Difference in the Response of neu and ras Oncogene-induced Rat Mammary Carcinomas to Early and Late Ovariectomy

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ABSTRACT

Rat mammary carcinomas were induced by directly inserting activated neu or ras genes into in situ rat mammary ductal cells using replication-defective retroviral vectors. neu was over 200 times more potent than ras in inducing rat mammary carcinomas. Ovariectomy 2 days postinfection dramatically reduced the occurrence of carcinomas induced by neu and extended their latency. In general, early ovariectomy had much less effect on the occurrence of carcinomas induced by ras and had no significant effect on their latency. Carcinomas induced by neu in ovariectomized rats had down-regulated estrogen receptor and progesterone receptor, while those induced by ras had only down-regulated progesterone receptor. Fully progressed mammary carcinomas in intact rats induced by both neu and ras had a similar response to ovariectomy, with an approximate regression rate of 60%. These data suggest that the activation of ras, but not neu, can replace at least some functions performed by ovarian hormones in the early phases of mammary carcinogenesis. These data also suggest a role for antiestrogen drug therapy in the prevention of neu-associated breast cancer.

INTRODUCTION

Estrogen plays an important role in both the etiology and treatment of breast cancer. Breast tumors rarely develop in ovariectomized women. Breast cancers developing in nonova-

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MATERIALS AND METHODS

Preparation of Recombinant Retroviruses. Recombinant retrovirus vectors JR-neu and JR-ras were constructed as described previously (12, 13). Briefly, the LNL6 retrovirus vector, provided by A. D. Miller (14), was modified to express neu from an internal simian virus 40 promoter. The v-Ha-ras and the activated rat neu oncogenes were cut out of pZipras6 and pSV2neuNT plasmids, respectively, and inserted into the unique BamHI restriction site of pJR.

In order to establish the retrovirus-producing cell line, pJR-ras plasmid DNA was transfected into V2 cells by the calcium phosphate precipitation method. The transiently expressed ecotropic viruses were harvested and used to infect the amphotropic packaging cell line PA317. G418-resistant colonies were expanded and characterized. JR-neu virus-producing cells were established in a similar fashion, except that &Delta;CRE and &Delta;CRIp packaging cell lines (15) were used instead of V2 and PA317. The virus stocks were further concentrated by spinning at 34,000 x g for 6 h through a 20% sucrose cushion and resuspending the pellets in 1/100 of the original volume (12, 13).

Infection of Mammary Epithelial Cells in Situ. Female Wistar-Furth rats (Harlan-Sprague-Dawley, Madison, WI), of 55 days old at the time of virus infusion, were maintained in temperature- and humidity-controlled facilities with a 12-h light/dark cycle. Food and water were available ad libitum. Starting 2 days before virus infusion, rats received three daily s.c. injections of an indirectly acting mammary mitogen, perphenazine (3 mg/kg) (Sigma Chemical) (16). Immediately before infusion, freshly thawed virus stocks were mixed with 80 µg/ml polybrene and 2 mg/ml indigo carmine (a vital tracking dye). The perphenazine-pretreated rats were anesthetized with ether, and the central duct of each gland was cannulated with a 27-gauge blunt-ended needle. About 15 µl virus suspension were infused into each gland (12).

Experimental Design and Hormonal Manipulation. The rats infected with either JR-ras or JR-neu were divided into three groups. Group one was left intact throughout the experiment. Rats from the second group were ovariectomized 2 days postinfection. Finally, individual rats from the third group were ovariectomized at least one of the mammary carcinomas was 25 mm² in the cross-sectional area (17). The mammary glands were harvested, fixed in 10% formalin, and processed by standard histological and histochemical procedures (18). Mammary tumors were scored according to the criteria of Breslow (19).

Receptor Binding Assay. Cytosolic ER and PgR levels were compared on the log scale using &Delta;² test. ER and PgR levels were compared on the log scale using &Delta;² tests.

