Transforming growth factors in urine from patients with primary brain tumors

HIROSHI KANNO, M.D., TAKEO KUWABARA, M.D., HIDETARO YASUMITSU, PH.D., AND MAKOTO UMEDA, M.D.

Department of Neurosurgery, School of Medicine, and Kihara Institute for Biological Research, Yokohama City University, Yokohama, Japan

Urine specimens obtained from 19 patients with primary brain tumors were examined for the activity of transforming growth factors (TGF's). Urine was assayed for TGF's by soft agar colony formation and iodine-125 (125I)-epidermal growth factor (EGF)-binding competition. Two nontransformed cell lines, clonal NRK49F and BALB/3T3 A31-1-1 cells, were used as indicator cells for the soft agar colony assay, while EGF receptor-rich A431 cells were used for 125I-EGF-binding competition assay. Urine samples were dialyzed against acetic acid, then lyophilized, prepared with gel-permeation chromatography, and assayed. All 19 patients and a control group of healthy individuals showed high levels of α-type TGF's with low molecular weight (4 to 8 kD) in all urine samples. In addition, α-type TGF's of high molecular weight (20 to 50 kD) were detected at high levels in urine from all 10 patients with high-grade astrocytoma; at intermediate levels in urine from one of two patients with low-grade astrocytoma and from two of four patients with meningioma; and at low levels in urine from one of two patients with low-grade astrocytoma, from two of four patients with meningioma, from one patient with oligodendroglioma, from two patients with neurinoma, and from all healthy control individuals. The high level of α-type TGF's with high molecular weight detected in urine from patients with high-grade astrocytoma could be useful as a tumor marker.

KEY WORDS • transforming growth factor • oligodendroglioma • astrocytoma • meningioma • brain neoplasm

Transforming growth factors (TGF's) are a family of polypeptides defined by their ability to reversibly cause anchorage-dependent cells to form colonies in soft agar medium, and are categorized into two functional classes: type α and type β. The former (TGF α) competes with epidermal growth factor (EGF) for binding to its receptor and shows moderate-sequence homology to EGF. This type has the ability to induce colony formation of NRK49F cells in soft agar medium by itself. The latter (TGF β) does not bind to the EGF receptor and induces colony formation of NRK49F cells synergistically with either EGF or TGF α in soft agar medium. This type also induces colony formation of AKR-2B or BALB/3T3 cells in soft agar medium in the absence of EGF or TGF α. Transforming growth factors have been isolated from culture media of cells transformed by viruses or chemicals, solid tumors of various origins, human cancer cell lines, embryo tissues, placenta, and urine. In addition, TGF β can be found in platelets and other adult tissues. The presence of TGF's with a high range of molecular weight (MW 30 to 35 kD) has been detected in the urine obtained from patients with disseminated cancer, and has proved useful as a tumor marker. Comparison of urinary TGF activity of normal and human tumor-bearing athymic mice revealed that urinary TGF's with an MW of 20 kD were increased four- to 10-fold in nude mice bearing human tumors and that the concentration of this factor was reduced to the basal level after removal of the tumor. A high level of β-type TGF activity has also been demonstrated in urine obtained from cancer patients. In this communication, TGF activity in urine from patients bearing primary brain tumors was examined.

