Hormone Dependency of a Serially Transplantable Human Breast Cancer (Br-10) in Nude Mice

Setsuo Hirohashi, Yukio Shimosato, Toru Kameya, Kanji Nagai, and Ryuichiro Tsunematsu

SUMMARY

The human breast cancer (Br-10) serially transplanted to nude mice (BALB/c-nu/nu) grew well in female mice but very slowly or not at all in untreated male mice and female mice treated with 1 mg of testosterone i.m. twice a week. The growth in female mice was arrested by ovariectomy, and that in male mice was accelerated by 0.1 mg of estradiol i.m. once a week.

Tumors in female and estrogenized male mice retained the original histology of duct carcinoma. Tumors in ovariectomized female, androgenized female, and male mice consisted of cells with smaller and more uniform nuclei, forming markedly dilated lumina in the first group and arranged in lobular patterns in the latter two groups.

High-affinity 8 S and 4 S estrogen receptors were present in tumors transplanted to female nude mice, but no progesterone receptors were detected.

These results provide experimental evidence for the hormone dependency of a human breast cancer in vivo and strongly suggest the important role of estrogen and androgen in the growth regulation of some estrogen receptor-positive human breast cancers.

INTRODUCTION

It is clinically well known that about one-third of human breast cancers respond to endocrine therapy, i.e., administration of hormones or ablation of the ovaries, adrenals, or hypophysis. However, it is difficult to predict the responsiveness of individual patients to such therapy. In vitro assay of estrogen receptors has allowed some progress in this field (9), and it is probable that estrogen is directly involved in the growth regulation of some human breast cancers through the mediation of its specific receptors. In addition there are suggestions that prolactin may play an important role in the growth of some human breast cancers, as in experimental mammary tumors (16). In this regard, there is considerable disagreement as to whether either estrogen or prolactin alone or both in concert are responsible for the growth of human breast cancers.

Research on the pathophysiology of human tumors has been hampered by the absence of adequate experimental models, but this problem has been partly overcome by successful heterotransplantations and serial passages of some human tumors in athymic nude mice (15, 19).

We previously reported briefly the establishment and properties of serially transplantable human breast cancers (Br-10 and -13) in nude mice (4, 19). The present report deals with details of the influence of the host's sex, ovariectomy, and administration of estrogen, androgen, and bovine prolactin on the growth and histology of Br-10, as well as assays of the estrogen and progesterone receptors, in order to confirm the hormone dependency of Br-10 and to determine the hormones involved in its growth regulation.

MATERIALS AND METHODS

Tumor. The tumor cells in the pleural effusion of a 43-year-old Japanese female with common ductal carcinoma of the breast were successfully heterotransplanted to female nude mice in July 1974, and the transplanted tumor was designated as Br-10 (19). Since then, it has been serially transplanted in our laboratory and is now in the 11th passage.

Animals. Male and female nude mice, 4 to 6 weeks old, with the genetic background of BALB/c were supplied from the Central Institute for Experimental Animals, Kawasaki, Japan, where mice were bred and maintained in vinyl isolators under specific-pathogen-free conditions (12). Mice were kept in the same way in our laboratory and were used for the experiments when they weighed 20 g or more. All the experiments were performed in vinyl isolators. Pellets (CLEA Japan, Tokyo, Japan) and tap water were given ad libitum. The bedding, pellets, and water were autoclaved. The number of animals used for the experiments is indicated in Tables 1 and 2.

Transplantation. When the transplanted tumor reached 1 cm or more at its greatest width, serial transfer was performed by transplanting several minced tumor tissue fragments (about 2 mm in diameter) with a trocar into the s.c. tissue of the backs of female mice. Transplantation for the experiments was done with the same method in male and female mice.

