Effect of v-rasH Oncogene Transfection on Estrogen-independent Tumorigenicity of Estrogen-dependent Human Breast Cancer Cells

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ABSTRACT

Spontaneous or therapeutically induced progression of hormone-dependent human breast cancer to a form not amenable to endocrine treatment has been frequently recorded in clinical settings. In an experimental model system, we have changed the estrogen-dependent tumorigenicity of a human breast cancer cell line, MCF-7, to an independent state by stably introducing a model oncogene, v-rasH1, into this cell line by means of DNA transfection. We now show that the oncogene-transfected hormone-independent MCF-7 cells may secrete diffusible tumorigenic factors that not only support their own tumor growth in vitro, but are also hormonally active in partially triggering the tumor growth of wild type previously nontumorigenic MCF-7 cells, even when the wild type cells are implanted at a distant anatomical site in the same animal. Estrogen-independent tumor formation by MCF-7 cells was also induced in 50% of animals given injection by continuous administration of conditioned media from MCF-7-ras cells. However, the wild type tumors had limited tumor growth. Tumors were verified as adenocarcinomas and by Southern blotting were shown to be derived from the cells injected. In an in vitro coculture assay, a 5- to 7-fold enhancement in anchorage-independent growth of MCF-7 cells was observed in the presence of MCF-7-ras feeder cell layer. These data suggest that v-rasH-induced estrogen-independent tumorigenicity of human breast cancer cells occurs by secretion of mitogens which may function in an endocrine manner.

INTRODUCTION

The mechanism of growth control in estrogen-dependent and -independent human breast cancer is not completely understood. The human breast cancer cell line, MCF-7, provides a model for estrogen responsiveness in vitro and estrogen dependence for tumorigenicity in vivo (1–6). In the absence of estradiol, MCF-7 cells are nontumorigenic in ovariectomized athymic mice. Estrogen-induced phenotypic changes in these cells, especially tumor formation, are reminiscent of alterations in cultured cells caused by oncogene activation, oncogene transfection, or growth factor stimulation. Some of the genes activated by estrogen treatment may have growth-enhancing functions analogous to those of known oncogenes (see Ref. 7 for review).

In an attempt to abrogate the estrogen dependence of MCF-7 cells, we transfected the cells with v-rasH1 gene of Harvey murine sarcoma virus in the presence of the pSV2-gpt plasmid, which functions as a dominant selectable marker (8). We were able to show that the transfected MCF-7 cells were rendered hormone independent for tumor formation when transplanted to athymic ovariectomized mice. Concomitant with the loss of estrogen dependence, the ras-transfected (MCF-7-ras) cells also became only minimally responsive to growth regulation by estradiol and antiestrogens in culture, although estrogen receptor and some estrogen responses such as induction of progesterone receptor and M, 52,000 protein persisted (8). The control clones transfected with the pSV2-gpt DNA alone, MCF-7-gpt, were nontumorigenic and still estrogen responsive similar to the wild type MCF-7 cells.

The molecular basis of ras-induced estrogen independence is unclear. At least two possible mechanisms may be considered to account for this phenomenon. The oncogene product may directly or indirectly stimulate growth by interacting with intracellular targets. The activated ras gene product itself is mitogenic in a variety of rodent cells; microinjection of M, 21,000 ras protein induces DNA synthesis and results in proliferation of quiescent cells (9, 10). Elevated expression of M, 21,000 protein appears sufficient to induce oncogenic transformation, since ligation of viral transcriptional promoter sequence results in activation of transforming activity of both rat and human normal cell rasH1 gene (7). Alternatively, transfection may result in the constitutive synthesis and secretion of growth factors necessary for in vivo proliferation. The latter possibility is supported by the fact that MCF-7-ras cells had augmented secretion of TGF-α3 and IGF-I in amounts comparable to estrogen stimulation of MCF-7 cells (11), and that several hormone-independent, highly tumorigenic breast cancer cell lines constitutively secrete higher levels of growth factors (12). Recent studies have suggested that TGF-α could be involved in an autocrine fashion in the development of malignancies in vivo (13).

