Heparin Binding Affinity of Rat Prostatic Growth Factor in Normal and Cancerous Prostates: Partial Purification and Characterization of Rat Prostatic Growth Factor in the Dunning Tumor

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ABSTRACT

The rat prostate contains two types of growth factors capable of stimulating DNA synthesis in BALB/3T3 cells. These rat prostatic growth factors (RPGF) were separable by a different affinity for heparin: low affinity type RPGF and high affinity (HIA) type RPGF. About 80% of the RPGF in the cytosol from normal prostates was low affinity type, whereas more than 80% in the cytosol from the Dunning tumors was HIA type. Elution profile of HIA-RPGF showed two peaks of activity eluted from the heparin-Sepharose column, one at 1.3-1.4 M NaCl (HIA1-RPGF) and the other at 1.6-1.7 M NaCl (HIA2-RPGF). HIA2-RPGF could be purified 1100-fold from the Dunning tumor (AT-3 subline) in about 20% recovery by heparin-Sepharose chromatography. The partially purified HIA-RPGF in the Dunning tumor has a molecular weight of about 19,000 and isoelectric point of about 3.8, and stimulated DNA synthesis at a concentration of about 0.25 nM. The activity was lost by heat treatment at 70°C for 5 min and by acid treatment, whereas it was stimulated by incubating with diethiothreitol. The HIA2-RPGF did not have transforming growth factor activity at a concentration of 250 ng/ml or lower in the presence of epidermal growth factor.

INTRODUCTION

There are several lines of evidence demonstrating the close relation between growth factors or their receptors and oncosomes or their products, e.g., human platelet-derived growth factor versus p28 (putative transforming protein of simian sarcoma virus) (1), and human EGF receptor versus v-erb-B oncogene of avian erythroblastosis virus (2). At present, in spite of several findings (3-8), it is difficult to conclude that there is a qualitative difference in growth factors in normal and cancerous prostatic tissues. In order to clarify the role of growth factor in the prostate cancer, it is no doubt necessary to determine whether there is a qualitative difference in growth factors in normal and cancerous prostates. Several growth factors, e.g., a tumor-derived CE cell growth factor (9), fibroblast growth factor (10, 11), and cartilage-derived growth factor (12), were found to have high affinity for heparin-Sepharose. We also found that a growth factor from the cytosol of human hypertrophic prostates capable of stimulating DNA synthesis in BALB/3T3 cells could be purified about 1000-fold by heparin-Sepharose chromatography (13). A rat prostatic adenocarcinoma, the Dunning tumor R 3327, is believed to have originated in the dorsolateral prostate (14). All the sublines of the tumor examined, including the parent tumor, lack biochemical markers of the dorsolateral prostate of the normal mature rat (15, 16). The present paper describes the first demonstration of intrinsic differences and changes of growth factors in normal and cancerous prostates of rats by use of affinity for heparin. We also report the account of partial purification and characterization of growth factor abundant in the AT-3 subline of the Dunning tumor.

MATERIALS AND METHODS

Animals and Tumor Tissues. For normal prostate tissues, male Sprague-Dawley rats (13-16 weeks of age) were purchased from CLEA Japan, Tokyo, Japan. Frozen tissues of HS, AT-2, and AT-3 sublines of the Dunning tumor (R 3327) (17) were used as cancerous prostate.

Preparation of Cytosol. All procedures were carried out at 4°C unless otherwise specified. The tissue was homogenized in 10 volumes (v/w) of buffer A containing 1 mM phenylmethylsulfonyl fluoride; buffer A was composed of 10 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose and 3 mM CaCl2. The homogenate was centrifuged at 1,500 x g for 10 min, and the supernatant was further centrifuged at 100,000 x g for 1 h. The resulting supernatant (cytosol) was used as the starting material.

Assay of Growth Factor Activity. Growth factor activity, DNA synthesis stimulating activity, was assayed on BALB/3T3 cells according to methods described previously (13, 18). All measurements were done in duplicate. Activity was expressed as cpm/well/3 h or unit, the latter being defined as the amount of growth factor equivalent to the activity of calf serum that can induce a half-maximal incorporation of labeled thymidine into 3T3 cell DNA.