Operation. Bilateral ovariectomy was performed through a single midback incision.
Hormone Treatment. For estrogen, estradiol dipropionate (0.1 mg in 0.2 ml of sesame oil) was injected i.m. once a week for several weeks. For androgen, testosterone propionate (1 mg in 0.1 ml of sesame oil) was injected i.m. twice a week for 10 weeks. For prolactin, bovine prolactin [0.2 mg (35 to 40 IU/mg) in 0.1 ml of 0.6% NaCl solution] was injected s.c. twice a day for 5 weeks. These hormones were supplied from Teikoku Hormone Manufacturing Co., Ltd., Tokyo, Japan.

Growth and Histology. The tumors were measured by calipers for 3 dimensions once a week, and their size was expressed by the product. They were weighed when experiments were completed. Excised tumors were fixed in 10% formalin solution and prepared for histological examination. Sections were stained with hematoxylin and eosin and Alcian blue-periodic acid-Schiff.

Estrogen and Progesterone Receptors. Receptor assays were performed with the use of sucrose density gradients (7) and DCC6 (10). The tumor was frozen in liquid nitrogen and shattered in a Thermovac Autopulverizer (Thermovac Industries Corp., Copiague, N. Y.). The tumor powder was homogenized in 4 volumes of cold 0.01 M Tris buffer, pH 7.4, containing 1.5 mM EDTA and 0.5 mM dithiothreitol, with the use of a glass-Teflon homogenizer. The homogenate was centrifuged at 2°C for 30 min at 210,000 x g to obtain the supernatant cytosol fraction. Protein was quantitated by the method of Lowry et al. (8).

For the sucrose gradient analysis of estrogen receptors, 3 150-μl portions of the cytosol were preincubated for 15 min with 50 μl of the homogenization buffer alone, with the buffer containing 250 nM estradiol, or with the buffer containing 250 nM testosterone, followed by the addition of 50 μl of 5.0 nM [2,4,6,7-3H]estradiol (specific activity, 98.5 Ci/m mole; New England Nuclear, Boston, Mass.) in the same buffer. For the sucrose gradient analysis of progesterone receptors, 2 150-μl portions of the cytosol were preincubated for 15 min with 50 μl of the buffer alone or with the buffer containing 15 μM cortisol, followed by the addition of 50 μl of 15 nM [1,2,6,7-3H]progesterone (specific activity, 103.7 Ci/m mole; New England Nuclear) in the same buffer. After incubation for an additional 60 min ice, each mixture was layered on 4.6 ml of a 5 to 20% sucrose gradient, and centrifuged at 2°C for 16 hr at 155,000 x g. The bottoms of the tubes were punctured, and each gradient was collected in 6-drop fractions into 34 scintillation vials. Radioactivity was determined in a toluene-Triton X-100 scintillation fluid at 35% efficiency. Bovine serum albumin (4.6 S) was run on a separate gradient to determine the approximate sedimentation coefficient.

For the saturation analysis of estrogen receptors with DCC, 100 μl of the cytosol were incubated with 100 μl of [2,4,6,7-3H]estradiol in increasing concentrations (0.15 to 9.6 nM) in duplicate for 18 hr in ice. Then, 50 μl of DCC suspension (2.5% Norit A and 0.025% dextran in Tris buffer, pH 7.4) were added, and after 30 min at 4°C the mixture was centrifuged for 10 min at 3000 x g. The radioactivity of 150 μl of the supernatant was assayed as above, and the data were analyzed according to the method of Scatchard (17).

RESULTS

Serial Transplantation. The growth of Br-10 serially transplanted in female mice varied slightly. Most tumors grew constantly and reached 1 cm in the greatest diameter in 8 weeks or so, but there were a few exceptions that showed some delay in growth. The serial transfers were performed every other month without failure.

Effect of Host's Sex. Transplantation experiments in male mice were performed twice. In the 1st experiment (4th passage), the tumors transplanted in male mice showed definitely retarded growth compared to the tumors in female mice (p < 0.05) (Table 1). In the 2nd experiment (6th passage), the growth discrepancy was greater, and the tumors became nonpalpable 3 weeks after transplantation and continued so thereafter (Chart 1). However, when examined after they were killed, very small tumors less than 2 mm in diameter were found in the s.c. tissue and were histologically verified (Table 2 and Fig. 3).

