High-Molecular-Weight Transforming Growth Factor Activity in the Urine of Patients with Disseminated Cancer

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ABSTRACT

Urine from 22 patients with a variety of disseminated cancers and from an equivalent number of nonmalignant controls of similar age and sex was tested for the presence of transforming growth factor (TGF) activity as measured by the ability to promote the growth in soft agar of nontransformed indicator cells. Cancer patients included those with carcinomas of the lung, breast, colon, and ovary, as well as melanomas and sarcomas. The nonmalignant controls included both normals and individuals with a variety of inflammatory and infectious disorders. Aliquots of unfrozen urine were acid extracted, chromatographed on a Bio-Gel P-30 column, and then tested for TGF activity using normal rat kidney fibroblasts and epidermal growth factor (EGF)-competing activity with human carcinoma A431 cells. These assays revealed that a high-molecular-weight TGF activity (M, 30,000 to 35,000) which coelutes with EGF-competing activity was present in 18 of 22 cancer patients but present in only five of 22 nonmalignant controls (p < 0.01). In contrast, a low-molecular-weight TGF activity (M, 6000 to 8000) which does not coelute with EGF-competing activity was found in all urines tested. These results indicate that an EGF-related, high-molecular-weight TGF activity is found in the urine of cancer patients and may be a useful tumor marker. Unlike other tumor markers described previously, high-molecular-weight TGF activity has a biological activity which is related to the expression of the transformed phenotype.

INTRODUCTION

TGFs2 are low-molecular-weight polypeptides produced by rodent and human tumor cells, which are capable of reversibly stimulating nontransformed cells to grow as colonies in soft agar (22, 24). TGFs have been identified in serum-free culture supernatants and cell extracts of virally and chemically transformed rodent cells (6, 11, 15, 20, 26) and in serum-free culture supernatants of various nonhuman tumor cell lines (24). TGF activity from human tumor cells and virus-transformed rat cells has been purified and partially characterized. The major component is a polypeptide with an apparent molecular weight of 7,400 (9, 26). Another form with an apparent molecular weight of 20,000 is also found, but larger forms have not been detected in the conditioned medium of tumor cells growing in culture. Although immunologically distinct from EGF, human TGFs can compete with EGF for binding to its receptor have been identified in both normal and transformed rodent cells and have been shown to lack soft agar growth-promoting activity except in the presence of added EGF (13, 14).

We have reported recently that TGF activity can be identified in acid-ethanol extracts of human urine (25). Our preliminary results indicated that different molecular weight species of TGF could be identified in human urine from different sources. All urines tested contained low-molecular-weight TGF activity in the M, 6,000 to 8,000 range, whereas pregnant female urine and urine from a patient with small-cell lung cancer were shown to have additional activities at an M, of approximately 10,000 and 20,000. In addition, the urine from the patient with lung cancer had high-molecular-weight TGF activity at an approximate M, of 30,000 to 35,000. This high-molecular-weight TGF was not found in the urine of the normal controls and also has not been found in the conditioned medium of human tumor cell lines. We now report that the high-molecular-weight TGF activity can, in fact, be found in the urine of a majority of patients with a variety of disseminated cancers and not in the urine of normal controls when assayed under the same conditions. The high-molecular-weight TGF activity in the urine of patients with disseminated cancers may be a useful tumor marker which, unlike other tumor markers identified previously, has a biological activity that may be directly related to the maintenance of the transformed phenotype.

MATERIALS AND METHODS

Patient and Control Populations. Urine specimens were collected from 22 patients with a variety of disseminated cancers including breast cancer (5 patients), lung cancer (5 patients), colon cancer (5 patients), malignant melanoma (4 patients), soft-tissue sarcoma (2 patients), and ovarian cancer (one patient). All patients had clinically detectable metastatic cancer and had not received any chemotherapy, hormonal therapy, or radiation therapy for at least 4 weeks prior to the time of specimen collection. In addition, the renal function of the patients was screened, and all patients were found to have normal serum creatinines (<1.5 mg/dl) and no proteinuria as determined by urinalysis.

Specimens were also collected from 22 nonmalignant controls of comparable age and sex. These controls included both normal volunteers and patients with nonmalignant inflammatory or infectious disorders including bronchitis, emphysema, pneumonia, pancreatitis, ulcerative colitis, and diverticulitis.

Urine Specimen Collection. Urines were collected from patients and normal controls over a 24-hr period and no more than 48 hr prior to testing. Patients were instructed to store their collection bottles at 4° during the collection period. Urine collections less than approximately 500 ml were considered inadequate and were not included for analysis. All urines were tested following storage at 4° without having been frozen previously.

