Abundant Expression of Immunoreactive Endothelin 1 in Mammary Phyllodes Tumor: Possible Paracrine Role of Endothelin 1 in the Growth of Stromal Cells in Phyllodes Tumor

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Abstract

Immunoreactive endothelin 1 (irET-1) concentrations were measured in extracts prepared from 4 phyllodes tumors and 14 fibroadenomas. irET-1 was detectable in all tissue extracts by specific radioimmunoassay, and the mean concentration of irET-1 was 18-fold and 27-fold higher in tissue extracts from phyllodes tumors than in those from intracanalicular fibroadenomas and pericanalicular fibroadenomas, respectively. Reverse-phase high-performance liquid chromatography coupled with radioimmunoassay in extracts from phyllodes tumors revealed one major irET-1 component corresponding to human standard ET-1. Furthermore, immunocytochemical staining for ET-1 revealed that numerous ET-1-immunoreactive cells were seen in the epithelial but not in the stromal cells, suggesting that ET-1 is synthesized by the epithelial component of phyllodes tumors. A possible paracrine role of ET-1 in the growth of this rare tumor which is characterized by its prominent stromal cellularity is discussed.

Introduction

ET-1 is a novel vasoconstrictive peptide originally isolated from the spent medium of cultured endothelial cells of porcine vessels (1). Recently, several investigators have demonstrated that ET-1 possessed a novel biologic activity which stimulates DNA synthesis in vascular smooth-muscle cells (2, 3), in rat mesangial cells (4), and in Swiss 3T3 fibroblasts (5, 6). Furthermore, ET-1 is produced by a wide variety of nonendothelial cultured cell types, including vascular smooth muscle cells (7), renal epithelial cells (8), endometrial glandular epithelial cells (9), and normal human breast epithelial cells (10) as well as several human tumor cell lines (11–13).

Mammary phyllodes tumor is a rare tumor representing only 2.5% of all fibroepithelial lesions of the breast (14). This tumor is characterized as a combined fibroepithelial tumor in which the mesenchymal or stromal component is much more hypercellular than fibroadenoma (15). Mammary phyllodes tumor tends to be large compared with any other type of breast tumor when patients come for treatment, because this tumor frequently grows rapidly. However, nothing is known about the mechanism for the rapid growth of this tumor.

Recently, Baley et al. (10) reported that specific cell-surface receptors for ET-1 were observed only in the stromal cells, but not in the epithelial cells of the breast. These observations prompted us to investigate the concentration of ET-1 in phyllodes tumor of the breast. We report here that tissue extracts from phyllodes tumors contained a considerably large amount of irET-1 compared to those from fibroadenomas and that this concentration is much higher than that from breast cancer tissues we previously reported (16).

Materials and Methods

Human Tumors. Eighteen fibroepithelial neoplasms (4 phyllodes tumors and 14 fibroadenomas) are included in this study. The diagnosis of phyllodes tumors was made if they had a characteristic leaflike and cystic gross appearance or if they contained both epithelium and fibrous stromal components as a part of the neoplasm together with a greater degree of stromal cellularity than fibroadenomas (17). These tumor tissues were stored at -80°C immediately after surgical removal until extraction. Each specimen was homogenized and extracted with 50 mM TE-HCI buffer (pH 7.4) containing 0.25% Triton X-100 as described previously (18).

Human ET-1 RIA. irET-1 concentration was determined by specific RIA for ET-1 as described previously (16, 19). irET-1 concentration in each extract was expressed as pg/mg protein. Protein concentration was determined by the method of Lowry et al. (20) with bovine serum albumin as a standard.

Reverse-Phase HPLC. Reverse-phase HPLC (high-performance liquid chromatography) was performed in tissue extracts from four phyllodes tumors using a column (0.46 x 25 cm; Capcell Pak C18 SG 120) eluted with a linear gradient of acetonitrile from 10 to 60% in 0.09% trifluoroacetic acid for 60 min at a flow rate of 1.0 ml/min. After evaporation, each elute was subjected to ET-1 RIA.

Immunocytochemistry for ET-1. Immunocytochemical staining for ET-1 was carried out in four phyllodes tumor tissues using the avidin-biotin complex method (21). ABC kits (Vector Laboratories, Inc.) were used. Briefly, after dewaxing, tissue sections were washed with 1% H2O2 in methanol for 20 min at room temperature in order to block the endogenous peroxidase activity. They were subsequently incubated with normal serum for 30 min to reduce nonspecific background staining and then incubated with rabbit polyclonal ET-1 antiserum (Peptide Institute, Inc., Osaka) at a dilution of 1:500 in humidified chambers overnight at 4°C. The control slides were treated with dilute rabbit serum. All the treated slides were exposed for 30 min to biotinylated goat anti-rabbit IgG (Vectorstain Elite Kit, Vector Laboratories, Bretton, England) and avidin-biotin peroxidase complex for 30 min at room temperature. They were washed in 0.01 M phosphate-buffered saline (pH 7.2) between incubation steps. Peroxidase activity was visualized with 0.01% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) in 0.05 M Tris-HCl buffer (pH 7.6). The sections were counterstained with Mayer’s hematoxylin.