Table 1

<table>
<thead>
<tr>
<th>Host's sex and treatment</th>
<th>No. of mice</th>
<th>Wt of tumors (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>3</td>
<td>489, 409, 289</td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>73, 45*</td>
</tr>
<tr>
<td>Male + E2</td>
<td>2</td>
<td>990, 230</td>
</tr>
</tbody>
</table>

* p < 0.05, f test.

† E2, 0.1 mg of estradiol dipropionate was injected i.m. once a week for 5 weeks, starting 5 weeks after transplantation.

Chart 1. Growth curve of human breast cancer (Br-10) in nude mice (4th passage). Tumors grown in female mice, male mice, and male mice treated with estradiol were weighed 10 weeks after transplantation.

- **Ox**: Ovariectomy; E2, i.m. injection of 0.1 mg of estradiol dipropionate once a week.

The tumor size was expressed by the product of 3 dimensions, which correlated well with the weight of tumors at the termination of experiments (correlative coefficient = 0.96). Each point represents the mean for tumors indicated in Table 2; bars, S.D. Tumors in female mice grew progressively, but their growth was completely arrested by ovariectomy. Tumors in male mice that once disappeared were stimulated to rapid growth by administration of estradiol.

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* The abbreviation used is: DCC, dextran-coated charcoal.
Tumors grown in female and male mice with or without the treatment indicated below were weighed 11 weeks after transplantation.

<table>
<thead>
<tr>
<th>Host's sex and treatment</th>
<th>No. of mice</th>
<th>Wt of tumors (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>8</td>
<td>476 ± 166*</td>
</tr>
<tr>
<td>Female + P</td>
<td>3</td>
<td>650, 510, 490</td>
</tr>
<tr>
<td>Female + Ox</td>
<td>3</td>
<td>145, 145, 66*</td>
</tr>
<tr>
<td>Female + Ox + P</td>
<td>3</td>
<td>152, 84, 34*</td>
</tr>
<tr>
<td>Female + T</td>
<td>5</td>
<td>Trace</td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>Trace</td>
</tr>
<tr>
<td>Male + E2</td>
<td>3</td>
<td>300, 222, 181</td>
</tr>
<tr>
<td>Male + P</td>
<td>3</td>
<td>Trace</td>
</tr>
</tbody>
</table>

* P, 0.2 mg of bovine prolactin was injected s.c. twice a day for 6 weeks, starting 5 weeks after transplantation; Ox, bilateral ovariectomy 5 weeks after transplantation; T, 1 mg of testosterone propionate was injected i.m. twice a week for 10 weeks, starting 1 week after transplantation; E2, 0.1 mg of estradiol dipropionate was injected i.m. once a week for the same period as prolactin.

Mean ± S.D.

† p < 0.01, t test.

Table 2

**Effect of Ovariectomy**. The tumor-bearing female mice (6th passage) were ovariectomized 5 weeks after transplantation, when the tumor size was about 110 cu mm. Ovariectomy suppressed the tumor growth completely (Chart 1), and at the termination of the experiment the tumors weighed significantly less than those of female mice without ovariectomy (p < 0.01) (Table 2).

**Effect of Estrogen on Tumors in Male Mice**. The tumor-bearing male mice (4th and 6th passages) were given injections of 0.1 mg of estradiol once a week for 5 or 6 weeks starting 5 weeks after transplantation. The weight of the tumors at the end of the experiments was comparable to that in female mice (Tables 1 and 2). In the 2nd experiment, the tumors that had disappeared became palpable again in 1 week and showed progressive growth thereafter (Chart 1).

**Effect of Androgen on Tumors in Female Mice**. The tumor-bearing female mice (10th passage) were given injections of 1 mg of testosterone twice a week for 10 weeks starting 1 week after transplantation. The tumors showed growth curves identical with those in untreated male mice, and when mice were examined after they were killed paper-thin tumors were found and histologically verified in 2 of 5 mice.

