Variant Forms of Rat Epidermal Growth Factor Present in the Urine of Nude Rats Bearing Human Tumors

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ABSTRACT

Epidermal growth factor (EGF) receptor-binding peptides from the urine of tumor patients have been reported to differ in molecular weight and relative hydrophobicity from those of normal individuals. Nude rats bearing human large cell lung carcinomas or chondrosarcomas and non-tumor-bearing sibling control rats were used to investigate the contributions of tumor and host to urinary EGF-related peptide growth factors. Peptides were adsorbed from urine onto methyl-bonded silica and eluted according to their relative hydrophobicity by a stepwise gradient of aqueous acetonitrile. Total EGF receptor-binding activity relative to urinary creatine was elevated in the urine of only one group of tumor-bearing rats. However, the proportion of relatively hydrophilic activity was increased markedly in all three groups of tumor-bearing rats. Rats bearing a large cell lung cancer excreted unusually hydrophilic M, 6000 peptides that were chromatographically similar to transforming growth factor α on reverse phase high performance liquid chromatography but proved to react with only by radiimmunoassay (RIA), EGF receptor-binding activity that was common to the urine of tumor-bearing animals regardless of tumor type, but more hydrophilic than that from control rats, had M, 60,000, 30,000, 12,000, and 4,000 to 7,000 components. All reacted fully in the rat EGF RIA and were negative for human EGF and transforming growth factor α by RIA. A more hydrophobic fraction of EGF receptor-binding activity, common to control and tumor-bearing animals, contained M, 33,000, 5,000 to 7,000, and 2,000 to 5,000 components. High performance liquid chromatography and gel electrophoresis of the M, 33,000 activity revealed a high molecular weight rat EGF comparable to that reported in human urine. No human EGF or transforming growth factor α was detected by RIA in any of the active fractions from tumor-bearing rat urine. Thus, all EGF receptor-binding activity appeared to derive from rat EGF produced by the rat host and not by the xenografted tumors.

INTRODUCTION

Some tumor cells are thought to escape normal growth regulation via autocrine mechanisms which involve inappropriate production of growth factors and/or growth factor receptors leading to self-stimulated proliferation (1). An example of this phenomenon is small cell lung carcinoma, which secretes a bombesin-like growth factor (2) that stimulates its growth (3); antibodies to bombesin inhibit small cell lung carcinoma growth in tissue culture and in nude mice (4). Autocrine stimulation was proposed as the mechanism by which peptide TGFs promote anchorage-independent growth and loss of density-dependent growth inhibition of cells in vitro and growth of tumors in vivo (1). Several TGFs are members of a family of peptides that bind to the EGF receptor, have structural homology to EGF, and, in the presence of the synergistic but functionally and structurally unrelated peptide TGF-β, cause anchorage-independent growth of normal rat kidney cells in soft agar (clonogenicity). Members of this clonogenic family of peptides include M, 6,000 EGF (5) and HMW EGF precursors (6), structurally related TGF-α (7) and its probable precursors (8, 9), a peptide encoded by vaccinia virus (10), and M, 15,000 and 20,000 to 22,000 peptide growth factors, secreted by A673 human tumor cells, that do not react with antibodies to either hEGF or TGF-α (11).

The v-sis oncogene is the only one known to encode a growth factor (12). The v-erb-β oncogene codes for a truncated EGF receptor (13), but one that codes for a peptide which binds to the EGF receptor has not been described. However, secretion of EGF receptor-binding growth factors in response to virus-induced cell transformation has been reported (14-17), and TGFs have been found in the urine of tumor-bearing patients (18-21). TGF-α and EGF bind to the same receptor (14) and both are clonogenic in the presence of TGF-β (22). Therefore, TGF assays that depend on competition for EGF receptor binding or clonogenicity cannot distinguish between EGF and TGF-α. However, RIAs make it possible to determine whether EGF radioreceptor activity is caused by TGF-α or EGF. Immunoreactive EGF is normally excreted in human urine at the rate of 30 to 100 µg/day (23) and appears to be a normal product of the kidney itself (24). Immunoreactive TGF-α is present in the urine of breast cancer patients at approximately 0.5% of the concentration of immunoreactive EGF (25) and has been reported to occur in greater amounts in hepatocellular cancer patients (26). HMW TGF in the urine of brain tumor patients has been identified as a HMW form of hEGF (6). Immunoreactive EGF has been reported to be elevated in the urine of many tumor patients (27). It is not yet clear whether the increased amounts of EGF and HMW EGF in the urine of tumor patients are produced by the tumor and excreted by the kidney or are the result of increased renal production of EGF stimulated by factor(s) produced by the tumor.