Assay of Transforming Growth Factor Activity. The activity of TGF-β was assayed by the methods of Huang et al. (19). In the assays, NRK cells were grown in 35- x 10-mm tissue culture dishes containing two layers of soft agar. The bottom layer contained 0.5% agar and 5% fetal calf serum, and the top layer contained 6.3 x 106 cells, 0.4% agar, 5% fetal calf serum, and 7 ng of EGF. Samples to be assayed were dissolved in 0.1 ml of phosphate-buffered saline and were applied to the top layer. After a 7-day culture, colonies larger than 6 μm diameter formed in the top layer were counted. The TGF-β activity was expressed as colony number/dish.

Stability Experiment. The final preparation of RPGF from Dunning tumor was used. In the heat treatment, RPGF dissolved in 10 mM Tris-HCl (pH 7.5) containing 1 mg/ml bovine serum albumin (2 μg RPGF preparation/ml) was incubated at 70°C for 5 min. In the treatment with disulfide-reducing agent, RPGF dissolved in 0.2 M NH4HCO3 (pH 9.0) was incubated at 25°C for 6 h in the presence of 50 mM dithiothreitol. With the acid treatment, RPGF samples were incubated overnight at 4°C in either 1 M acetic acid or HCl. After incubation the pH was adjusted to 7.5 with NaOH.

Determinations of Physicochemical Properties. The molecular weight of RPGF was determined by molecular sieve chromatography on a Sephadex G-75 column (2.5 x 90 cm) equilibrated and developed with 10 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl. The reason for inclusion of 0.5 M NaCl during this step is detailed in "Results." The molecular weight markers used were bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), α-chymotrypsinogen A (25,000), myoglobin (17,000), and RNase A (13,700). Mouse submaxillary gland EGF was purchased from Toyobo. Fetal calf serum was obtained from GIBCO Laboratories. Isoelectric focusing in the sucrose density gradient column was carried out according to the method of Vesterberg and Svensson (20) with Ampholine carrier ampholytes (pH 3.5-10). Protein was determined by the method of Lowry.
et al. (21) using bovine serum albumin as a standard. For dialysis, Spectra/Por tubing (Spectrum Medical Industries, Inc.) with cutoff molecular weight of 3500 was used. NaCl concentration was determined by specific conductivity measured at 25°C by a conductivity meter, CDM 3 (Radiometer, Copenhagen, Denmark).

RESULTS

Growth Factor Activity in Cytosols of Normal Rat Prostates and the Dunning Tumor. Growth factor activities were examined in cytosols prepared from the prostates of normal rats and the Dunning tumor (Fig. 1). At a dose of about 10 μg/ml, the cytosol from the dorsolateral prostate was as active as the ventral prostate. However, the cytosol from the ventral prostate was not adsorbed on the heparin-Sepharose, whereas about 87-98% of the activity in the cytosol from the Dunning tumor was adsorbed; the former type of growth factor was designated LoA-RPGF and the latter HiA-RPGF. All the sublines of the Dunning tumor showed the high content of HiA-RPGF; the content of HiA-RPGF in the AT-3 subline was the highest (Table 1). To analyze HiA-RPGF in detail, the cytosol from normal rat prostates were subjected to chromatography on a heparin-Sepharose column in the presence of 0.5 M NaCl. More than 95% of the total protein (A280) was eluted in the void volume fractions, where about 80% of the activity (units) in the cytosol from the dorsolateral prostate was recovered as LoA-RPGF (Fig. 2). By contrast, only about 7% of the activity in the cytosol from the ventral prostate was recovered as LoA-RPGF from the void volume fractions where two small peaks of the activity were detected (Fig. 3). Fractions 6-10 showed activity significantly lower than the basal activity because of cytotoxicity. The cytosol from the ventral prostate, when previously dialyzed overnight against buffer A at 4°C, gave a broad single peak containing about 80% of total activity (units) in the void volume fractions on heparin-Sepharose chromatography. These results were in accordance with the data shown in Table 1.