**Effect of Prolactin**. Tumor-bearing females, ovariectomized females, and male mice (6th passage) were given injections of 0.2 mg of bovine prolactin for 6 weeks starting 5 weeks after transplantation. However, administration of bovine prolactin did not influence the growth of tumors or the histology of host breast tissue in any of these groups (Table 2).

**Histology of Tumors**. Tumors transplanted in female mice retained the histology of the original tumor without exception for the duration of the experiment as of this writing (23 months), although cells and nuclei increased somewhat in size and stroma decreased in amount. The tumors consisted of trabeculae and small nests of polygonal cells with inconspicuous lumina containing mucin (Fig. 1). Ovariectomy did not change the basic structure of the tumor, but tumor cells became atrophic, possessing smaller, more uniform nuclei, while lumina became markedly dilated and conspicuous (Fig. 2). Fig. 3 displays the histological features of paper-thin tumors found 11 weeks after transplantation in male mice, the individual cells of which were similar to those of the tumor in ovariectomized female mice except that small clusters of the cells were arranged in lobular patterns. The same histological features were found in the tumors of androgenized female mice. Tumors in male mice treated with estradiol were histologically identical with those in female mice (Fig. 4), revealing frequent mitoses corresponding to the progressive growth induced by estradiol. Administration of bovine prolactin, which failed to stimulate the growth, did not influence the histology of the tumors at all in any group.

**Estrogen and Progesterone Receptors**. Sucrose gradient analyses for estrogen and progesterone receptors were performed on 2 occasions with the same results, by using 6th and 8th passage tumors in female mice. Chart 2 presents patterns of sucrose density gradient ultracentrifugation for estrogen receptors. The characteristic 8 S and 4 S peaks bound to [3H]estradiol are clearly shown. Preincubation with a 50-fold excess of unlabeled estradiol eliminated both peaks, but they were unaffected by preincubation with unlabelled testosterone. Therefore, both 8 S and 4 S peaks were regarded to represent specific cytoplasmic estrogen receptors. On the other hand, sucrose density gradient ultracentrifugation for progesterone receptors disclosed no peak bound to [3H]progesterone.

Estrogen receptors in a 10th passage tumor in a female mouse were quantitatively analyzed by the DCC method. Chart 3 represents a saturation curve and a Scatchard analysis of the data. The total bound [3H]estradiol might include [3H]estradiol bound to nonspecific proteins. However, addition of 5 nm [3H]estradiol saturated most of the binding sites, indicating that unsaturable nonspecific binding was negligible. In addition, the Scatchard analysis showed a linear relationship, indicating a single class of binding sites. From these results, the amount and dissociation constant...
Hormone-dependent Human Breast Cancer in Nude Mice

Successful heterotransplantation of human breast cancers to nude mice has been reported by several investigators (3, 4, 13, 14, 19, 20). However, hormone dependency of heterotransplanted human breast cancer has not been reported except from our laboratory (19). Some transplantable tumors established by others grew too slowly to be utilized in experimental studies, and others appeared to be hormone independent. Giovannella and Stehlin (3) reported that the growth of Br-10, which we established, was shown to be an adequate experimental model for study of the mechanism of hormone dependence in human breast cancers. The transplantability was 100% in female mice, and the tumor growth was rapid enough to perform experimental studies in vivo, although very occasionally tumors with slower growth were noted.

Br-10 showed preferential growth in female nude mice, growth arrest as a result of ovariectomy, and progressive growth in male mice treated with estradiol. These results clearly indicate that Br-10 is hormone dependent and that its growth is stimulated by estrogen. On the other hand, Br-10 showed very slow or no growth in male and androgenized female mice, suggesting the antagonistic effect of androgen to estrogen in the growth regulation of Br-10.