Extraction of Urine Specimens. In selected patients, large volumes...
>1000 ml of urine were subjected to acid-ethanol extraction according to a modification of the procedure of Roberts et al. (15) and as reported previously (25). Clarified urine was extracted with acidified 95% ethanol, centrifuged to remove acid-insoluble material, and then reextracted with cold ether and ethanol. The resulting precipitate was collected by centrifugation, redissolved in 1 m acetic acid, dialyzed against 0.2 m acetic acid, and chromatographed on a Bio-Gel P-100 system (2.5 x 75 cm) at room temperature with 1 m acetic acid as the eluent. Column fractions of 3.5 ml were collected, and aliquots were lyophilized and tested for the presence of soft-agar growth-promoting activity and EGF-competing activity as described below.

We also developed a more rapid, smaller scale extraction procedure for urinary TGF activity, which could be used to screen the urines of large numbers of patients and controls. This procedure consisted of clarifying by centrifugation an aliquot of urine not frozen previously containing 10 mg of urine protein. The clarified aliquot of urine was then acidified with acetic acid (final concentration, 1 m), and the resulting precipitate of acid-insoluble material was removed by centrifugation. The supernatant was then lyophilized, resuspended in 0.5 ml of 1 m acetic acid, and chromatographed on a Bio-Gel P-30 system (1.6 x 20 cm) at room temperature with 1 m acetic acid as the eluent. The column was calibrated with standard markers including carbonic anhydrase (M, 29,000), ribonuclease (M, 13,900), and insulin (M, 6,000). Column fractions of 1.0 ml were collected, and aliquots were lyophilized prior to testing for soft-agar growth-promoting or EGF-competing activity as described below.

**Soft-Agar Growth Assay.** Column fractions were tested for the presence of factors capable of stimulating nontransformed NRK (clone 49F) fibroblasts to grow as 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% fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.) plus 0.3% agar (Difco Laboratories, Detroit, Mich.) 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 consisting of more than 6 cells were scored in 4 random low-power fields at both 7 and 14 days. NRK cells do not grow as colonies in soft agar when using a nonparametric Wilcoxon 2-sample test.

**EGF-binding Competition Assays.** Column fractions were also tested in selected cases for the presence of factors capable of competing with 125I-radiolabeled mouse EGF for binding to receptors on formalin-fixed human carcinoma A431 cells according to methods published previously (6, 21).

**Statistical Analysis.** A comparison of M, 30,000 to 35,000 TGF activity in the urine of cancer patients was made with normal controls using a nonparametric Wilcoxon 2-sample test.

**RESULTS**

**Comparison of TGF Activity in Cancer and Normal Urine.** In initial experiments, 1- to 2-liter volumes of urines from a cancer patient and a normal control were extracted by the acid-ethanol method described above, chromatographed on a Bio-Gel P-100 chromatography system, and tested for the presence of TGF activity and EGF-competing activity. As reported previously (25), these experiments revealed that the urine from the cancer patient contained a high-molecular-weight TGF activity (M, of approximately 30,000 to 35,000) which coeluted with EGF-competing activity and which was not found in the normal control. In contrast, both urines contained low-molecular-weight TGF activity (M, of approximately 6,000 to 8,000) that did not coelute with EGF-competing activity. To determine whether the presence of the high-molecular-weight TGF activity could, in fact, be observed in a large percentage of patients with disseminated cancer, the more rapid screening assay for the presence of this activity in the urine was utilized. Column fractions obtained following Bio-Gel P-30 chromatography of acid-extracted urine were assayed for TGF activity by testing for factors capable of stimulating soft agar colony growth of NRK cells and for EGF-competing activity using human A431 cells. Chart 1 shows the results of a typical experiment in which urine from a patient with alveolar cell soft-tissue sarcoma was assayed using the Bio-Gel P-30 system. Two peaks of TGF activity were seen with molecular weights of approximately 30,000 to 35,000 and 6,000 to 8,000, which corresponded to the major TGF activity peaks seen previously on the Bio-Gel P-100 system. The low-molecular-weight TGF activity at M, 6,000 to 8,000 was found in every urine tested. EGF-competing activity in the sarcoma patient's urine again appeared to coelute with the high-molecular-weight TGF activity and not with the low-molecular-weight activity. Moreover, protein determinations on chromatographic fractions measured by absorbance at 280 nm were similar for cancer patients and normal controls. In both cases, protein was detected by this method in fractions corresponding to a molecular weight of less than 6,000 and was not detected in the M, range of 30,000 to 35,000. Since this more rapid assay could effectively identify the high-molecular-weight urinary TGF activity, this methodology was used to survey a large number of urines collected from patients with a variety of metastatic cancers.

