 25,000 to 30,000 and one of approximately M, 6,000. Both demonstrated EGF-competing activity and soft-agar colony-forming activity. It was therefore of further interest to determine whether the M, 6,000 TGF had arisen from the 30K TGF. Accordingly, a M, 30,000 TGF pool was isolated by gel filtration and, following storage for 4 days at 4° in the column eluant (1 M acetic acid), was rechromatographed by gel filtration over Bio-Gel P-30. The results

![Chart 2. Comparisons of ratios of TGFₐ/TGFₑ for normal controls and cancer patients.](chart)

<table>
<thead>
<tr>
<th>No. of colonies of NRK cells in soft agar/starting 10 mg of urinary protein analyzed</th>
<th>TGFₐ</th>
<th>TGF₀</th>
<th>TGFₑ</th>
<th>TGF₀/Cancer Patients</th>
<th>TGFₑ/Cancer Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>31-yr-old male with colon carcinoma</td>
<td>300 (155)</td>
<td>110 (22)</td>
<td>270 (88)</td>
<td>275 (144)</td>
<td>0 (11)</td>
</tr>
<tr>
<td>36-yr-old normal male</td>
<td>5 (28)</td>
<td>154 (22)</td>
<td>580 (66)</td>
<td>0 (0)</td>
<td>388 (50)</td>
</tr>
<tr>
<td>41-yr-old female with breast carcinoma</td>
<td>930 (230)</td>
<td>240 (40)</td>
<td>220 (280)</td>
<td>1100 (1150)</td>
<td>0 (10)</td>
</tr>
<tr>
<td>49-yr-old normal female</td>
<td>16 (25)</td>
<td>380 (80)</td>
<td>185 (56)</td>
<td>0 (0)</td>
<td>244 (220)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, ng equivalents of EGF-competing activity per liter of urine.
Chart 3. Reverse-phase HPLC of tumor-associated high-molecular-weight TGF. Reverse-phase HPLC of M, 30,000 activity isolated by Bio-Gel P-30 chromatography, as described by Sherwin et al. (23), from an acid extract of urine from a female patient with breast carcinoma. Soft-agar growth-promoting activity (•) represented as number of colonies containing 8 to 20 cells in 4 low-power microscope fields. EGF-competing activity (Ο) represented as percentage of inhibition of binding of 125I-EGF. •, MeCN gradient used, as described in "Materials and Methods."

Chart 4. Rechromatography of TGFA by gel filtration. TGFA, isolated after HPLC, was applied to a Bio-Gel P-30 column (2 x 40 cm) equilibrated in 1 M acetic acid and run at a flow rate of 0.2 ml/min. Two-ml fractions were taken, lyophilized, and then tested for colony-promoting activity (•) and EGF-competing activity (Ο). Revealed that the M, 30,000 TGF had been converted exclusively to a M, 6,000 TGF (Chart 5). These results suggest that there is a correlation between the M, 30,000 tumor-associated TGF and TGFA and further suggests that the M, 6,000 urinary TGF is at least in part derived from the M, 30,000 urinary TGF.

Two other issues which were of concern were how the elution from HPLC of urinary TGF differed from that of TGF secreted in vitro by tumor cells and which of the various urinary EGF-related TGF reverse-phase fractions (TGFA) might actually contain urogastrone (HEGF). Accordingly, a sarcoma growth factor preparation (6) and homogeneous recombinant HEGF were separately chromatographed under the same conditions as was the urinary TGF (Chart 6). The tissue culture-derived sarcoma growth factor activity eluted earlier in the gradient, approximately 26% MeCN, and hence is less hydrophobic than is urine-derived TGF. Also, as shown, the HEGF eluted with the same retention time as did TGFC (see Chart 1) and we tentatively conclude that some, if not all, of the EGF-competing activity in that region of the chromatogram may be due to EGF.