Materials and Methods

Preparation of Urine Samples

Morning void urine was collected from 19 patients with primary intracranial tumors who were admitted to
Yokohama City University Hospital between December, 1985, and May, 1986. These included 10 patients with high-grade astrocytoma (grades III and IV), two with low-grade astrocytoma (grade II), one with oligodendroglioma, four with meningioma, and two with neurinoma. The patients ranged in age from 21 to 69 years. These patients had normal renal function and were receiving neither surgical treatment for brain tumors nor anticancer therapy when the urine was collected. Urine was obtained from five healthy persons to establish normal control levels. Urine samples were extracted by a method basically described by Sherwin, et al. Glacial acetic acid was added to the samples to a final concentration at 0.1 M, and the samples were stored at 4°C for more than 24 hours. After centrifugation at 2000 rpm for 15 minutes at 4°C, 100 ml of the supernatant was dialyzed against 0.2 M acetic acid, using 3000-cutoff dialysis tubing, and then lyophilized. Macroscopically, the samples were neither turbid nor hemorrhagic. The lyophilized materials were dissolved in 5 ml of 1 M acetic acid and chromatographed on a 2.6 × 60-cm Bio-Gel P-60 column at a flow rate of 7 ml/hr using 1 M acetic acid as the eluent. Portions of each fraction were lyophilized and tested in the soft agar colony assay and iodine-125-(125I)-EGF binding competition assay. The column was calibrated with ferritin (Vo, MW 450 kD), egg albumin (MW 45 kD), chymotrypsinogen A (MW 25 kD), ribonuclease (MW 14 kD), and insulin (MW 6 kD) as molecular markers.

**Soft Agar Colony Assay**

Transforming growth factor activity was examined by the soft agar colony assay using nontransformed cloned NRK49F cells and BALB/3T3 A31-1-1 cells as indicator assay cells according to the method described by Morita, et al. Briefly, a single-cell suspension of indicator cells in 0.5 ml of 0.3% agar in medium consisting of 10% serum in Dulbecco's modified Eagle's medium was overlaid onto 0.5 ml of 0.6% agar medium prepared in wells of 24 well plates. Then, 0.1 ml vol-
Transforming growth factors associated with brain tumors

umes of fractionated test samples in NaHCO₃-free minimum essential medium (MEM) were added on top of the agar medium. Each aliquot was prepared by lyophilization of 0.5 ml of each fraction from the Bio-Gel column. When NRK49F cells were used as indicator cells with or without the addition of 2 ng/ml EGF, the number of cells in each well was adjusted to 1000 in a medium of calf serum. When BALB/3T3 cells were used, 5000 cells were placed in a medium of fetal calf serum. After 2 weeks of cultivation in a 5% CO₂ incubator at 37°C, each well was examined under a stereoscopic microscope for colonies consisting of over 40 cells. The potency of TGF activity was expressed as follows (based on the number of colonies found in each well): more than 120 colonies, 61 to 120 colonies, 31 to 60 colonies, 16 to 30 colonies, six to 15 colonies, and five colonies or less.

125I-EGF-Binding Competition Assay

125I-EGF-binding competition was examined for α-type TGF activity, since TGF α competes with EGF for binding to their receptors. A431 cells, derived from human epidermal carcinoma, were subconfluently cultured on coverslips in each well of 24-well plates. A 0.5-ml volume of each Bio-Gel fraction was lyophilized, dissolved in 0.1 ml of the binding buffer solution (consisting of 0.1% bovine serum albumin and 1% 4-(2-hydroxyethyl)-1-piperazinetanesulfonic acid in MEM adjusted to pH 6.8) which contained 1 ng/ml of 125I-EGF; this mixture was added to each well. After incubation at room temperature for 1 hour, the coverslips were washed three times with the binding buffer solution, and the radioactivity of each coverslip was counted with a gamma counter. The potency of EGF-binding competition of samples was expressed as follows (based on 125I-EGF-binding inhibition rate): more than 60%, 31% to 60%, 16% to 30%, 6% to 15%, and below 6%.

Results

Urine samples were chromatographed on a Bio-Gel P-60 column equilibrated in 1 M acetic acid. Each column fraction was examined for TGF activity by soft agar colony formation and 125I-EGF-binding competition. Figure 1 left shows a representative pattern of the TGF activities of a urine sample obtained from a patient with high-grade astrocytoma (Case P1). Colony formation of NRK49F cells in soft agar medium without EGF was observed at high-MW (20 to 50 kD) and at low-MW (4 to 8 kD) ranges. Strong EGF-binding competition was also shown at both high- and low-MW ranges. The samples, however, had little potentiation for soft agar colony formation of NRK49F cells with the addition of EGF and a very weak ability to form soft agar colonies of BALB/3T3 cells, indicating that the activities observed here were mainly due to α-type TGF and not β-type TGF.