RESULTS

Tumor Induction. Fifty-five-day-old female Wistar-Furth rats pretreated with perphenazine were infected with 2 x 10⁷
CFU/ml of JR-ras or $1 \times 10^5$ CFU/ml JR-neu replication-defective retrovirus vectors. As expected from our previously established titer-response relationship (12, 13), about an equal number of mammary carcinomas per rat developed in rats infected with the two different vectors (Fig. 2). Tumors induced by neu oncogene had shorter average latencies than those induced by ras oncogene (Fig. 1). The time to first tumor in neu-infected rats averaged 5.4 weeks, while for ras-infected rats the time to first tumor averaged 8.2 weeks. The tumor incidences started to plateau 9 weeks after infection with JR-neu, and a nearly linear increase in tumor multiplicity continued up to 16 weeks postinfection with JR-ras. As shown previously, all the induced tumors are histologically classified as mammary carcinomas (12, 13).

Response to Early Ovariectomy. When rats infused with the neu retroviral vector were ovariectomized 2 days postinfusion, a significant increase in tumor latency was found ($P = 0.047$). The median time to first tumor was 5.4 weeks in the control, while less than one-half of the rats in the ovariectomized group developed tumors by 15 weeks (Fig. 1A). In contrast, rats that were infused with JR-ras did not respond to ovariectomy in terms of changes in latency time ($P = 0.8$). The median times to first tumor were 7.2 weeks and 8.2 weeks for ovariectomized and control, respectively (Fig. 1B).

In addition to analyzing latency (Fig. 1), the average number of tumors developing following early ovariectomy was also analyzed (Fig. 2). A significant difference in the average number of neu-induced tumors between control and early ovariectomized rats were observed at all time periods analyzed between 8 and 19 weeks postinfection ($P < 0.01$). In contrast, there were no significant differences between the average number of ras-induced tumors at time points analyzed between 8 and 17 weeks postinfection. However, at the termination of the experiment at week 22 a significant difference between control and ovariectomy was found ($P = 0.03$).

ER and PgR Levels. The ER and PgR levels in the ras- and neu-induced rat mammary carcinomas developing in intact and ovariectomized rats were measured. Table 1 demonstrates that the ER and PgR levels in mammary carcinomas from the intact control groups were over 20 fmol/mg cytosol protein on average and did not differ quantitatively between the two oncogenes. The ER and PgR contents in carcinomas induced by neu oncogene were significantly lowered by ovariectomy ($P < 0.001$). Interestingly, tumors developing in the ras vector-infected and ovariectomized rats also contained reduced levels of PgR ($P < 0.001$); however, no changes in ER contents were observed ($P = 0.7$) (Table 1).

Response to Late Ovariectomy. We next examined the response of established (>25 mm$^2$ cross-sectional area) neu or ras-induced mammary carcinomas to ovariectomy. Tumor growth or regression was then monitored by measuring the changes in tumor size relative to the size at the time of ovariectomy as compared to a matched group of tumors from the control rats. Most of the carcinomas from the intact rats infected with either ras or neu vector continued to grow after reaching 25 mm$^2$. In contrast, tumors from ovariectomized rats could be divided into two groups, those that regressed and those that did not. During the 5-week postovariectomy observation period, over 60% of the carcinomas induced by the neu oncogene regressed to less than 80% of cross-sectional area measured at the day of ovariectomy, with three (38%) complete remissions (Table 2). Contrary to the lack of clear response to early ovariectomy, ras-induced established mammary carcinomas were as responsive to late estrogen withdrawal as those...
greater than 25 mm² in cross-sectional area, measured as the product of the two long axes.

...characterization of these two tumor types has previously been selected so that they would cause a similar number of mammary carcinomas per rat (12, 13). The present study thus permitted direct comparison of the transforming potencies between the two vectors in a single experiment. At 1 × 10⁵ CFU/ml, the neu virus induced rat mammary carcinomas with a frequency similar to that of the ras virus at 2 × 10⁷ CFU/ml. Thus the ras-expressing retrovirus vector was 200 times more tumorigenic than the neu-expressing, but otherwise identical vector.