Statistical Analysis. The statistical significance of differences in irET-1 concentration was tested by means of the t test.

Results

Clinical Features. Table 1 shows the clinical and pathological findings of 18 female patients with fibroepithelial tumors of

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1 To whom requests for reprints should be addressed.
2 The abbreviations used are: ET-1, endothelin 1; irET-1, immunoreactive endothelin 1; RIA, radioimmunoassay; HPLC, high-performance liquid chromatography; IGF-1, insulin-like growth factor 1.
Table 1  Clinical and pathological findings in 18 patients studied

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Age of patient (yr)</th>
<th>Size of tumor (cm)</th>
<th>Duration of symptoms</th>
<th>Recurrence</th>
<th>irET-1 (pg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phyllodes (benign)</td>
<td>42</td>
<td>13.0</td>
<td>2 yr 9 mo</td>
<td>No</td>
<td>10.85</td>
</tr>
<tr>
<td>2</td>
<td>Phyllodes (benign)</td>
<td>24</td>
<td>14.5</td>
<td>6 mo</td>
<td>Yes</td>
<td>24.46</td>
</tr>
<tr>
<td>3</td>
<td>Phyllodes (benign)</td>
<td>21</td>
<td>4.5</td>
<td>15 day</td>
<td>No</td>
<td>19.12</td>
</tr>
<tr>
<td>4</td>
<td>Phyllodes (benign)</td>
<td>36</td>
<td>9.0</td>
<td>3 mo</td>
<td>No</td>
<td>31.29</td>
</tr>
<tr>
<td>5</td>
<td>Fibroadenoma (intracanal)</td>
<td>17</td>
<td>3.0</td>
<td>3 yr</td>
<td>No</td>
<td>1.02</td>
</tr>
<tr>
<td>6</td>
<td>Fibroadenoma (intracanal)</td>
<td>26</td>
<td>2.5</td>
<td>20 days</td>
<td>No</td>
<td>0.31</td>
</tr>
<tr>
<td>7</td>
<td>Fibroadenoma (intracanal)</td>
<td>33</td>
<td>3.8</td>
<td>5 yr</td>
<td>No</td>
<td>0.81</td>
</tr>
<tr>
<td>8</td>
<td>Fibroadenoma (intracanal)</td>
<td>13</td>
<td>8.5</td>
<td>1 yr 2 mo</td>
<td>No</td>
<td>2.36</td>
</tr>
<tr>
<td>9</td>
<td>Fibroadenoma (intracanal)</td>
<td>19</td>
<td>4.3</td>
<td>5 mo</td>
<td>No</td>
<td>1.75</td>
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<tr>
<td>10</td>
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<td>22</td>
<td>2.2</td>
<td>21 days</td>
<td>No</td>
<td>0.77</td>
</tr>
<tr>
<td>11</td>
<td>Fibroadenoma (intracanal)</td>
<td>37</td>
<td>5.8</td>
<td>18 yr</td>
<td>No</td>
<td>1.04</td>
</tr>
<tr>
<td>12</td>
<td>Fibroadenoma (pericanal)</td>
<td>25</td>
<td>3.5</td>
<td>8 mo</td>
<td>No</td>
<td>0.26</td>
</tr>
<tr>
<td>13</td>
<td>Fibroadenoma (pericanal)</td>
<td>21</td>
<td>4.0</td>
<td>2 mo</td>
<td>No</td>
<td>1.61</td>
</tr>
<tr>
<td>14</td>
<td>Fibroadenoma (pericanal)</td>
<td>30</td>
<td>3.5</td>
<td>1 yr</td>
<td>No</td>
<td>0.84</td>
</tr>
<tr>
<td>15</td>
<td>Fibroadenoma (pericanal)</td>
<td>24</td>
<td>2.0</td>
<td>7 mo</td>
<td>No</td>
<td>0.62</td>
</tr>
<tr>
<td>16</td>
<td>Fibroadenoma (pericanal)</td>
<td>33</td>
<td>3.2</td>
<td>11 mo</td>
<td>No</td>
<td>1.06</td>
</tr>
<tr>
<td>17</td>
<td>Fibroadenoma (pericanal)</td>
<td>40</td>
<td>1.5</td>
<td>9 yr</td>
<td>No</td>
<td>0.33</td>
</tr>
<tr>
<td>18</td>
<td>Fibroadenoma (pericanal)</td>
<td>27</td>
<td>2.8</td>
<td>3 mo</td>
<td>No</td>
<td>0.68</td>
</tr>
</tbody>
</table>

* Intracanal, intracanalicular type; pericanal, pericanalicular type.