Table 1 Contents of prostatic growth factor in the cytosols of normal prostates, Dunning tumors, and benign hypertrophie prostates

<table>
<thead>
<tr>
<th>Prostate</th>
<th>Growth factor activity (units/mg protein)</th>
<th>Relative content of LoA-RPGF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventral*</td>
<td>74 (141)</td>
<td>75.0</td>
</tr>
<tr>
<td>Dorsolateral</td>
<td>117</td>
<td>77.0</td>
</tr>
<tr>
<td>Dunning tumor subline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS</td>
<td>180</td>
<td>9.3</td>
</tr>
<tr>
<td>AT-2</td>
<td>215</td>
<td>12.8</td>
</tr>
<tr>
<td>AT-3</td>
<td>278</td>
<td>2.3</td>
</tr>
<tr>
<td>Human benign prostatic hypertrophic tissue</td>
<td>175</td>
<td>8.1</td>
</tr>
</tbody>
</table>

* Cytosol from the ventral prostate was previously dialyzed overnight against 10 mM Tris-HCl (pH 7.5) containing 0.25 mM sucrose and 3 mM CaCl2 at 4°C.

The activity of the cytosol from the dorsolateral prostate and the Dunning tumor was neither enhanced nor reduced by dialysis. As compared to the cytosol from the dorsolateral prostate of normal rats, dose-response curves for the activity of the cytosol from the Dunning tumor showed a slightly higher level of growth factor; the Dunning tumor contained about two times as much growth factor activity as did normal prostate (Fig. 1; Table 1).

Three Types of RPGF with Different Affinity for Heparin-Sepharose. As reported previously (13), 90-95% of the growth factor activity in the cytosol of human hypertrophie prostates was bound to heparin-Sepharose in the presence of 0.5 M NaCl. The affinities of RPGF for heparin were analyzed with cytosols from normal rat prostates and the Dunning tumor. The cytosol was mixed with heparin-Sepharose suspended in 10 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl, and then the mixture was centrifuged to obtain the supernatant and the heparin-Sepharose (Table 1). It was found that about 80% of the activity in the cytosol from the ventral and dorsolateral prostates of normal rats was not adsorbed on the heparin-Sepharose, whereas about 87-98% of the activity in the cytosol from the Dunning tumor was adsorbed; the former type of growth factor was designated LoA-RPGF and the latter HiA-RPGF. All the subline of the Dunning tumor showed the high content of HiA-RPGF; the content of HiA-RPGF in the AT-3 subline was the highest (Table 1). To analyze HiA-RPGF in detail, the cytosol from normal rat prostates were subjected to chromatography on a heparin-Sepharose column in the presence of 0.5 M NaCl. More than 95% of the total protein (A280) was eluted in the void volume fractions, where about 80% of the activity (units) in the cytosol from the dorsolateral prostate was recovered as LoA-RPGF (Fig. 2). By contrast, only about 7% of the activity in the cytosol from the ventral prostate was recovered as LoA-RPGF from the void volume fractions where two small peaks of the activity were detected (Fig. 3). Fractions 6-10 showed activity significantly lower than the basal activity because of cytotoxicity. The cytosol from the ventral prostate, when previously dialyzed overnight against buffer A at 4°C, gave a broad single peak containing about 80% of total activity (units) in the void volume fractions on heparin-Sepharose chromatography. These results were in accordance with the data shown in Table 1.
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Fig. 3. Affinity chromatography of ventral prostate RPGF on a heparin-Sepharose column. Cytosol of the ventral prostate (45 ml; 5.9 mg protein/ml) was supplemented with 6.4 ml of 4 M NaCl and applied to a heparin-Sepharose column. Other experimental conditions are the same as those described in the legend to Fig. 2.