The most common cancers of both men and women are lung carcinomas, of which about 75% are classified as NSCLC type. Currently there is no effective treatment for NSCLCs. If it were possible to detect these tumors at an earlier stage, perhaps surgical intervention, radiotherapy, and/or multiagent chemotherapy might be effective. Some NSCLCs produce EGF receptors (28, 29) and thus might respond to the autocrine production of EGF-related growth factors. Therefore, it is possible that urinary peptides of the EGF/TGF-α family might derive from NSCLCs or be produced by normal tissue(s) in the host in response to the presence of these tumors. We sought to establish human xenograft NSCLCs in nude rats as a model in which to test this hypothesis. Although the success in establishing tumor transplants in nude rats is less than in nude mice (30), a rat xenograft model has the advantage that rats produce greater volumes of urine which can be conveniently collected in metabolic cages. This study concerns the two of five NSCLCs and a CS which grew successfully in nude rats.
VARIANTS OF EGF IN URINE OF RATS BEARING HUMAN TUMORS

This study was designed to determine whether the 2 human NSCLCs and the CS caused a detectable increase in the excretion of EGF receptor-binding growth factors in the urine of the nude rats bearing the tumors and to ascertain whether the tumors secreted the growth factors [i.e., the human peptide(s) was found in the urine of the rats] or stimulated their excretion indirectly due to some tumor-host interaction [i.e., the rat peptide(s) was found increased in the urine]. In either case, measurable increases in urinary excretion would have the potential of perhaps providing detection of a currently untestable malignancy.

MATERIALS AND METHODS

Tumor Propagation, Urine Collection, and Preparation. Cells from a human large cell lung carcinoma with squamous metaplasia (NSCLC I), undifferentiated large cell lung carcinoma (NSCLC II), and a CS (all generously provided by Drs. Henry Azar and Jose Costa) were injected s.c. with a trocar into nude rats (19 Cr:NIH nu). Tumors arising at the site of injection were assumed to derive from the human cells injected and were not karyotyped. Rats were housed in metabolic cages which allowed the collection of urine separate from feces. Urine from nude rats bearing tumors 1 cm or more in diameter was collected daily and stored frozen at −40°C until between 8.000 and 1.3 liters was obtained over a period of about 1 month. The tumors of the rats in each group were increasing in size during the period of collection. A comparable volume of urine (750 ml) was collected from sibling control nude rats. Before processing, urine was thawed and pooled. Volume, total creatinine, and total protein were determined (NSCLC I, 0.940 ml of 1 M acetic acid, and centrifuged at 100,000 × g for 30 min. The supernatants were applied to a column (1.5 x 85 cm; 125 ml bed volume) of Bio-Gel P-100 (100 to 200 mesh; Bio-Rad Laboratories, Richmond, CA), equilibrated with 1 M acetic acid and transferred to a Chromatographie column containing a lower "cushion" layer of Sepralyte in a ratio of 3 g of cushion/g of urinary protein. Free suspension adsorption of select urinary proteins to Sepralyte is achieved by circulating Sepralyte in urine with a magnetic stirring bar for 45 min. Sepralyte was allowed to settle for 30 min, urine was decanted, and Sepralyte was suspended in 12% acetonitrile and transferred to a Chromatographie column containing a lower "cushion" layer of Sepralyte in a ratio of 1 g of cushion Sepralyte per 3 g of adsorbed Sepralyte. Sequential, stepwise batch elution of C-1 Sepralyte beads with 12, 15, 20, 25, 30, 35, and 50% acetonitrile in 0.1 M NaCl and 0.1% TFA was then carried out. Five ml acetonitrile were used per g of total Sepralyte.