Since substantial soft-agar colony-forming activity was consistently observed with TGFC, and because in our experience, at doses ≤10 mg/ml, neither mouse EGF nor HEGF intrinsically caused the formation of colonies by NRK cells, it seemed likely that some of the activity might be due to the presence of non-EGF-related TGF. Therefore, in order to further characterize the...
Accordingly, suboptimal amounts (2 ng) of HPLC-purified mouse EGF was added to urinary HPLC fractions in a soft-agar colony assay. Results of a representative HPLC experiment with a urine sample taken from a normal female control are in Chart 7. In the absence of exogenous EGF, most colonies contained only 8 to 20 cells and coeluted with EGF-competing activity (α-TGF activity). The addition of purified mouse EGF (2 ng/ml) to the cultures enhanced the numbers of colonies for all fractions that contained EGF-competing activity and increased the size of those colonies to greater than 40 cells. Mouse EGF alone caused the formation of only low numbers of small colonies (<6 cells). In addition, when in the presence of added mouse EGF large colonies were found to be promoted by certain HPLC fractions (approximately, Fractions 65 and 91), eluting as broad, heterogeneous peaks with 30 and 43% MeCN. These fractions had shown no EGF receptor-binding activity and no colony-forming activity in the absence of EGF supplements. Hence, these fractions probably contain β-TGF-like activity, as described previously (1, 18, 20). In contrast to the distinctions in α-TGF-like profiles between normal and cancer urine, no differences in β-TGF-like activity profiles between normal controls and cancer patients (not shown) could be discerned.

**DISCUSSION**

Human urine has previously been shown to be a source of both HEGF (4, 9), which has limited soft-agar colony-promoting activity (6, 23) and EGF-related transforming growth factor activity (23, 27). Gel filtration of acid extracts of urine showed that normal donors expressed low-molecular-weight EGF-related TGF activity (M, 6,000 to 8,000) and that the majority of cancer patients demonstrated an additional high-molecular-weight EGF-related TGF activity (M, 30,000 to 35,000) (23, 27). The present study was undertaken to further analyze urinary TGF activity.

We now report that reverse-phase HPLC analyses of acid extracts of urine from normal donors and cancer patients revealed the presence of a total of 5 EGF-related growth factors with soft-agar colony-promoting activity and 2 non-EGF related factors which had colony stimulating activity only in the presence of added EGF. Of the 5 EGF-related activities observed, one (TGF4) was elevated in the urine from cancer patients and correlated with previously reported M, 30,000 to 35,000 tumor-associated TGF. A second (TGF5) occurring mainly in the urine from cancer patients, and a third, (TGF6), found at high levels only in normal control urines, were also identified.

The relative proportions of the various α-TGF-like activities observed within each specimen tested were in general the same when tested for either soft-agar growth-promoting activity or EGF-competing activity. However, the EGF competition assay is a rapid, 1-day radioreceptor assay, vis-à-vis the soft-agar bioassay which requires 7 to 14 days. Moreover, the solid-phase EGF assay is as effective and sensitive as the soft-agar bioassay for distinguishing the HPLC-separated TGFs. Thus, we were able to demonstrate that ultrafiltration, reverse-phase HPLC, and solid-phase EGF-competition assay techniques could be combined to provide a rapid, convenient procedure for monitoring EGF-related growth factor activity expression in the urine. Using this technology, we were able to discriminate between urinary EGF-related TGFs produced by cancer patients and normal controls.

The soft-agar colony formation assay warrants further comment. The colonies formed by the NRK cells were reported originally to consist of greater than 20 cells (5, 6) when sarcoma growth factor was used as a colony promoter. Other investigators report that purified EGF-related TGF (α-TGF) promotes formation of only very small colonies (18, 20). Similarly, there have been reports that EGF does not promote colonies (5, 6) or promotes large colonies (11, 18). These differences may, in part, be due to differences in growth factor purity but may also reflect qualitative differences in the colonies which resulted from the different growth factors examined. Also, the NRK cell line has an increased sensitivity to growth factors and heightened susceptibility to spontaneous transformation when cells from higher passages are used (11).