Fig. 4. Affinity chromatography of Dunning tumor RPGF on a heparin-Sepharose column. Cytosol of AT-3 subline of the Dunning tumor (29.5 ml; 4.1 mg protein/ml) was supplemented with 4.2 ml of 4 M NaCl and applied to a heparin-Sepharose column. Other experimental conditions are the same as those described in the legend to Fig. 2.

1. When a gradient elution with NaCl was applied to the column, two peaks of growth factor activity were recovered: one was eluted at 1.3-1.5 M NaCl (designated HiA₁-RPGF) and the other at about 1.6-1.7 M NaCl (designated HiA₂-RPGF). The ratios of HiA₁-RPGF/HiA₂-RPGF calculated from activity units were about 1 and ½ for the ventral prostate and the dorsolateral prostate, respectively. On the other hand, about 85% of the activity (units) in the AT-3 subline was adsorbed by the column (Fig. 4). HiA₁-RPGF in the AT-3 subline appeared to be separated into HiA₁-RPGF and HiA₂-RPGF. The higher ratio of HiA₂-RPGF/HiA₁-RPGF in the Dunning tumor did not vary among the sublines of the tumor; HiA₁-RPGF was found to be minor in the tumors tested (less than 5% of total HiA-RPGF).

These results indicate that there are three types of growth factor (LoA-RPGF, HiA₁-RPGF, and HiA₂-RPGF) with different affinities for heparin-Sepharose and that the content ratio of HiA/LoA was remarkably higher in the Dunning tumor.

Partial Purification of RPGF in the Dunning Tumor. To clarify the characteristics of RPGF in the Dunning tumor, the cytosol (75 ml) prepared from the AT-3 subline (7.5 g) was subjected to affinity chromatography on a heparin-Sepharose column. Chromatographic patterns were essentially the same as those shown in Fig. 4. More than 98% of the total protein (A₂₈₀) was eluted in the void volume fractions, whereas a negligible amount of protein was eluted with NaCl gradient. The active fractions (42-48 in Fig. 4) were diluted to decrease NaCl concentration to about 0.8 M with 10 mM Tris-HCl (pH 7.5) and subjected to the second affinity chromatography on a newly packed column of heparin-Sepharose. Thus, the two-step procedure resulted in about 1100-fold purification, and the recovery was about 20%. The dose-response curve of the final preparation of HiA₂-RPGF in the Dunning tumor shows that it distinctly stimulated DNA synthesis at a concentration as low as 5 ng/ml (about 0.25 nM) (Fig. 5).

Properties of Partially Purified HiA₂-RPGF in the Dunning Tumor. The partially purified HiA₂-RPGF in the Dunning tumor was subjected to isoelectric focusing with Ampholine carrier ampholytes (Fig. 6). One major peak with a pl of about 3.8 in addition to two minor peaks with pl's of 8.6 and 10.5 was recovered in a yield of 30%. About 89% of the total activity was focused as a major peak at pH 3.8. The activity was more unstable at acidic pH than at neutral and alkaline pH (data not shown).
shown). Accordingly, the major growth factor in the Dunning tumor seemed to be represented by a component with pl of about 3.8. The molecular weight of RPGF in the Dunning tumor was estimated to be about 19,000 by molecular-sieve chromatography on a Sephadex G-75 column in the presence of 0.5 M NaCl (data not shown). When HiA<sub>2</sub>-RPGF was subjected to molecular-sieve chromatography in the presence of NaCl lower than 0.1 M, the active fractions were not eluted within two bed volumes from the column of either Sephadex G-75 or TSK 2000SW. The activity of the growth factor was almost completely destroyed by heating at 70°C for 5 min (Table 2; Fig. 5), whereas the activity was stimulated in the presence of dithiothreitol at pH 9.0 (Table 2). Treatment with either 1 M acetic acid or 1 M HCl destroyed most of the activity. As shown in Table 3, the partially purified HiA<sub>2</sub>-RPGF did not stimulate the colony formation of NRK cells in soft agar at a concentration of 250 ng/ml or lower in the presence of EGF (5 ng/ml), indicating the HiA<sub>2</sub>-RPGF did not have TGF-β activity.