Gel Filtration Chromatography. The acetonitrile eluates were desalted by dialysis for 48 h against 2 changes of 1 M acetic acid [Spectrophor tubing, molecular weight cutoff 3500] (Creative Biomolecules, Hopkinton, MA) were added to 750 ml of human urine that contained <0.05 μg of endogenous immunoreactive human TGF-a and rat TGF-α equally well (11). The sensitivity and half-maximal displacement were 3 pg of purified TGF-α kindly provided by Dr. Michael B. Sporn, added per 35-mm dish. A 0.5% agar base layer and 0.3% agar overlay containing the test sample were used. Normal rat kidney cells, a clone of 49F, were seeded at 3 x 10⁶ cells/dish, overlaid with medium containing the test sample, incubated at 37°C in a humidified 5% CO₂ atmosphere, and refed with medium on day 5. The number of colonies containing either 20 or more cells, or 50 or more cells per 8 low-power fields, was scored on day 15.

RIA for hEGF, rEGF, and TGF-α. Homologous hEGF RIA was performed by modifications (37) of a method (38) using an antiserum raised in a rabbit to highly purified hEGF, generously provided by Dr. Yukio Hirata, National Cardiovascular Institute Research Center, Osaka, Japan. Highly purified hEGF (39) was used for a reference standard and labeled tracer. The sensitivity (10% displacement) was 2 pg hEGF/tube, and half-maximal displacement was 10 pg hEGF/tube. A heterologous rEGF RIA was performed using an antiserum (R10) raised in a rabbit to highly purified mEGF (40) that was shown to bind highly purified rEGF. Highly purified rEGF prepared from male rat submandibular glands (40) and having the same sequence reported by Simpson et al. (41) was used as radiiodinated tracer and reference standard. The sensitivity and half-maximal displacement were 3 pg and 30 pg of rEGF/assay tube, respectively. The TGF-α RIA was performed (8) using a commercial kit (Biotope, Inc., Seattle, WA). Full-length bioactive synthetic TGF-α (42) was used as radiiodinated tracer and reference standard. The antiserum was raised in a rabbit to the COOH-terminal 17-amino acid fragment of TGF-α. This TGF-α RIA detected human TGF-α and rat TGF-α equally well (11). The sensitivity and half-maximal displacement were 0.2 and 1.0 ng TGF-α/assay tube, respectively. Proteins in samples were reduced (40 mg/ml, heated (100°C for 1 min) prior to assay. To confirm that the TGF-α RIA detected TGF-α extracted from urine, 3 μg of fully bioactive recombinant hTGF-α (Creative Biomolecules, Hopkinton, MA) were added to 750 ml of human urine that contained <0.05 μg of endogenous immunoreactive TGF-α. The urine was subject to the usual extraction procedure, and the immunoreactive TGF-α in the denatured, reduced extract was determined.

RESULTS

EGF-related Growth Factors in the 20% Acetonitrile Eluate. Two growth factors known to bind to the EGF receptor, EGF and TGF-α, can be concentrated from urine by adsorption to

3 C. D. Mount, T. J. Lukas, and D. N. Orth, unpublished data.
microparticulate methyl-bonded silica; adsorption of radioiodinated growth factors is 99% for hEGF and 87% for rTGF-α (31). Total EGF radioreceptor activity elutes with 15 to 30% acetonitrile. Stepwise elution is performed to separate component peptides according to their relative hydrophobicity. Approximately 50% of 125I-rTGF-α added to and subsequently extracted from urine eluted in the 15 to 20% acetonitrile fraction (31). The EGF RRA activity per g urinary creatinine in the 20% eluate was 6- to 7.5-fold greater in the urine of rats bearing tumor NSCLC I than the other tumors and 30-fold greater than the control rats (Table 1). To determine whether the 20% acetonitrile elutes from rats bearing tumors NSCLC I might contain TGF-α, this fraction was chromatographed on a Bio-Gel P-10 column (Fig. 1). EGF radioreceptor activity eluted as a single peak, just after the M, 6,000 marker, which is similar in molecular weight to EGF. Active fractions were pooled and lyophilized and contained less than 2 mg of protein.