The breast. All phyllodes tumors were regarded as histologically benign according to the criteria proposed by Norris and Taylor (17). The 14 fibroadenomas comprised 7 intracanalicular types and 7 pericanalicular types.

Four phyllodes tumors occurred between 21 and 42 years of age at the time of diagnosis with an average age of 31 years, which was slightly older than the average age of 26 years for the 14 fibroadenomas. The median size of phyllodes tumors was 10.3 cm with a range of 4.5-14.5 cm in greatest diameter, much larger than that of fibroadenomas (3.6 cm). The clinical duration of symptoms, based on the histories obtained from the patients, varied from less than 1 month to 18 years. With respect to phyllodes tumor, tumors of Case 1 and Case 2 had enlarged rapidly after an initial period (2 years 5 months, 5 months, respectively) of slow growth, and tumors of Case 3 and Case 4 grew rapidly from first appearance until removal. In contrast, the growth of all fibroadenomas was uniformly slow. All tumors were excised. One (Case 2) of the four phyllodes tumors recurred locally. In this case, the recurrence presented 5 months after the initial operation and was successfully treated by reexcision.

irET-1 in Tumor Extracts. The reverse-phase HPLC profile of ET-1 in the tissue extracts from Case 1 phyllodes tumor is depicted in Fig. 1 and shows a major peak in the position of human standard ET-1. Similar results were obtained in the tissue extracts from three other cases of phyllodes tumor. A serial dilution curve of tissue extracts from phyllodes tumors exhibited parallelism with that of standard ET-1 in the RIA (data not shown), indicating that these extracts contain irET-1.

As shown in Table 1 and Fig. 2, a more than 18-fold higher mean concentration of irET-1 was found in tissue extracts from phyllodes tumors (21.43 pg/mg protein, ranging from 10.85 to 31.29 pg/mg protein) than in those from intracanalicular fibroadenomas (1.15 pg/mg protein, ranging from 0.31 to 2.36 pg/mg protein; P = 0.0001). Similarly, a more than 27-fold higher irET-1 concentration was found in tissue extracts from phyllodes tumors than in those from pericanalicular fibroadenomas (0.77 pg/mg protein, ranging from 0.26 to 1.61 pg/mg protein; P < 0.0001). No significant difference was found in irET-1 concentration between two types of fibroadenomas.

Immunocytochemical Staining. As shown in Fig. 3, irET-1 was localized to epithelial cells in phyllodes tumor tissues, while...
components but not in the stromalal components. The immunoreactivity, local

gave negative results. The results were similar in the tissue from all four

no stromal cells showed positive immunostaining. The immu

appearance. The results were similar in the tissue from all four patients studied. Control studies with nonimmune rabbit serum gave negative results.

Discussion

In the present study, we demonstrated that irET-1 concentra
tion in the extracts from phyllodes tumors is significantly higher than that from two types of fibroadenomas. This irET-1 concentration of phyllodes tumor tissues is significantly higher ($P = 0.001$) than that of breast cancer tissues (3.634 pg/mg protein) previously reported (16). Furthermore, immunocy

tochemical study revealed the presence and localization of irET-1 in epithelial cells of phyllodes tumors.

ET-1 is a potent vasoconstrictor through its activity on vascular smooth muscle cells. Like other vasoconstrictor, ET-1 has been reported to stimulate the formation of inositol phosphates and $\mathrm{Ca}^{2+}$ mobilization in these cells (22, 23). Recently, several investigators have reported that ET-1 shows other biological effects in nonvascular systems (24–27) than vasoconstriction. Specific receptors for ET-1 are distributed not only in the cardiovascular system, but also in a wide variety of tissues (28), suggesting its diverse physiological functions. However, Baley et al. (10) reported that ET-1 receptors were not detectable in breast epithelial cells and that breast stromal cells possessed ET-1 receptors which are coupled to an inositol lipid-specific phospholipase C, suggesting a possible paracrine role for this peptide in the breast. Very recently, Schrey et al. (29) showed that ET-1 alone caused a modest stimulation of DNA synthesis in human breast fibroblasts and that ET-1 synergizes very strongly with IGF-1 to stimulate DNA synthesis (29). Human fibroblasts produce IGF-1 (30), and indeed, human breast stromal cells express IGF-1 mRNA (31). ET-1 and IGF-1 may thus function as a growth factor on breast stromal cells. Our present results suggest that ET-1 may play an important role in stimulating the growth of stromal cells of phyllodes tumor in a paracrine fashion, causing the rapid growth of this tumor.

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