Tissue-targeted Antisense c-fos Retroviral Vector Inhibits Established Breast Cancer Xenografts in Nude Mice

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ABSTRACT

The c-fos proto-oncogene has been implicated as a regulator of estrogen-mediated cell proliferation. We have tested the tissue specificity and antitumor efficacy of a mouse mammary tumor virus-regulated antisense c-fos retroviral vector. Systemically administered vector could be detected in several tissues but was only expressed in breast epithelium, thus supporting targeting to mouse mammary tumor virus-regulated tissues. In vivo transduction of 30-70% of MCF-7 human breast cancer cells produced expression of antifos RNA, decreased expression of the c-fos target mRNA, induction of differentiation, and inhibition of s.c. tumor growth. In vivo transduction of established i.p. MCF-7 tumors with a single injection of XM6:antifos inhibited tumor growth in athymic nude mice with a corresponding inhibition of c-fos, transforming growth factor β1 and transforming growth factorα expression. Four daily injections with the antifos RNA induced a much larger MCF-7 i.p. tumor inhibition, with a marked prolongation of survival in the absence of any host tissue toxicity. These results indicate that inhibition of key nuclear genes such as c-fos may lead to disruption of paracrine factors and an antitumor effect, providing a strategy for cancer gene therapy.

INTRODUCTION

Retroviruses have evolved as effective agents that can introduce DNA into specific cells and use molecular mimicry to cause cancer in animals. The MMTV1 causes a transmissible breast cancer in certain strains of mice as a consequence of efficient transcription in breast cells and activation of oncogenes. The transcriptional unit that produces efficient transcription of the MMTV genome in breast cells is the MMTV-LTR, which is a hormone-responsive promoter. The hormone-responsive nature and breast specificity of the MMTV promoter have been exploited in expression vectors to produce regulated expression in cultured cells and in transgenic mice. The use of a breast-targeted promoter could provide selectivity so that an antioncogene is expressed predominantly in breast cancer cells.

Breast cancer cells are influenced by external signals including estrogen and paracrine factors (1). The mechanisms through which these factors contribute to malignancy are uncertain, but several studies have proposed that estrogen signals through transcription factors like c-fos, which in turn regulate genes that contribute to malignancy. Numerous studies have implicated c-fos in estrogen-mediated cell signaling (2-5). Estrogen is believed to be important in the development of progression of breast cancer and is required for tumor formation by MCF-7 human breast cancer cells in nude mice (6-8). Estrogen stimulates growth and induces DNA synthesis in MCF-7 cells, providing a mechanistic model system for study of hormone-responsive malignancies (6-7). Although the precise mechani

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3 The abbreviations used are: MMTV, mouse mammary tumor virus; LTR, long terminal repeat; TGF, transforming growth factor; RT-PCR, reverse transcription-PCR.

anisms through which estrogen contributes to tumorigenesis are unknown, this hormone induces the growth-associated transcription factors c-fos and c-myc, suggesting a hypothesis that estrogen-mediated tumor formation is mediated through these nuclear proto-oncogenes. There is some molecular evidence for interactions between steroid hormone receptors and fos/jun/AP-1 since these transcription factors can interact at certain target sites (9-12). Functional transcriptional roles for the c-fos gene have been identified by antisense inhibition studies, which have identified a number of genes whose transcriptional induction requires c-fos (13). These genes include proteases, such as stromelysin (14) and collagenase (15), and paracrine factors such as TGF-β1 (16).

We have tested the role of c-fos and the utility of antisense retroviral vectors using a breast-targeted antisense retroviral vector to transduce human breast MCF-7 tumors in nude mice. Transduced tumors expressed antifos RNA, with a resulting decreased expression of endogenous c-fos mRNA and c-fos-regulated gene targets, and inhibition of tumor growth and invasiveness. In addition, in vivo transduction of established i.p. tumors by anti-fos vector increased survival compared with control vector-treated animals.

MATERIALS AND METHODS

Vector Construction and Production. The breast-targeted retroviral vector XM6:antifos was constructed from XM5 (a derivative of N2) by the following steps: (a) XM5 was digested with HindIII and BamHI, blunted with Klenow, and then ligated to produce XM6, a retroviral vector that lacked HindIII and BamHI sites; (b) the Xhol-Sall fragment of pBl βI globin was cloned into Xhol-digested XM6, and we selected for the orientation that retained the 3′ Xhol site; and (c) the Xhol-BamHI fragment from our MMTV antisense plasmid (13) was cloned into Xhol-BamHI-digested XM6:βI globin.