These results confirm the reproducibility of our in situ gene expression of v-H-ras from the Moloney murine leukemia virus long terminal repeat, while in chemically induced tumors ras is regulated by its own promoter. We are currently constructing vectors in which ras is controlled by its own promoter in order to address this difference in gene regulation in relation to response to ovariectomy.

Although ovariectomy resulted in a reduction in PgR in ras carcinomas, the expected reduction in tumor ER in ovariectomized animals was not observed. No clear explanation is currently available for the sustained ER expression in these carcinomas. More importantly, more than one-half of the well-established mammary carcinomas regressed after ovariectomy, suggesting that most of the ras-induced fully progressed tumors were dependent on estrogen for optimal growth. These latter results are consistent with those reported by Sukumar et al. (11).

We demonstrated here, for the first time, the estrogen dependency of activated neu oncogene-induced mammary carcinomas. All the rat mammary carcinomas induced by neu oncogene were positive for ER and PgR. Ovariectomy immediately after infection reduced the tumor frequency by almost 90%. Therefore, hormonal intervention at early stages could prevent rat mammary epithelial cells containing the activated neu oncogene from progressing to a frank mammary carcinoma. This requirement for estrogen also provides further support for the hypothesis that neu oncogene product by itself is not sufficient to predispose an infected cell to full malignancy (13). Other endogenous factors, including estrogen, are required to facilitate transformation. However, escape from estrogen dependency did occur at low frequency, as indicated by the tumors arising in the infected rats despite the early withdrawal of estrogen. In addition, estrogen removal also constituted an effective treatment for fully progressed mammary carcinomas in that a majority of these tumors regressed upon ovariectomy. Together these observations suggest that the activation of the neu gene does not generally lead to the abrogation of hormonal requirements during mammary carcinoma progression. Acquisition of estrogen-independent growth in selected carcinomas is

### DISCUSSION

In this report, parallel experiments were conducted to determine the hormone requirements of the tumors induced by ras and neu oncogenes. A detailed molecular and histopathological characterization of these two tumor types has previously been published (12, 13). The titers for the two retrovirus vectors were selected so that they would cause a similar number of mammary carcinomas per rat (12, 13). The present study thus permitted direct comparison of the transforming potencies between the two vectors in a single experiment. At 1 × 10⁵ CFU/ml, the neu virus induced rat mammary carcinomas with a frequency similar to that of the ras virus at 2 × 10⁷ CFU/ml. Thus the ras-expressing retrovirus vector was 200 times more tumorigenic than the neu-expressing, but otherwise identical vector. These results confirm the reproducibility of our in situ gene transfer system (12, 13) and complement the data obtained from transgenic mouse studies demonstrating mammary tumor induction by targeted oncogene expression (19).

Previous reports have dealt with the issue of ras protooncogene activation and hormone dependency of mammary carcinomas (10, 11). Transfection of the MCF-7 human breast cancer cell line by v-H-ras oncogene under the transcriptional control of three tandem repeats of Ha-MuSV long terminal repeats bypasses dependence on estrogen for tumor growth (10). In contrast, the activation of c-H-ras oncogene in the rat mammary carcinomas induced by N-nitroso-N-methylurea or 7,12-dimethylbenz(a)anthracene was unrelated to the acquisition of hormone-independent growth (11).

Our results demonstrate that carcinomas induced by *in situ* introduction of the ras oncogene expressed both ER and PgR. Interestingly, these tumors displayed a unique response to estrogen deprivation. Unlike the situation for chemically induced mammary carcinomas in the rat, ovariectomy failed to increase tumor latency or lower the overall frequency of carcinoma induction by ras oncogene. While many chemically induced mammary cancers have activated ras, these cancers differ from the ones described here. Specifically, the carcinomas described here have an unrelated product expression of v-H-ras, while in chemically induced tumors ras is regulated by its own promoter. We are currently constructing vectors in which ras is controlled by its own promoter in order to address this difference in gene regulation in relation to response to ovariectomy.