In the studies with human urine-derived growth factor activity, NRK cells were taken at passages 17 to 19 for soft-agar assays. Under these conditions, most of the colonies formed consisted of approximately 20 cells after a 7- to 14-day incubation period. Purified mouse EGF and HEGF caused the formation of few colonies, if any, containing no greater than 6 cells (23) when the EGF alone was used at concentrations as high as 10 ng/ml. In
the present study, an EGF supplement (2 ng/ml) was able to enhance the size and number of colonies promoted by some HPLC fractions that had EGF-competitive activity and, in addition, was able to promote colony formation by HPLC fractions which otherwise had shown neither colony-forming nor EGF-competitive activity. This is a novel discovery, since EGF-dependent growth-promoting activity in urine has not been described. Because there are reports that α-TGF colony-forming activity is not enhanced by EGF supplements (18, 20), this result suggests that suboptimal amounts of non-EGF-related TGF (β-TGF) may be a contaminant in those α-TGF fractions examined and is consistent with the demonstration that 3 orders of magnitude less EGF than α-TGF is required to augment the promotion of β-TGF colony formation (18, 20). Further purification of the components in urine with growth promoting activity is required to further resolve this issue. Accordingly, we have purified recently a M, 6,000 EGF-related TGF from melanoma patient urine which retained EGF-competitive activity and EGF-independent soft-agar growth-promoting activity, and which upon isoelectric focusing revealed a single peak that was distinct from the isoelectric point of urogastrone.3

The presence of high levels of tumor-associated TGFα, after HPLC purification of urine from cancer patients is in agreement with earlier observations by Sherwin et al. (23) of a unique M, 30,000 to 35,000 TGF activity found after gel filtration to be predominantly in urine from cancer patients. Our results indicate that the M, 30,000 TGF and TGFα are equivalent and that the M, 30,000 TGF will also convert to an active M, 6,000 TGF under conditions which have been reported not to promote conversion of high-molecular-weight HEFG (10). In addition, tumor-associated TGFA did not elute on reverse phase at the same retention as did HEFG. Thus, TGFα and M, 30,000 TGF are not HEFG, although the possibility exists that they may be either precursors or consist of altered forms of EGF.

Finally, we observed that these urinary TGFS appear to be chemically distinct from tissue culture-derived TGF by displaying increased hydrophobic character. If the urinary tumor-associated TGF is, in fact, a tumor-derived substance and not the result of a host response, its increased hydrophobicity over that of a tissue-culture-derived TGF suggests that it may have undergone chemical alteration in vivo. This was shown to be the case in another study involving urine from tumor-bearing mice.4

The dramatically elevated levels of TGFA and TGFα, which were found exclusively in the urine from cancer patients could be the result of changes in normal TGF metabolism. Thus, the high-molecular-weight tumor-associated TGF, corresponding to TGFA, may be present in the urine from cancer patients because it is not as efficiently metabolized as it is in normal controls. This interpretation is based on analogy to the conversion of high-molecular-weight EGF to low-molecular-weight EGF (10, 25) and is consistent with the demonstration that partially purified high-molecular-weight TGF (M, 30,000) will gradually convert to low-molecular-weight TGF (M, 6,000). We are examining currently the chemistry of M, 30,000 TGF conversion to M, 6,000 TGF. Immunological and biochemical comparisons to HEFG have also been undertaken to further resolve this aspect of our studies, and we are in the process of completely characterizing these various TGFS in order to understand the biochemical relationships which exist between them, to compare them to TGFS produced in vitro, and to ultimately understand their biological function. Finally, the observation that urine from cancer patients contains TGF activities which, via reverse-phase HPLC and a solid-phase EGF-competitive binding assay, can be distinguished from TGF activity found in normal controls further supports the possibility that TGF activity may be a clinically useful biomarker for certain types of cancer.

REFERENCES


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