The pooled lyophilized material from P-10 fractionation was applied to an analytical reverse-phase HPLC column to distinguish between EGF and TGF-α, which is more hydrophilic than EGF (31). Using a linear gradient of 18 to 36% acetonitrile, 2 peaks of EGF radioreceptor activity and clonogenicity eluted from the HPLC column, one at 19% acetonitrile (peak I) and the second at 21% acetonitrile (peak II) (Fig. 2). Synthetic rTGF-α elutes at 22% acetonitrile (31), but no immunoreactive TGF-α was detected in any fraction (Fig. 2). Fractions 1 to 4 (peak I) and 7 to 9 (peak II) that had EGF radioreceptor activity were pooled and subjected to EGF RRA and rEGF and TGF-α RIAs (Table 2A). Both peaks had comparable rEGF immunoreactivity and EGF radioreceptor activity; neither had detectable TGF-α immunoreactivity in an RIA assay that detects rat and human TGF-α equally well. Therefore, these peptides appeared to be derived from the nude rat host, not from the human tumor.

In a reconstruction experiment, this RIA measured 2.9 of 3 μg of exogenous, bioactive TGF-α added to normal urine indicating that if any rTGF-α or hTGF-α were present, it would have been detected by our assay method.

Fig. 2. Reverse-phase HPLC of the M, 6,000 EGF RRA activity from the Bio-Gel P-10 column eluate (Fig. 1). Fractions containing RRA activity were pooled, lyophilized, and reconstituted in 1 ml of 0.1% TFA and injected into a C18-Bondapak (Waters Associates) analytical column. The peptides were eluted with a linear 18 to 35% acetonitrile gradient in 0.05% TFA over 120 min. Flow rate was 0.7 ml/min. Fraction volume was 2 ml. Absorbance was monitored continuously at 206 nm. Fractions were assayed for EGF RRA activity, clonogenicity, and immunoreactive TGF-α.

Table 2 Total radioreceptor activity and immunoreactivity of selected chromatographic peaks derived from acetonitrile eluates of the urine of animals bearing tumor NSCLC I

<table>
<thead>
<tr>
<th>Acetonitrile elute (%)</th>
<th>Chromatographic source</th>
<th>RRA* (ng)</th>
<th>RIA* (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>HPLC (peak I)</td>
<td>352</td>
<td>493</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>450</td>
<td>495</td>
</tr>
<tr>
<td>B</td>
<td>Bio-Gel P-100 (peak II)</td>
<td>1700</td>
<td>1190</td>
</tr>
<tr>
<td></td>
<td>Bio-Gel P-100 (peak III)</td>
<td>5080</td>
<td>4267</td>
</tr>
</tbody>
</table>

Table 1. EGF radioreceptor activity in the 20, 25, and 30% acetonitrile eluates from methyl-bonded silica

<table>
<thead>
<tr>
<th>Acetonitrile elute (%)</th>
<th>ng EGF equivalent/g total urinary creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>NSCLC I 6,000 NSCLC II 1,000 CS 800 Control 200</td>
</tr>
<tr>
<td>25</td>
<td>17,000 58,000 182,000 5,000</td>
</tr>
<tr>
<td>30</td>
<td>100,000 42,000 111,000 118,000</td>
</tr>
<tr>
<td>Total</td>
<td>123,000 101,000 293,800 123,200</td>
</tr>
</tbody>
</table>

Fig. 1. Bio-Gel P-10 gel chromatography of the hydrophilic 20% acetonitrile eluate derived from the urine of rats bearing tumor NSCLC I. Fraction volume was 3.5 ml. After determination of absorbance at 280 nm of alternate fractions, 175-μl aliquots of selected fractions were lyophilized and reconstituted in 100 μl RRA buffer prior to EGF RRA. The elution positions of molecular weight standards [carbonic anhydrase (M, 30,000), RNase (M, 13,700), and bovine trypsin inhibitor (M, 6,000)] are indicated. K, thousands.