The chromatographic pattern of a urine sample from a healthy individual (Case C1) is depicted in Fig. 1 right. Colony formation of NRK49F cells in soft agar medium and EGF-binding competition were observed at the low-MW range at high potency, but at the high-MW range at a very weak level. Again, little potentiation for colony formation of NRK49F cells was observed with the addition of EGF. Soft agar colony formation was scarcely visible when BALB/3T3 cells were used.

Table 1 summarizes the findings in urine samples obtained from patients bearing various types of brain tumor. Urine samples from all 10 patients with high-grade astrocytoma and from one of the two patients with low-grade astrocytoma had a potent ability to cause formation of high- and low-MW ranges which was independent of the presence of EGF. Urine samples from two of four meningioma patients exhibited an intermediate level of activity at the high-MW range as well as a high level at low-MW range. Samples from other patients, including one with low-grade astrocytoma, one with oligodendroglioma, two with meningioma, and two with acoustic neurinoma, revealed very little TGF activity at the high-MW range when tested with NRK49F cells. However, these samples always showed high activity at the low-MW range. The examination of fractions from healthy individuals revealed that all samples had weak TGF activity at the high-MW range (Table 2). None of the samples, including those from patients with malignant tumors, showed enhancement of colony formation of NRK49F cells by the addition of EGF, and all formed few colonies when BALB/3T3 cells were used.

The results of 125I-EGF-binding competition assays are summarized in Table 3. Strong competition was observed at the high-MW range in all three samples tested from malignant glioma-bearing patients, but competition was very weak in all three samples tested from healthy individuals. A high percentage of inhibition of 125I-EGF-binding was demonstrated in low-MW fractions of all samples from both malignant glioma-bearing patients and healthy individuals.

Discussion

Transforming growth factor activity in urine from patients with primary brain tumors and from healthy individuals was examined by soft agar colony formation and 125I-EGF-binding competition assays. The activity detected in our studies should be ascribed to α-type TGF's because of the ability of samples to cause formation of NRK49F colonies in soft agar medium and to inhibit EGF-binding to A431 cells. Since no potentiation was observed in the colony formation of NRK49F cells with the addition of EGF, and also since little BALB/3T3 colony formation was detected, β-type TGF's appear to have been minimal in the urine samples examined.

Twardzik, et al., have demonstrated that urine from patients with disseminated cancer contains high-MW α-type TGF activity in addition to low-MW TGF activ-
TABLE 1
Transforming growth factor (TGF) activity in urine of patients with primary brain tumors

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Pathology</th>
<th>Assay Method</th>
<th>TGF Activity</th>
<th>(V_o) 20-50 kD</th>
<th>4-8 kD</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>53</td>
<td>F</td>
<td>astrocytoma grade IV</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P2</td>
<td>47</td>
<td>F</td>
<td>astrocytoma grade IV</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P3</td>
<td>56</td>
<td>F</td>
<td>astrocytoma grade IV</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P4</td>
<td>36</td>
<td>M</td>
<td>astrocytoma grade III</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P5</td>
<td>63</td>
<td>M</td>
<td>astrocytoma grade III</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P6</td>
<td>66</td>
<td>M</td>
<td>astrocytoma grade III</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P7</td>
<td>46</td>
<td>M</td>
<td>astrocytoma grade III</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P8</td>
<td>21</td>
<td>M</td>
<td>astrocytoma grade III</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P9</td>
<td>42</td>
<td>M</td>
<td>astrocytoma grade III</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P10</td>
<td>34</td>
<td>F</td>
<td>astrocytoma grade III</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P11</td>
<td>33</td>
<td>F</td>
<td>astrocytoma grade II</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P12</td>
<td>36</td>
<td>M</td>
<td>astrocytoma grade II</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P13</td>
<td>30</td>
<td>M</td>
<td>oligodendro-glioma</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P14</td>
<td>65</td>
<td>F</td>
<td>meningioma</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P15</td>
<td>49</td>
<td>F</td>
<td>meningioma</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P16</td>
<td>51</td>
<td>F</td>
<td>meningioma</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P17</td>
<td>49</td>
<td>F</td>
<td>meningioma</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P18</td>
<td>52</td>
<td>F</td>
<td>acoustic neurinoma</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>P19</td>
<td>40</td>
<td>F</td>
<td>acoustic neurinoma</td>
<td>NRK EGF(+)</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
</tbody>
</table>