In the studies reported here, we have addressed directly the role of MCF-7-ras secretions in the acquisition of the estrogen-autonomous tumorigenic phenotype. By using a novel in vivo tumorigenic assay in nude mice, we show that the hormone-independent MCF-7-ras cells can partially stimulate tumor formation by wild type MCF-7 cells in an estrogen-depleted host, even when the wild type cells were injected at a different anatomical site from the test cells, MCF-7-ras. Further, in an in vitro coculture assay, a feeder layer of MCF-7-ras cells stimulated the anchorage-independent growth of the wild type MCF-7 cells by 5- to 6-fold. The results suggest production of diffusible, systemically active tumor-stimulating factors by the hormone-independent MCF-7-ras cells.

MATERIALS AND METHODS

Cells. MCF-7 cells (1) were originally obtained from the Michigan Cancer Foundation (Detroit, MI). MCF-7-ras cells were obtained by transfecting v-rasH1 oncogene from Harvey murine sarcoma virus into MCF-7 cells in the presence of the pSV2-gpt plasmid, which functions as a selectable gene marker (8). MCF-7 cells transfected with the PSV2-gpt DNA alone, MCF-7-gpt, were used as a control for all the studies with MCF-7-ras cells. Cells were cultured in 10% fetal calf serum or in 5% sulfatase and CCS in IMEM (GIBCO) as described (4).

Tumorigenicity Experiments. Cultured cells in log phase of growth were harvested with trypsin-EDTA and resuspended in medium (IMEM) containing 5% CCS at a density of 2 x 10^7 cells/ml. Approximately 4 x 10^6 cells of each type were inoculated in two anatomically distant mammary fat pads of each ovariectomized athymic mouse (Ncr-}

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Received 4/21/87; revised 7/23/87; accepted 7/30/87.

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The abbreviations used are: TGF-α, α-transforming growth factor; IGF-I, insulin-like growth factor I; CCS, charcoal-treated calf serum; IMEM, improved minimal essential medium; PBS, phosphate-buffered saline; TGF-β, β-transforming growth factor.
Autoradiography was at −70°C for 1-3 days by using X-ray films and 10% dextran sulfate. Blots were normally washed to a final stringency of 0.5 x 0.15 M NaCl-0.015 M sodium citrate at 60°C.

Tris (pH 7.5), 20 μg/ml sheared and denatured salmon sperm DNA, 3 x Denhardt’s solution, 0.5% sodium dodecyl sulfate, 10 mM citrate, and an intensifying screen. For 16 h at 42°C in 50% formamide, 3 x 0.15 M NaCl-0.015 M sodium citrate that contains the -ras” sequences (8). Hybridization was performed separated by electrophoresis on a 0.7% agarose gel, transferred to nitrocellulose paper (15), and hybridized to a 32P-labeled insert probe from dispersed tumor cells. The restriction enzyme-digested DNA was isolated directly from tumors and also from secondary cultures derived with a 1-ml layer of IMEM containing 0.8% Bacto agar, 10% CCS, of IMEM plus 5% CCS in the mammary fat pad area.

Cell monolayers used for collection of conditioned medium were harvested in PBS containing 0.04% EDTA, sonicated, and aliquots were taken for DNA determination by a fluorescence assay (14). Cellular DNA concentration was used to calculate the volume of conditioned medium for filling the same equivalents of producer cell DNA.

Anchorage-independent Growth Assay. MCF-7-ras or MCF-7-gpt cells were plated in triplicate in 35-mm tissue culture dishes at a density of 10² cells/well in IMEM containing 5% CCS. After the cells were attached, medium was aspirated and the monolayer was gently overlaid with a 1-ml layer of IMEM containing 0.8% Bacto agar, 10% CCS, and 2 mM glutamine. After the agar hardened, a 0.8-ml top layer of IMEM containing 0.36% agar, 10% CCS, and 3 x 10⁶ MCF-7 cells was added. Cultures were incubated at 37°C in 5% CO₂ atmosphere for 6-10 days. Colonies were counted with a Bausch & Lomb stem cell colony counter. Colonies greater than 60 μm (approximately equivalent to more than 50 cells) were scored as positive. The data are representative of three experiments each done in triplicate and shown as mean ± SE.