Fig. 3. Bio-Gel P-100 chromatography of the HMW clonogenic Bio-Gel P-10 peak from the 25% acetonitrile eluate derived from the urine of rats bearing tumor NSCLC I. Fractions 40 to 48 from the Bio-Gel P-10 chromatography of this 25% eluate were pooled, lyophilized, and reconstituted in 2 ml of 1 M acetic acid and centrifuged for 30 min at 100,000 × g. The supernatant was applied to the Bio-Gel P-100 column. Fraction volume was 1.8 ml. Absorbance at 280 nm was determined on odd-numbered fractions, and 90-μl aliquots of even-numbered fractions were lyophilized and assayed for clonogenicity. Arrows, elution positions of molecular weight standards bovine serum albumin (68K), carbonic anhydrase (30K), RNase A (13.7K), and insulin (6K).
Table 3 Comparison of EGF RRA activity and immunoreactivity of medium molecular weight (M, 4000 to 7000) peptides in the 30% acetonitrile eluates of urine from tumor-bearing animals

<table>
<thead>
<tr>
<th>Tumor</th>
<th>RRA (ng)</th>
<th>RIA (ng)</th>
<th>TGF-α (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSCLC I</td>
<td>19,000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9,500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;50&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NSCLC II</td>
<td>50,000</td>
<td>47,000</td>
<td>&lt;50</td>
</tr>
<tr>
<td>CS</td>
<td>60,000</td>
<td>45,000</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as ng equivalents of mEGF reference standard.
<sup>b</sup> Expressed as ng equivalents of the rEGF, hEGF, or TGF-α reference standard, respectively.
<sup>c</sup> ND, not done.

EGF-related Growth Factors in the 25% Acetonitrile Eluate

We next examined the more hydrophobic 25% acetonitrile eluate. EGF radioreceptor activity in this fraction was 3.4- to 36-fold greater in the tumor-bearing animals than in the controls (Table 1). There was a total of only 4 μg of EGF radioreceptor activity in the 25% acetonitrile fraction of the control rat urine pool. Therefore, the control sample was not further examined. Bio-Gel P-10 chromatography of all three 25% acetonitrile fractions derived from the urine of animals bearing each of the three different tumors revealed HMW peaks of clonogenic activity that eluted in the void volume and a clonogenic activity with apparent molecular weight of between 4,000 and 7,000 (data not shown). HMW TGFs have been reported in urine of tumor patients (6, 18, 19), so we further characterized the HMW component from tumor NSCLC I by Bio-Gel P-100 chromatography. At least 3 peaks of clonogenic activity were observed, 2 major ones with apparent molecular weights of approximately 60,000 and 33,000 and a minor peak with a molecular weight of about 15,000 (Fig. 3). Fractions 12 to 24 (peak I), 25 to 39 (peak II), and 40 to 45 (peak III) were pooled, lyophilized, and reconstituted in the appropriate assay buffer and subjected to EGF RRA and rEGF RIA. Most of the EGF radioreceptor activity in all 3 peaks was accounted for by a rEGF-related molecule (Table 2B). Active 25% acetonitrile eluate fractions in the M, 4,000 to 7,000 range were pooled, lyophilized, and reconstituted in the appropriate assay buffer and subjected to EGF RRA and RIAs for rEGF, hEGF, and TGF-α. In the urine of rats bearing all 3 tumors, the EGF RRA activity was accounted for in large part (50 to 94%) by immunoreactive rEGF; no immunoreactive hEGF or immunoreactive TGF-α was detected (Table 3).