Table 1: Levels of ER and PgR in JR-ras or JR-neu retrovirus vector-induced mammary carcinomas in Wistar-Furth rats in the presence or absence of Ovx.

<table>
<thead>
<tr>
<th>Receptors (fmol/mg cytosol protein)</th>
<th>JR-neu Control</th>
<th>JR-neu Ovx</th>
<th>JR-ras Control</th>
<th>JR-ras Ovx</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>89.3 ± 13.9</td>
<td>22.0 ± 12.8*</td>
<td>80.9 ± 8.7</td>
<td>79.3 ± 13.9*</td>
</tr>
<tr>
<td>No. of Tumors tested</td>
<td>14</td>
<td>5</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Range</td>
<td>29-203</td>
<td>0-60</td>
<td>26-137</td>
<td>21-153</td>
</tr>
<tr>
<td>PgR</td>
<td>311.3 ± 69.3</td>
<td>34.4 ± 11.7*</td>
<td>284.2 ± 33.3</td>
<td>32.1 ± 11.2*</td>
</tr>
<tr>
<td>No. of Tumors tested</td>
<td>14</td>
<td>5</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Range</td>
<td>57-1107</td>
<td>0-67</td>
<td>82-561</td>
<td>0-118</td>
</tr>
</tbody>
</table>

* Significantly different from control (P = 0.0009).
* Not significantly different from control (P = 0.9).
* Significantly different from JR-neu Ovx (P = 0.01).
* Significantly different from control (P = 0.0001).

Table 2: Response to ovariectomy of carcinomas induced by v-H-ras or neu oncogene.

<table>
<thead>
<tr>
<th>Response to ovariectomy</th>
<th>ras-induced tumors</th>
<th>neu-induced tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regressors</td>
<td>Control</td>
<td>Ovx</td>
</tr>
<tr>
<td>1/22 (4.5%)</td>
<td>6/11 (54.5%)</td>
<td>3/19 (15.8%)</td>
</tr>
<tr>
<td>Progressors</td>
<td>21/22 (95.5%)</td>
<td>5/11 (45.5%)</td>
</tr>
</tbody>
</table>

* Significantly different from control (P < 0.01).
likely to be associated with other distinct molecular events occurring during tumor progression. It is important to point out that the neu oncogene used here is activated by point mutation in contrast to activation by overexpression in human breast cancer. Recently, it has been shown that overexpression of normal neu oncogene is able to transform an immortalized human mammary epithelial cell line, in vitro and in vivo, although in not as potent a fashion as the mutated neu oncogene (20). We thus plan to extend their studies by replacing the mutated neu gene used in these studies with the wild-type gene. These studies will be important in assessing the relevance of these findings to human malignancies.

In summary, we have found that the induction of rat mammary carcinomas by the direct introduction of activated ras and neu oncogenes responds differently to ovariectomy. Tumors induced by neu respond to early and late ovariectomy as would be expected based on both human epidemiology and results obtained with chemically induced experimental rat mammary cancer models. Specifically, early ovariectomy reduces the frequency and extends the latency of developing carcinomas. The lack of estrogen results in the down-regulation of both estrogen and progesterone receptors. In addition, fully progressed tumors in intact rats regress following ovariectomy. In contrast, the induction of ras-induced tumors generally is not affected by early ovariectomy. While tumors developing in ovariectomized rats have down-regulated PgR, they do not have down-regulated ER. Fully progressed ras-induced tumors in intact animals, however, do respond to ovariectomy. v-Ha-ras is thus able to replace some biological functions of estrogen but not others that were studied here. Activated neu, however, was not able to replace these estrogen-related cellular effects.

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