The PA317 master cell bank was produced by transfecting PA317 cells with twice-cesium banded XM6:antisense plasmid DNA. Following transfection, the PA317 cells were split and then treated with G418 until individual clones could be identified and expanded. Each clone was then screened for its ability by analyzing its ability to transfer G418 resistance (since the retroviral vector contains a neomycin resistance gene). Vector supernatant derived from this master cell bank has been shown to be free of replication competent retroviruses by assay on PG4 S+ L− cells with and without amplification for 1 week on Mus Dunni fibroblasts.

High-titer vector for the multiple dosing protocol was collected from cells grown within the extracapillary space of a chamber in which cells grow on multiple filters (CellCo, Germantown, MD). Cells were initially placed in a 34°C incubator and allowed to adjust to this temperature for 48 h while the CellCo type B artificial capillary system was perfused with DME and 10% calf serum for 2 days in a humidified 34°C incubator with 5% CO2. Ten million cells are then trypsinized from plates or flasks and then inoculated into the extracapillary space of the CellCo cartridge in DME and 10% serum at 34°C. Medium was then changed daily, and lactate production and glucose consumption were measured daily. Once the lactate production or glucose consumption were over 60 mg/day, we collected supernatant as long as the lactate and glucose levels assured optimal vector production. The titers of the vector preparations was determined by quantifying the number of particles present that conferred G418 resistance to transduced MCF-7 cells, using appropriate dilutions.

MCF-7 Xenografts. Female nu/nu mice were housed in microisolator cages, fed only autoclaved food and water, and handled only with gloves. MCF-7 cells were cultured in Iscoves minimal essential medium with 5%
Nuclease protection assays were performed by our published methods (17, 18). RT-PCR was performed by our published methods (18) using the following basic method. RNA samples were reverse transcribed for 1 h at 37°C using 2 μg of total RNA, 1 μg random hexamers (Boehringer Mannheim), 1X first-strand buffer (GIBCO-BRL), 0.01 mM DTT, 0.5 mM each dATP, dCTP, dGTP, and dTTP, and 200 units Superscript II RNaseH-reverse transcriptase (GIBCO-BRL). The RNA:DNA duplexes were used as templates for 20-cycle PCR reactions using the following conditions: denaturation at 94°C, 20 s; annealing at 52°C, 45 s; and elongation at 75°C, 90 s. The following primers were used for RT-PCR studies: c-fos, 5'-AGCCCGCGCTCTCGTCTCCFCTGT-3' and 5'-TGCCCCFCCTGCCAATGCTCTG; MMTV, 5'-AAGGGAATGTGGGAGGTCAGTG-3' and 5'-GCAGGATAAGCCAAATGGGTAGTG-3' (this primer detects correctly terminated RNAs in the 3′ globin region of the antisense transcript); TGFα, 5'-CGTGGCCCTAGCCAAATGGGTAGTG-3' (this primer detects correctly terminated RNAs at the polyadenylation site). The expected product for the MMTV-regulated transcript. The primers and conditions for RT-PCR are presented in “Materials and Methods.”

RESULTS

Tissue Specificity of Antisense fos RNA. We first tested whether the MMTV-based XM6:antifos retroviral vector would be specifically expressed in mammary tissues when given systemically, since studies of the MMTV promoter in transgenic mice indicate breast-targeted expression in vivo (19–22). Two × 10⁸ vector particles were injected i.p. into nude mice over a 4-day period (four divided doses) and 12 different tissues from 12 animals were analyzed to determine the distribution of retroviral vector. Although the majority of these were negative for vector DNA by a sensitive PCR method, vector was detectable in the blood at 4 h, and a total of 8 tissues (not including blood samples) contained vector DNA 4 h after injection (8 of 144 specimens; 6%). Of these vector-positive samples, only the mammary gland sample expressed the antisense mRNA, as determined by RT-PCR (Fig. 1, compare Lane 9 to Lanes 1–8). The RT-PCR result from the mammary gland sample must represent transcribed RNA for two reasons: (a) treatment with RNase destroys the signal (Fig. 1, compare Lanes 9 and 10); and (b) the PCR band detects correctly terminated antisense mRNA at the polyadenylation site.