EGF-related Growth Factors in the 30% Acetonitrile Eluate

The relatively hydrophobic 30% acetonitrile eluates from the urine of all 3 tumor-bearing and control nude rat groups would be expected to contain most of the EGF that was present in the urine (31). Bio-Gel P-10 chromatography of each of these fractions revealed an apparent HMW peak of clonogenic activity eluting in the void volume and a broad region of clonogenic activity with an apparent molecular weight distribution of 3,000 to 7,000 (Fig. 4). RRA of the M, 3,000 to 7,000 fractions from NSCLC I and II indicated that the clonogenic activity probably resulted from two incompletely resolved peaks of EGF receptor-binding activity, one with an apparent molecular weight of 6,000 and a second at about M, 4,000. HMW EGF has been found in urine of brain tumor patients (6), so we purified and characterized the apparent HMW (M, 30,000) component. Bio-Gel P-100 chromatography of the pooled Bio-Gel P-10 HMW

Fig. 4. Bio-Gel P-10 gel chromatography of the 30% acetonitrile eluate derived from the urine of tumor-bearing and control animals: (A) tumor NSCLC I; (B) tumor NSCLC II; (C) tumor CS; and (D) control animals. Fraction volume was 3.5 ml. Aliquots (150 μl) of even-numbered fractions were lyophilized prior to assay for clonogenicity and EGF radioreceptor activity. RRA values greater than 3000 ng exceeded the standard curve and are plotted as greater than 3000 ng.

Fig. 5. Reverse-phase HPLC of the HMW fractions from Bio-Gel P-100 gel chromatography of the 30% acetonitrile eluate derived from the urine of tumor-bearing and control animals: (A) tumor NSCLC I; (B) tumor NSCLC II; (C) control animals. Active fractions eluting from a Bio-Gel P-100 column (not shown) were pooled, lyophilized, and reconstituted in 1 ml of 0.1% TFA and injected into a C<sub>18</sub>-Bondapak reverse-phase HPLC analytical column. Peptides were eluted with 10 ml of 0.05% TFA, 10 ml of 25% acetonitrile (23% in C), and a linear gradient of 25 to 35% acetonitrile (23 to 35% in C) over 120 min. Flow rate was 0.7 ml/min. Fraction volume was 2 ml. A 100-μl aliquot of each fraction was diluted with 50 μl of 0.1% bovine serum albumin in 1 M acetic acid, lyophilized, and reconstituted in 100 μl of RRA buffer prior to assay of EGF radioreceptor activity. Absorbance at 206 nm was monitored continuously. Arrows, point at which each gradient reached 29% acetonitrile.
eluate fractions of all 3 xenografts, and the control urine produced single $M_r$ 25,000 to 35,000 peaks (data not shown). These peaks were further purified by reverse-phase HPLC. RRA activity eluted from the column between 27 and 30% acetonitrile, with the larger peaks in all 3 tumors and the control eluting with 29% acetonitrile (Fig. 5). Aliquots of the 29% acetonitrile peak of each urine extract containing approximately 100 ng of RRA activity were lyophilized and subjected to SDS-PAGE (Fig. 6). Samples from the CS and the control animal urine contained a single predominant peptide component with an apparent molecular weight of 33,000, as did a sample of HMW weight hEGF (23, 40). Urine from NSCLC I and NSCLC II tumor-bearing rats also contained $M_r$ 33,000 components, as well as smaller components with molecular weights of about 20,000 and 10,000, on SDS-PAGE. The lower molecular weight peptides observed in the electrophoretic pattern in Fig. 6 presumably resulted either from digestion by acid-active exopeptidase(s) present in the urine extract or, more likely, from contaminating endopeptidase cleavage of disulfide loops prior to fractionation resulting in 2 or more strands being released after reduction by β-mercaptoethanol. Aliquots of RRA-active HPLC eluate fractions were then pooled and subjected to hEGF and rEGF RIAs (Table 4). In each eluate, most of the HMW and medium molecular weight RRA activity (data not shown) could be accounted for by immunoreactive rEGF; no immunoreactive hEGF was detected in any of the samples.