DNA Isolation and Southern Blot Analysis. Genomic DNA was isolated directly from tumors and also from secondary cultures derived from dispersed tumor cells. The restriction enzyme-digested DNA was separated by electrophoresis on a 0.7% agarose gel, transferred to nitrocellulose paper (15), and hybridized to a ³²P-labeled insert probe that contains the v-ras™ sequences (8). Hybridization was performed for 16 h at 42°C in 50% formamide, 3 x 0.15 M NaCl-0.015 M sodium citrate, 3 x Denhardt’s solution, 0.5% sodium dodecyl sulfate, 10 mM Tris (pH 7.5), 20 μg/ml sheared and denatured salmon sperm DNA, and 10% dextran sulfate. Blots were normally washed to a final stringency of 0.5 x 0.15 M NaCl-0.015 M sodium citrate at 60°C (16). Autoradiography was at −70°C for 1-3 days by using X-ray films and intensifying screen.

RESULTS

Estrogen-independent Tumorigenicity of MCF-7 Cells in Nude Mice. We developed an in vivo bilateral tumor model system in nude mice in which tumorigenicity assays were performed by injecting, simultaneously, hormone-independent (MCF-7-ras) and hormone-dependent cells (MCF-7 or MCF-7-gpt) into two anatomically distant sites (opposite mammary fat pads). Bilaterally injected wild type MCF-7 or MCF-7-gpt cells served as control. In the absence of any exogenous estrogenic sources, animals were monitored for tumor formation at both sites of injection. As shown in Fig. 1 and further summarized in Table 1, s.c. tumors became apparent within 2-3 weeks of implantation of MCF-7-ras cells at a 100% incidence, and the tumors

![Image](cancerres.aacrjournals.org)
grew progressively in an estrogen-autonomous manner. Interestingly, under these conditions tumors also developed contralaterally in 50-55% of the injection sites of MCF-7 or MCF-7-gpt cells (Table 1). The MCF-7 or MCF-7-gpt tumors grew steadily for 4-7 weeks, after which these ceased to grow.

Control animals receiving an inoculum of either MCF-7 cells or MCF-7-gpt cells in both sites, did not develop tumors in the absence of estrogen supplementation to the animals. One control animal developed a tumor in one of the sites of MCF-7 implantation. This tumor, however, regressed 4 weeks after its appearance; by contrast, the induced MCF-7 tumors were still present and maintained their size in the animals which received an inoculum of MCF-7-ras cells in the opposite site. Wild type MCF-7 cells were highly tumorigenic in both inoculation sites when animals were treated with a 0.5-mg estradiol pellet (Table 1). These tumors appeared with an incidence of 85-90% as early as 2-3 weeks after inoculation and grew progressively in size at a rate comparable to MCF-7-ras-induced tumors. These results are consistent with the idea that MCF-7-ras cells produce diffusible, systemically active tumor stimulating factors which are capable of conferring partial tumorigenicity in nontumorigenic MCF-7 cells, even when MCF-7 cells are injected at a distance from MCF-7-ras cells.

Upon histological examination, the malignant nature of the tumors was indicated by invasiveness of neoplastic cells (Fig. 2). Tumors formed either by the wild type MCF-7 cells or by MCF-7-ras cells were poorly differentiated invasive adenocarcinomas and were indistinguishable from each other. Intense diffuse and cord-like infiltration of pleomorphic neoplastic cells with a dense connective tissue stroma was clearly evident in both the tumor types.

While wild type MCF-7 tumors were induced in the presence of MCF-7-ras cells, uterine growth of animals remained unaffected. This excludes production of a true estrogen by the MCF-7-ras cells. On an average, after 4-6 weeks of tumor formation, the uterine wet weight was $10.7 \pm 2.9$ mg (SD) in animals bearing MCF-7-ras/MCF-7 tumors, comparable to uterine wet weight of $8.4 \pm 2.7$ mg in unstimulated ovariectomized animals. Uteri in animals bearing MCF-7 tumors induced by estradiol pellets had mean uterine weights of $86 \pm 13.2$ mg. Furthermore, uterine histology showed no hyperplasia (data not shown). Undetectable levels of radioimmunoassayable estradiol and estrone were observed in the blood of animals bearing MCF-7-ras/MCF-7 tumors. Thus the tumor stimulus produced by MCF-7-ras cells which induces the wild type MCF-7 cells, is apparently not generally estrogenic.