For examination of the role of progesterin in the growth of Br-10, the unavailability of human prolactin led us to use bovine prolactin, although the effectiveness of nonprimate prolactins on human breast tissues is controversial at present (1, 2, 5, 16, 18). In contrast to results in prolactin-dependent, 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors (11), the bovine prolactin at the dose level that we used did not produce any effects on the growth and histology of either Br-10 or the breast tissue of mice. The results do not indicate prolactin independency of Br-10, since one would not expect the prolactin preparation to stimulate the human tissue when it did not stimulate the breast tissue of mice. It is possible that human prolactin or a larger dose of the bovine prolactin may produce different results on the growth and histology of Br-10. However, Br-10 grew well in nude mice in the absence of human prolactin.

Tumors transplanted in male mice showed very slow but definite growth in the 4th passage, but they did not grow at all in the 6th passage. The reason for the difference is not clear, but it might be due to the selection of more highly hormone-dependent clones during serial transfers.

Histology of the tumors in nude mice showed some changes, depending on the hormonal condition of the hosts. Tumors exhibiting retarded growth in ovariectomized female mice, male mice, and androgenized female mice were composed of atrophic tumor cells with smaller and more uniform nuclei, forming markedly dilated lumina in the 1st group, and arranged in lobular patterns in the latter 2 groups. These changes are at present regarded as a simple atrophic or dormant state, although the lobular arrangement observed requires ultrastructural studies of the cells concerning their differentiation toward either acinic cells or ductal cells.

Sucrose density gradient analyses of Br-10 transplanted in female mice revealed characteristic 8 S and 4 S receptors specific for estrogen, and the binding affinity of the receptors was shown to be very high (Kd = 1.6 x 10^{-10} M) by a Scatchard analysis of results obtained using the DCC method. The presence of estrogen receptors gave the biochemical basis for the direct action of estrogen. We consider that the growth of Br-10 transplanted to nude mice is directly stimulated by estrogen through the mediation of its specific cytoplasmic receptors.

Binding of [3H]estradiol to the cytosol of Br-10 was not inhibited at all by preincubation with a 50-fold excess of testosterone. Therefore, there must be a mechanism other than competitive inhibition for the antagonistic effect of androgen to estrogen in the growth regulation of Br-10. It might be possible that androgen directly inhibited the tumor growth or reduced the amount of estrogen receptors or prolactin receptors. These possibilities must be studied.

Horwitz et al. (6) suggested that progesterone receptors induced by estrogen might be better markers of hormone dependence. However, in Br-10, progesterone receptors were not detected on 2 different occasions in tumors possessing estrogen receptors. This suggests that estrogen-receptor complexes, which stimulated the biochemical processes required for cell proliferation, did not induce the progesterone receptors in Br-10. However, there remains the possibility that further administration of estrogen or other hormones may induce progesterone receptors in this tumor, and this problem must be studied further.

The patient from whom Br-10 was derived was a premenopausal woman, and ovariectomy did not protect her from distant metastases, but bone metastases responded to androgen therapy. The original tumor was an infiltrating duct carcinoma of scirrhous type. Br-10, serially transplanted in nude mice, retained not only the original histology but also...
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the biological function to respond to estrogen and androgen. The results presented herein suggest strongly that the growth of some breast cancers in premenopausal women is directly regulated by estrogen.

Br-13 (established by us) is another human breast cancer transplanted in nude mice in both ascites and solid forms, which was described in detail previously (4). This tumor, in which estrogen receptors were not detected, grew in mice of both sexes and appeared unresponsive to sex hormones. These 2 transplantable tumors will provide good experimental models for the study of both the biological and the therapeutic aspects of human breast cancer.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Histology of the tumor in female mice, showing trabeculae and small nests of polygonal cells with scanty stroma. H & E, x 175.
Fig. 2. Histology of the tumor in ovariectomized female mice, showing smaller and more uniform nuclei and markedly dilated lumina. H & E, x 175.
Fig. 3. Histology of the paper-thin tumor in male mice, in which small nests arranged in lobular patterns are made up of cells similar to those shown in Fig. 2. H & E, x 175.
Fig. 4. Histology of the tumor in male mice treated with estradiol, which is identical with that shown in Fig. 1. H & E, x 175.
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