**Survey of Urine from Patients with Disseminated Cancer for M, 30,000 to 35,000 TGF Activity.** Urine specimens from patients with different types of cancer were tested for the presence of high-molecular-weight TGF activity using the Bio-Gel P-30 chromatography system described above. An equal number of urines from nonmalignant controls of similar age and sex distributions were tested under identical conditions. All urines tested, from both cancer patients and controls, had low-molecular-weight TGF activity in the M, 6,000 to 8,000 range. However, whereas a majority of cancer patients were found to have high-molecular-weight TGF activity at M, 30,000 to
This activity was not seen in most of the nonmalignant controls. Chart 2 shows a scattergram representation of these findings. Since low-molecular-weight TGF activity is present in all urines tested, these results are expressed as a ratio of high- to low-molecular-weight TGF activity to control for the variability in the NRK colony assay and to eliminate false negatives. This ratio was calculated by dividing the number of colonies in 3 peak fractions at M, 30,000 to 35,000 by the number of colonies in 3 peak fractions at M, 6,000 to 8,000. As shown in Chart 2, 18 of 22 cancer patients and only 5 of 22 nonmalignant controls scored as positive. Nonparametric statistical analysis of these results by the Wilcoxon 2-sample test indicated that this result is highly significant with a p value of <0.01. Moreover, these 5 positive nonmalignant controls had fairly low levels of high-molecular-weight TGF activity that were exceeded by 9 of the 18 cancer patients. It is noteworthy that the control population includes 10 individuals with nonmalignant infectious and inflammatory conditions including bronchitis, pneumonia, colitis, pancreatitis, and diverticulitis. Four of the 5 nonmalignant controls scoring as positive in this assay were individuals one of these conditions.

Table 1 shows the types of patients with disseminated cancer that were tested for high-molecular-weight TGF activity in these experiments. All patients were tested at least 4 weeks after their last radiation or chemotherapy treatment. Patients with disseminated cancer arising in the lung, breast, colon, and ovary, as well as patients with soft-tissue sarcoma and malignant melanoma, were all found to have this activity in their urine. The 2 highest positives with TGF activities of 1.35 and 1.45 were patients with small-cell lung cancer and soft-tissue sarcoma, respectively.

Possible reasons for the apparent lack of high-molecular-weight urinary TGF activity in the majority of normal controls were further explored in the following experiments. To exclude the possibility that normal (nonmalignant) urine contains a factor which inhibits or degrades high-molecular-weight TGF activity, urine from normal controls was mixed in varying proportions, extracted, and cochromatographed with urines from known positive cancer patients. In no instance was there any apparent inhibition of the M, 30,000 to 35,000 TGF activity in the cancer patient’s urine by the addition of normal urine (data not shown). An alternative possibility that was considered was that, although the high-molecular-weight TGF activity appeared to be tumor associated with the above assay conditions, it might, in fact, be possible to detect this activity at very low concentrations in normal urine. Chart 3 shows the results of experiments in which increasing concentrations of input urine protein from both a nonmalignant control and a cancer patient were assayed for TGF activity. As shown, this activity can be detected in the cancer patient’s urine at protein concentrations as low as 5 mg, whereas this activity is not detected in the normal control’s urine until a protein concentration of 50 mg is used. This suggests that the M, 30,000 to 35,000 TGF activity from urine might, in fact, be present in very low concentrations in normal urine, although these urines did not score as positive using the assay conditions described above. Chart 3 also reveals an apparent plateau in the level of high-molecular-weight TGF activity in the positive urine when the input protein concentration exceeds 10 mg.

**DISCUSSION**

TGFs which are capable of stimulating the growth of nontransformed indicator cells in soft agar have been identified in serum-free culture supernatants of rodent and human tumor cells and in cell extracts of virally and chemically transformed
rodent cells (6, 11, 15, 20, 22, 24). We reported previously that similar activities could be identified in human urine and that a patient with lung cancer appeared to have at least one high-molecular-weight species of TGF activity at M, 30,000 to 35,000 which could not be identified in the urine of normal controls (25). We now show that most patients with disseminated cancer have a similar high-molecular-weight TGF activity in their urine and that this activity is lacking in the majority of normal individuals when assayed under the same conditions. In contrast, low-molecular-weight TGF activity at M, 6000 to 8000 is found in all urines tested. This low-molecular-weight soft-agar growth-promoting activity, however, does not correspond to the peak of EGF-competitiveness activity (Chart 1), suggesting that more than one peptide may be involved in producing this effect.