DISCUSSION

It is generally difficult to measure growth factor content with high accuracy without immunological techniques because biological samples contain unknown inhibitors including cytotoxic substance. As a matter of fact, the present study showed that the ventral prostate of normal rat contains a dialyzable cytotoxic substance (Fig. 3), whereas the dorsolateral prostate and the Dunning tumor did not. Dose-response curves for growth factor activity indicate that the growth factor content in the Dunning tumor is not remarkably higher than that of the normal prostate, as already demonstrated in normal and diseased prostates in humans (3, 4, 7, 8). These results led us to examine a qualitative difference in growth factor between normal and cancerous prostatic tissues. Klagsbrun and Shing (22) reported that tumor-derived CE cell growth factor had a special affinity for heparin without correlation between heparin binding affinity and isoelectric point of growth factor. They also suggested that the various CE cell growth factors have in common a functional domain that is involved in binding to heparin. Nishi et al. (13) demonstrated that human prostatic growth factor in benign hypertrophie prostates with high affinity for heparin (Table 1), indicate the result that about 90% of the activity in human hypertrophie tumor than in normal prostates. These findings, together with the result that about 90% of the activity in human hypertrophie prostate shows high affinity for heparin (Table 1), indicate that the increase of HiA type growth factor is closely related to neoplastic or hypertrophie growth of the prostate, although it may not be necessary for malignant growth of the prostate. Dose-response curves of RPGF partially purified from the Dunning tumor (HiA<sub>2</sub>-RPGF) demonstrated that it could stimulate DNA synthesis in BALB/3T3 cells at a concentration as low as 5 ng/ml. TGF-β is known as a mitogenic factor for a variety of fibroblastic monolayer cultures. Since RPGF was isolated from tumor tissue, it inevitably is interesting to ask whether RPGF in the Dunning tumor does or does not have TGF-β activity. HiA<sub>2</sub>-RPGF did not stimulate the colony formation of NRK cells in the presence of EGF at a concentration as high as 250 ng/ml (Table 3), indicating that the RPGF did not have TGF-β activity. Heparin-binding activity and stability properties indicate that HiA<sub>2</sub>-RPGF in the Dunning tumor could be grouped into an acidic heparin-binding growth factor in the light of a new classification proposed by Lobb et al. (23). Presently, we do not know whether RPGFs are specific for the prostate or are also present in normal and cancerous tissues other than those of the prostate. Although there remains uncertainty regarding the nature of determinants that affect the heparin affinity of RPGF subclasses, further characterization of these components using other refined approaches such as immunological methods is necessary not only for understanding the significance of RPGF in prostatic tissue but also for establishing the role of HiA-RPGF in diseased prostates.

Table 2 Stability of partially purified Dunning tumor RPGF

<table>
<thead>
<tr>
<th>Condition</th>
<th>Remaining activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Heat, 70°C for 5 min</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Acid</td>
<td>7</td>
</tr>
<tr>
<td>1 M HCl</td>
<td>20</td>
</tr>
<tr>
<td>1 M acetic acid</td>
<td>20</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>20</td>
</tr>
<tr>
<td>RPGF</td>
<td>260</td>
</tr>
<tr>
<td>EGF</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 3 TGF-β activity of partially purified HiA<sub>2</sub>-RPGF from the Dunning tumor

<table>
<thead>
<tr>
<th>Sample</th>
<th>Colony no./dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-buffered saline</td>
<td>29 (17)</td>
</tr>
<tr>
<td>Fetal calf serum</td>
<td>224 (43)</td>
</tr>
<tr>
<td>HiA&lt;sub&gt;2&lt;/sub&gt;-RPGF</td>
<td>50 (3.1)</td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>100 (3.4)</td>
</tr>
<tr>
<td>250 ng/ml</td>
<td>86 (3.0)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, relative colony no./dish.

REFERENCES

13. Nishi, N., Matuo, Y., Muruguma, Y., Yoshitake, Y., Nishikawa, K., and
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