Although MCF-7 cells were initially derived from metastatic effusions of patients with breast cancer, we have not seen macroscopic metastases from MCF-7-ras or the wild type MCF-7 cells in nude mice. To prove that the tumors induced at the site of MCF-7 or MCF-7-gpt inoculation by transinoculated MCF-7-ras cells did not arise from metastases, we analyzed DNA from the cultured tumor-derived cells for the presence of v-ras sequences. Southern blots (Fig. 3) of PstI- and XhoI-digested DNA revealed v-ras-related sequences integrated in MCF-7-ras tumor-derived cells which were absent in MCF-7 or MCF-7-gpt tumor-derived cells. These results thus confirm that contralaterally induced tumors were derived from the cells injected. We also analyzed the tumor DNA directly for the v-ras-related sequences and confirmed that the wild type tumors were devoid of v-ras-related sequences (data not shown).

Detection of Tumorigenic Activity in Conditioned Medium. MCF-7 cells as well as other breast cancer cells secrete a number of polypeptide growth factors including IGF-1 (17), transforming growth factors of both $\alpha$ and $\beta$ type (12, 18, 19), platelet-derived growth factor, an epithelial cell colony-stimulating factor, and an autocrine motility factor (7). Secretion of some of the growth factors in MCF-7 cells is increased both by estradiol stimulation and v-ras transfection (11). Consistent with this finding is the fact that certain murine cell lines transformed with oncogenes, specifically the v-ras, secrete TGF-$\alpha$, TGF-$\beta$, and platelet-derived growth factor-like molecules (20-23). These growth factors enhance cellular growth in vitro, and also cellular tumorigenicity in vivo as shown recently (12, 13).

To test the biological activity of the culture supernatants, we analyzed the medium conditioned by the wild type MCF-7 and MCF-7-ras cells for stimulation of in vivo tumorigenicity of recipient animals. Tumors initiated at both the sites were excised and treated with a double enzyme mixture (collagenase, 100 units/ml; hyaluronidase, 50 units/ml) for 8 h at room temperature. The dispersed tumor cells were next cultured. Genomic DNA (10 $\mu$g) from cells derived from MCF-7-ras tumors (Lane 1), MCF-7 tumors (Lane 2), and MCF-7-gpt tumors (Lane 3) was digested with Pst I or Xho I, subjected to electrophoresis, Southern blotted, and hybridized to $^{32}$P-labeled v-ras probe. The blots were washed in 1 $\times$ 0.15 M NaCl-0.015 M sodium citrate containing 0.5% sodium dodecyl sulfate at 60°C for 90 min with three changes and once in 0.5 $\times$ 0.15 M NaCl-0.015 M sodium citrate for 30 min at 60°C and exposed to Kodak XAR-5 films. Kb, kilobase.
MCF-7 cells in an estrogen-independent manner. Concentrated serum-free conditioned medium was infused into ovariectomized animals via Alzet miniosmotic pumps, and MCF-7 cells were inoculated s.c. into the animals. After a period of 3 weeks tumors appeared at the site of inoculation in 50% of inoculated animals receiving conditioned medium from MCF-7-ras cells (Table 2). Control animals receiving MCF-7 cells either alone or in combination with pumps containing PBS or concentrated nonconditioned medium did not develop tumors. Conditioned medium collected from MCF-7 cells or MCF-7-gpt cells also induced tumors but at lower incidence. MCF-7-ras-conditioned medium was apparently more potent in stimulating MCF-7 cellular tumorigenicity than the MCF-7 or MCF-7-gpt-conditioned medium (2 of 12 tumors induced by MCF-7 and MCF-7-gpt-conditioned medium versus 8 of 16 tumors induced by MCF-7-ras-conditioned medium). The tumors, although histologically similar to MCF-7-ras-induced adenocarcinomas, had limited tumor growth. The conditioned medium-induced tumors reached maximum size in 3-4 weeks (approximately one-tenth the size of MCF-7-ras tumors), usually declining in size thereafter. We have previously reported a similar observation with MCF-7 tumors induced with conditioned medium from estrogen-stimulated cells (24). The reason for incomplete tumorigenesis in the presence of conditioned media is not clear; perhaps certain other factors required for angiogenesis are lacking. The results, however, do suggest that conditioned medium from MCF-7-ras cells contain elevated levels of tumor factors which may humorally act to induce tumorigenicity of the parental MCF-7 cells.