When cancer patients and controls are compared with regard to the ratio of high- to low-molecular-weight TGF activity, 18 of 22 cancer patients but only 5 of 22 controls were positive, a result which is highly significant statistically ($p < 0.01$). The cancer patients tested included patients with cancer of the lung, breast, colon, and also sarcomas and melanomas. All had clinically evident tumor, had not received anti-cancer therapy for a minimum of 4 weeks prior to testing, and had normal renal function without proteinuria. The nonmalignant controls shown for the most part to lack the M, 30,000 to 35,000 TGF activity included 10 individuals with inflammatory disorders, such as bronchitis, pneumonia, pancreatitis, colitis, and diverticulitis. Individuals with one of these disorders accounted for 4 of the 5 positives among the control group, thus suggesting the possibility that inflammatory disorders characterized by cell proliferation and hyperplasia may result in increased levels of high-molecular-weight TGF activity. The presence of increased levels of other tumor markers such as CEA or AFP in inflammatory and hyperplastic conditions is well recognized (1, 7, 10, 27). Moreover, although the data presented demonstrate that high-molecular-weight TGF activity in urine is tumor-associated, the experiment in which this activity is titrated against increasing amounts of input protein (greater than 10 mg) indicates that very low levels of this activity may, in fact, be present in normal urines which score as negative under the usual assay conditions. In this respect, high-molecular-weight urinary TGF activity further resembles other tumor markers, such as CEA, which can be found in large percentages of the normal population (e.g., cigarette smokers) at low concentrations (19). In any case, as shown by the mixing experiments described above, the absence of this activity in most of the control urines under the usual assay conditions was not due to the presence of an inhibitory factor in normal urine.

The exact nature of the high-molecular-weight TGF activity found in the urine of patients with disseminated cancer remains to be defined. Preliminary analysis indicates that, like other TGF molecules described previously, the M, 30,000 to 35,000 species is an acid-stable polypeptide, as evidenced by the complete loss of soft-agar growth-stimulating activity in chromatographic aliquots treated with trypsin and the stability of this activity in 1 M acetic acid. This TGF also appears to lack the glycoprotein moiety required for binding to concanavalin A. Moreover, as shown here, the high-molecular-weight TGF activity coelutes with EGF-competitively active activity, suggesting that it can compete for binding to EGF receptors and may therefore be related to EGF. In contrast, the low-molecular-weight urinary TGF activity at M, 6000 to 8000 does not coelute with an EGF-competitively active TGF, thus suggesting that human cancer patient urine may contain both EGF-related and EGF-unrelated TGFs analogous to those described recently in rodent cells (13, 14). The lack of a concanavalin A-related glycoprotein moiety in M, 30,000 to 35,000 urinary TGF is important and indicates that it is distinct from the M, 27,000 glycoprotein found in greater than 100-mg quantities in the urine of patients with leukemia and disseminated cancer (16). This protein, termed "EDC1," is a protease inhibitor and is thought to be a fragment of the larger plasma molecule, inter-α-trypsin inhibitor (2). The high-molecular-weight urinary TGF activity appears to be distinct by virtue of size and other biochemical properties from the M, 18,000 tumor angiogenesis factor-like activity found in the urines of patients with transitional cell tumors of the bladder (3).

The M, 30,000 to 35,000 TGF activity in the urine of patients with disseminated cancer may be a clinically useful tumor marker. However, as is true with other tumor markers like CEA and AFP, this activity appears to be only tumor associated and not tumor specific. Thus, high-molecular-weight TGF activity can be identified, at least at low concentrations, in the urines of certain nonmalignant controls including individuals with nonmalignant inflammatory or infectious disorders. The lack of complete specificity for the high-molecular-weight urinary TGF activity suggests that its usefulness as an early diagnostic marker may be limited, but its quantitative expression may be related to the extent of tumor burden which would make it useful in following the course of patients with cancer and their response to treatment. CEA, which has the same lack of complete tumor specificity, has been shown to be valuable in this way for the management of patients with colorectal and other cancers (7, 12).

The high-molecular-weight urinary TGF activity is unique among tumor markers with regard to its specific biological properties. In contrast to CEA and AFP, which appear to be oncifetal antigens without a precise functional role in the malignant state, the urinary M, 30,000 to 35,000 TGF activity is biologically active and can reversibly promote the growth in soft agar of nontransformed indicator cells. To the extent that soft agar growth is a property of transformed cells (4, 8), urinary TGF activity may represent a tumor cell product which in turn supports the growth of the cells producing it. Sporn and Todaro (18) have postulated recently that "autocrine secretion" of growth factors, such as TGF, by tumor cells may play a role in the development or maintenance of the neoplastic state (8, 17, 23). If, in fact, the urinary TGF activity described here is a direct product of the patient's tumor, the experiments presented above provide preliminary evidence supporting the hypothesis that such autocrine secretion may be occurring in vivo in patients with disseminated cancer.

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REFERENCES


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