* NRK EGF(−) = activity to cause formation of NRK49F colonies in soft agar medium without the addition of epidermal growth factor (EGF); NRK EGF(+) = activity to cause formation of NRK49F colonies in soft agar medium with the addition of EGF; 3T3 = activity to cause formation of BALB/3T3 colonies in soft agar medium; \(V_o\) = MW 450 kD (with ferritin). TGF activity: ++++ = more than 120 colonies; +++ = 61 to 120 colonies; ++ = 31 to 60 colonies; + = 16 to 30 colonies; ± = six to 15 colonies; − = five colonies or less.
Transforming growth factors associated with brain tumors

healthy persons cannot be easily explained. Recently, however, Samsoonar, et al.,21 demonstrated that untransformed bovine anterior pituitary cells produce α-type TGF’s in culture. This suggests that even in the body of healthy individuals some tissues might be producing TGF α, which are then excreted in the urine. A fairly good correlation was observed between the excretion of high-MW α-type TGF’s and malignancy. The production of TGF colonies at high levels was observed in all 10 samples from patients with high-grade astrocytoma. Among specimens from nine patients with more benign tumors, one sample contained TGF’s at a high level, two at an intermediate level, and the other six at very low levels. All samples from five healthy individuals contained weak activity at the high-MW range. The reason why cancer patients excrete high-MW α-type TGF’s is not clear and needs explanation. The MW of TGF α is 5616 D. Therefore, TGF activity eluted at the low-MW range may be due to TGF α. As to activities at the high-MW range, Kimball, et al.,8 reported that high-MW α-type TGF’s associated with tumors can be recovered at the low-MW level after acid treatment. Thus, high-MW TGF may be related to TGF α, either as an aggregate form or a form bound to some protein. Another possibility is that high-MW α-type TGF is a molecule somewhat different from authentic TGF α.

Nishimura, et al.,15 have reported that high or intermediate activity of β-type TGF can be detected in patients with advanced cancer. Although our procedure of detection was the same as theirs, we could not detect β-type TGF in urine samples obtained from malignant glioma patients. However, we demonstrated β-type TGF as well as α-type TGF in the culture media of an established human glioma cell line and rat glioma C6 cell (unpublished data). The MW of TGF β is 25 kD, larger than that of TGF α.4,5,29 Moreover, the β-type TGF which Nishimura, et al., demonstrated was detected mostly at the Vα range by Bio-Gel P-100 fractionation. Therefore, one possibility is that the very large β-type TGF cannot pass through the blood-brain barrier. In order to understand the precise reason, further clarification is necessary.

There is no clinically useful tumor marker of malignant glioma. Before surgical treatment, it is occasionally difficult to diagnose whether a lesion is a glioma or a cerebral infarction, or whether it is a recurrence of malignant glioma or radiation necrosis. Urinary high-MW α-type TGF might be useful as a tumor marker to diagnose malignant glioma and to evaluate the effect of chemotherapy and radiation therapy.

References


Manuscript received July 7, 1987.

This work was supported in part by Grants-in-Aid for Cancer Research (59010068, 60015055) from the Ministry of Education, Science, and Culture of Japan.

Address reprint requests to: Hiroshi Kanno, M.D., Department of Neurosurgery, Odawara City Hospital, 46 Kuno Odawara, Kanagawa 250, Japan.