TG-F-α does not elute in 30% acetonitrile (31); therefore, TG-F-α RIA was not performed; however, any $M_r$ 6000 hEGF produced by the xenograft tumors should be found here. Again, the medium molecular weight RRA activity, and the low molecular weight RRA activity as well, in the pooled peak fractions (data not shown) was accounted for by immunoreactive rEGF; no immunoreactive hEGF was detected (Table 4).

### DISCUSSION

Failure to recover either TG-F-α or hEGF from the urine of tumor-bearing rats suggests that the tumors did not secrete EGF and TG-F-α, that the peptides are produced in amounts too low to be detected; that they are not released into the circulating blood; that they have some modification, such as a COOH-terminal extension of TG-F-α, that renders them less detectable by RIA (11); or that, if present in blood, they are not filtered or otherwise excreted by the kidney. The tumors used in these experiments have not yet been grown in tissue culture, and their ability to secrete growth factors is unknown. However, a human tumor known to secrete TG-Fs (11) caused increased urinary excretion of mEGF when implanted in nude mice but released little or no hEGF into the urine (43). TG-F-α recovered from the urine of these animals was low, about 50 ng/liter of a HMW form versus about 900,000 ng/liter of total EGF radioreceptor activity. We should have detected even this small amount of TG-F-α in the most hydrophilic 20% acetonitrile fraction (31). Moreover, in only two experimental samples was the immunoreactive EGF result substantially lower than the EGF radioreceptor result (200 versus 100 ng in Table 2, 25% eluate, Bio-Gel P-100 Peak III; and 19,000 versus 9,500 ng in Table 3, NSCLC I). We attribute this to experimental variation rather than to the presence of novel EGF receptor competing polypeptides that are not detected by our RIAs for rEGF, hEGF, or human TG-F-α.

Although we did not detect hEGF or TG-F-α, we found that tumor-bearing rats excreted more hydrophilic forms of rEGF than did control rats. EGF/urogastrone is synthesized as a part of a $M_r$ 128,000 precursor molecule in which the $M_r$ 6,000 EGF sequence is located near the COOH-terminal end of the putative extracytoplasmic domain, adjacent to the transmembrane sequence (44, 45). The biological effects of EGF are mediated by binding to its receptor and stimulating the protein kinase activity of the receptor (46), an action that is shared by other peptides (10, 47). The structural properties that these peptides share are limited to the number and placement of disulfide bonds, the size of the corresponding intervening peptide loops, and the placement of about 15 critical residues (7, 41, 48). In view of the wide structural variation in peptides that bind to and activate the EGF receptor, it seems probable that any one of them could sustain some modification without loss of bioactivity. In...
rats bearing tumor NSCLC I, M, 6,000 rEGF was converted from a peptide eluting in reverse-phase HPLC in 27% acetonitrile to peptides that eluted in 18 and 21% acetonitrile, both of which retained bioactivity and immunoreactivity. The extent of modification required to produce this hydrophilic shift is difficult to estimate. Interestingly, a similar hydrophilic modification was observed in EGF recovered from the urine of colon cancer patients. This modified hEGF eluted from a C18-Bondapak column in 20% acetonitrile.

The association of hydrophilic modifications of EGF and the presence of a tumor may relate to the capacity of transformed cells and tumors to release proteases (for reviews see Ref. 49). Mouse skin treated with phorbol ester released proteolytic activity (50), and mouse cells in culture released a sulfhydryl-protease termed "major excrated protein," in response to phorbol ester (51). Tumor cells have also been reported to release collagenase (52), elastase (53), and plasminogen activator (54). Proteases could act to make EGF more hydrophilic by cleaving 1 or more of the 3 peptide loops between disulfide bonds, thereby exposing additional free carboxyl- and amino-terminal ends.