In Vitro Assay of Transforming Activity in Conditioned Medium. We next used an independent assay as an in vitro correlate of tumorigenicity to test the biological activity of the diffusible factor secreted by MCF-7-ras cells. Loss of anchorage dependence and acquisition of the ability to grow in a semisolid growth medium have been shown to be associated with cellular tumorigenicity (12). We developed a coculture soft agar colony formation assay to test the anchorage-independent growth of MCF-7 cells in the presence of MCF-7-ras feeder layer (Fig. 4). A 5- to 7-fold higher efficiency of colony formation by MCF-7 cells was observed when MCF-7-ras cells acted as the conditioner in feeder cultures. If, on the other hand, MCF-7 or MCF-7-gpt cells were used in the feeder layer, a low but detectable number of MCF-7 colonies was identified. We have also seen that in the absence of any feeder layer, MCF-7 cells per se were poorly clonogenic in soft agar under these culture conditions (Fig. 4). Physiological concentrations of estradiol stimulated colony formation by MCF-7 cells to the same extent as seen with MCF-7-ras cells in feeder layer. Surprisingly, MCF-7 cells demonstrated no augmented response to estradiol in the presence of MCF-7-ras feeder layer. We conclude that MCF-7-ras cells secrete a diffusible activity(s) which cross-feeds and thereby replaces estradiol in conferring anchorage-independent growth of MCF-7 cells.

Table 2  Estrogen-independent tumor formation by MCF-7 cells in athymic mice in response to concentrated conditioned medium

<table>
<thead>
<tr>
<th>Pump contents</th>
<th>Tumor incidence</th>
<th>Tumor cross-section area (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td>IMEM</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td>Conditioned medium (MCF-7 cells)</td>
<td>1/6</td>
<td>0.05</td>
</tr>
<tr>
<td>Conditioned medium (MCF-7-gpt cells)</td>
<td>1/6</td>
<td>0.04</td>
</tr>
<tr>
<td>Conditioned medium (MCF-7-ras cells)</td>
<td>8/16</td>
<td>0.08 ± 0.01*</td>
</tr>
</tbody>
</table>

* Tumors formed/mice inoculated.

DISCUSSION

Breast cancer has been observed in individual patients over time to change from an estrogen-dependent to a hormonally independent tumor. Commonly, the estrogen-independent derivatives pursue a more aggressive growth pattern in the patient. MCF-7 cells represent a model for estrogen-dependent breast cancer, relevant to clinical settings. These cells show continuous growth in culture, as is true of other transformed cells. However, these cells per se are insufficient for tumorigenesis in vivo. Exogenous estrogen is necessary for these cells to express the fully malignant phenotype. The v-rasH oncogene converts this cell line from absolute hormone dependence of in vivo tumor formation to an estrogen-independent, fully tumorigenic phenotype. In a bilateral tumor model system, we now show that the in vivo-induced MCF-7-ras tumors and also the media conditioned by these cells, can partially stimulate tumor formation by wild type MCF-7 cells, even when the wild type cells are injected at a different anatomical site. Contralateral tumors by MCF-7 cells, although smaller in size, were verified as adenocarcinomas and were shown not to contain the v-rasH sequences. Corresponding observations were made in a soft agar assay with MCF-7-ras cells growing in an attached feeder layer, that anchorage-independent colony formation of MCF-7 cells was induced by MCF-7-ras cells. These data are consistent with the possibility that MCF-7-ras cells secrete growth factors that may stimulate their own growth and also provide a humoral network for tumor growth of distantly implanted cells in vivo.