The moderately hydrophilic 25% acetonitrile eluate from methyl-bonded silica yielded EGF RRA activity that could be resolved into 4 molecular weight groups that may represent different levels of cleavage of the M, 128,000 EGF precursor. Rat salivary gland contains EGF (41) and may have contributed some of the EGF we found in urine, although evidence in the mouse suggests that this is not the case (55). EGF from an acid-ethanol extract of athymic mouse urine contained only M, 6,000 and M, 20,000 forms of EGF (43). The HMW form of EGF found in the 25% acetonitrile eluate of tumor-bearing animals may correspond to the HMW EGF we observed to be increased in the urine of human astrocytoma patients (6). In both rat and human, these hydrophilic HMW EGFs probably represent modification of the M, 33,000 HMW previously reported in trace amounts in normal human urine (23). HMW EGF in an extract of pooled human urine has been shown to be a glycosylated fragment of the EGF precursor (56). Changes in the carbohydrate side chains of HMW EGF could greatly affect the net charge and apparent size of the molecule. The M, 60,000 EGF RRA activity found in the 25% acetonitrile eluate has no counterpart in either the 30% acetonitrile eluate or any other isolate of EGF previously reported. This moieity may represent an aggregation or early cleavage product in the processing of pro-EGF.

In the more hydrophobic 30% acetonitrile eluate, an approximately M, 30,000 EGF common to both tumor-bearing and control animals appeared to have a molecular weight of 33,000 by SDS-PAGE. This material comigrated with purified HMW hEGF and appeared to be similar or identical to M, 33,000 and 29,000 EGF forms previously isolated from human urine (23). HMW EGF from tumor-bearing rats had no hEGF immunoreactivity and appeared to be a HMW form of rEGF analogous to the HMW hEGF in human urine (23, 40). An approximate molecular weight of 20,000 was reported for a HMW form of mouse urinary EGF, suggesting that mouse precursor EGF may have an alternate cleavage site from rat and human EGF precursor (43).

The more abundant form of EGF in human (23) and mouse (43) urine corresponds to the fully processed M, 6000 peptide. While both the control and tumor-bearing rat urine yielded a relatively large proportion of M, 5000 to 7000 immunoreactive

rEGF in the 30% acetonitrile fraction, only tumor-bearing animals produced more than 4 µg of M, 5,000 to 7000 RRA activity eluting in the more hydrophilic 25% acetonitrile fraction. Heterogeneity occurs in M, 6000 hEGF in human urine through the loss of 1 or more COOH-terminal residues (39). The loss of these amino acids affected the retention time on reverse-phase HPLC but did not affect the affinity of the altered EGF for the EGF receptor. Mouse EGF lacking the NH2-terminal asparagine residue also retains full receptor binding activity (57). Rat salivary gland EGF is reported to be shorter than mEGF and hEGF by 5 COOH-terminal residues, having only 48 amino acids (39). Heterogeneity of the NH2-terminal end of rat salivary gland EGF also occurs (41, 58), yet these rEGFs are equipotent in RRAs. It appears possible that the tumor-related hydrophilic, 25% acetonitrile eluting rEGF results from the loss of NH2- and/or COOH-terminal residues from the more hydrophobic parent rEGF.

An rEGF with an apparent molecular weight of 3000 to 5000 was recovered from the 30% acetonitrile eluate fraction of urine from both tumor-bearing and control animals. Much less low molecular weight activity was found in the more hydrophilic 25% acetonitrile fraction. Whether this low molecular weight rEGF actually represents a smaller active peptide or results from modification causing reduced mobility on Bio-Gel P-10 is not known. Similarly apparent low molecular weight EGF activity has been recovered from human urine (6).

We conclude that neither immunoreactive TGF-α nor immunoreactive hEGF were excreted into the urine in detectable amounts from any of these human tumor-bearing nude rats. It is possible that other tumors might cause the excretion of these growth factors or elicit their secretion as a host response. In support of this latter possibility, recent evidence by RIA indicates that TGF-α is released into the conditioned medium of human alveolar macrophages and that human alveolar macrophages have increased levels of TGF-α mRNA following antigenic stimulation. However, in our study the major difference between rats bearing tumors NSCLC I, NSCLC II, and CS and non-tumor-bearing rats was in the greater extent of apparent alteration of the rEGFs that were excreted, possibly as an effect of tumor-related proteolysis.

REFERENCES

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