Members of ras gene family have been frequently found as transforming genes in a broad spectrum of human cancers (7). However, their role has not been thus far clearly understood in mammary carcinomas. c-rasH activation (mutated at the codon 12) has been reported in a cell line, Hs578T, derived from a carcinosarcoma in the breast (25). Increased expression of ras encoded M, 21,000 protein has been shown in some malignant mammary tumors. c-rasH activation (mutated at the codon 12) has been reported in a cell line, Hs578T, derived from a carcinosarcoma in the breast (25). Increased expression of ras encoded M, 21,000 protein has been shown in some malignant mammary tumors. c-rasH activation (mutated at the codon 12) has been reported in a cell line, Hs578T, derived from a carcinosarcoma in the breast (25). Increased expression of ras encoded M, 21,000 protein has been shown in some malignant mammary tumors.

Further-
more, expression of the ras oncogene in human breast cancer is not hormonally regulated (8). At the present time there is no definitive correlation between aberrant expression of an oncogene with oncogenicity in breast cancer.

Our studies show that an exogenous activated oncogene can bypass the hormonal requirement for tumor formation and convert a previously estrogen-dependent cell line into an independent tumorigenic line. From these studies, it does not necessarily follow that ras activation is the only mechanism by which primary breast cancer in vivo becomes estrogen independent. Given the ability of activated ras genes to fully transform many types of cells, including normal cells when linked to strong transcriptional enhancers (30), it should not be surprising that MCF-7 cells become hormone independent. The results, however, do suggest that a single gene mutation or gene amplification in a cell which already has malignant potential, can radially alter its tumor phenotype. In vivo, such a biological event may be related with the proliferation of a variant of the original breast cancer clone with a newly activated oncogene which could provide a selective growth advantage and eventually result in its growth autonomy.

It is interesting to note that the mammary tumors induced by injection of nitrosomethylurea into rats, which contain a transforming ras gene, still remain estrogen dependent for growth in vivo (31). This may possibly be explained by differences in biological activity between the -ras and activated c-ras genes. The ras gene encoded M, 21,000 protein differs from the normal human cellular homolog (c-ras) at 2 amino acids, 12 and 59 (8). Further, in our studies quantitative changes in ras expression due to experimentally induced amplification of the ras by transcriptional enhancers may also be important.

One mechanism by which ras transfect MCF-7 cells become fully tumorigenic in nude mice may be the induction of growth stimulatory molecules. Induction of several molecular species with growth-promoting activity including IGF-I, TGF-α, and TGF-β have been identified in MCF-7 ras cells (11). Some of these growth factors when delivered to athymic mice via minipumps partially confer hormone-independent tumorigenicity of MCF-7 cells (24). Although the wild type MCF-7 wild type MCF-7 cells did form tumors when inoculated into mice in presence of MCF-7-ras tumors or MCF-7-ras-conditioned media released via minipumps, the wild type tumors were smaller in size. The growth factors produced by MCF-7-ras cells may not be adequate to stimulate complete tumor formation by the wild type cells. It is also likely that the growth factors may not be the sole mediators of hormone-independent tumorigenicity. Perhaps certain other factors may be lacking, including those required for angiogenesis.

Recently, several examples of establishment of growth factor-independent, fully tumorigenic systems by oncogenes have been reported for a number of factor-dependent, nontumorigenic cell lines (32–35). The system clearly resembling the one described here is the conversion of hormone-responsive murine mammary cells to the hormone-independent state by coinoculation with hormone-independent cells (36). Although generation of an autocrine system as the mechanism of transformation is plausible (37–42), it may not address the whole process of in vivo induction of tumors. We report a new finding suggesting that growth factors secreted from autonomous cells confer hormone-independent potential in nontumorigenic, hormone-dependent cells implanted at a distance. This observation underscores production of endocrine mitogen(s) by the hormone-independent cells. Further progress on the nature of these factors is central to our understanding of growth regulation of breast cancer and its escape to hormone-independent cancer.

REFERENCES


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