Ex Vivo Transduction of Breast Cancer Cells Inhibits Tumor Growth. Exponentially growing MCF-7 breast cancer cell monolayers were ex vivo transduced overnight with 10⁸ particles of either XM6:antisos (an MMTV-regulated antisense retroviral vector; Fig. 2A) or XM6:sensefos (a control sense vector differing only in the orientation of the c-fos fragment as described; Ref. 13) and then injected s.c. into estrogen-supplemented female nu/nu mice (23). Southern blot demonstrated that up to 70% of cells were transduced by this ex vivo transduction protocol. Although the animals injected with MCF-7 cells transduced with XM6:sensefos control vector developed highly invasive tumors within 2 weeks, none of the XM6:antisos transduced MCF-7 cells developed palpable tumors within 2 weeks. Tumors did eventually develop in 10 of 12 animals treated with the XM6:antisos vector but were smaller and less invasive (Tables 1 and 2). By RNase protection assays, both XM6:antisos and control vector (XM6:sense) produced MMTV-regulated transcripts, but only the XM6:antisos vector reduced c-fos mRNA levels (Fig. 2B). Histological examination of these tumors showed that the XM6:antisos transduced tumors were predominantly encapsulated, compared with control XM6:sensefos transduced tumors, which consistently invaded skeletal muscle or s.c. lymphatics (Fig. 2C). The XM6:antisos-transduced tumors appeared more differentiated by morphological criteria and stained with alcian blue, a marker for mammary differentiation (Fig. 2C).

In Vivo Transduction of Established Tumors Inhibits Tumor Growth and Gene Expression. We next tested the effect of XM6:antisos on established human breast tumors in an animal model. Previous studies had shown that vector DNA was undetectable in the blood more than 4 h after i.p. administration. Because of these data and the reported rapid inactivation of human retroviruses by circulating human complement (24, 25), we chose an i.p. tumor model in which the vector would be administered into the same body cavity. To document that the effect of XM6:antisos was on established tumors, we used magnetic resonance imaging to identify MCF-7 tumors 10 days after inoculation of 5 × 10⁶ tumor cells (Fig. 3A). The radiographic abnormalities were then confirmed by autopsy. All five randomly tested mice showed established peritoneal adenocarcinomas, ranging from 1–4 mm in diameter, 10 days after tumor cell inoculum (Fig. 3B).

We next studied the effect of a single injection of the antisense fos vector on MCF-7 tumors in estrogen-supplemented athymic mice. Ten days after inoculation of 5 × 10⁶ tumor cells, a single dose of 5 × 10⁸ particles of XM6:antisos or XM6:sensefos was administered i.p. At 3 weeks, there was an 80% reduction in tumor mass in the antisense-treated animals compared to those treated with the control vector (Fig. 4A). To assess tumor cell specificity and the molecular mechanism(s) of tumor inhibition, we analyzed DNA and RNA from tumor specimens and tumor-free peritoneum. There was no detectable gene transfer into peritoneal host cells (Fig. 4B, P1 and P2) whereas 25–40% of tumor cells contained control or experimental vector DNA (Fig. 4B, S1 through A2). By RT-PCR, levels of c-fos as well as the c-fos-
Fig. 2. Tumor formation by ex vivo-transduced MCF-7 cells. A, structure of the XM6:antifos retroviral vector. Small arrow, the direction of LTR transcription; larger arrow, the direction of MMTV-regulated transcription. B, nuclease protection assay measuring c-fos mRNA (Fos) and the MMTV transcript (MMTV) in peritoneum and in tumor samples. Probes were generated with T7 polymerase and hybridized with 1 µg of total mRNA. The human c-fos probe detects the 260-bp protected fragment shown (Fos); the human β globin probe detects the 220-bp fragment corresponding to exon 3, denoting correctly terminated antisense mRNA in the MMTV transcript (MMTV) by methods we have used previously (16). Endogenous c-fos mRNA is decreased in tumors from mice treated with XM6:antifos (AS1 and AS2) compared with control tumors (ST1 and ST2). Control is an RNA sample from peritoneal tissue (Un). C, histopathology of s.c. MCF-7 tumors after ex vivo transduction with XM6:antifos or control XM6:sensefos retroviral vector. Left panel, XM6:sensefos control-transduced tumor showing skeletal muscle invasion at low power (upper) and high power (lower); right panel, sparing of skeletal muscle by XM6:antifos-transduced tumor (low power) and alcian blue positivity in differentiating tumor cells (high power).

Dependent genes TGF-α and TGF-β1 were significantly lower in the antisense-treated tumors compared to the controls, whereas glyceraldehyde-3-phosphate dehydrogenase expression levels were the same in both groups (Fig. 4C).

To better assess the impact of antisense fos on tumor burden, we tested the effect of XM6:antifos on survival of tumor-bearing animals. Vector titer was increased by collecting it at 34°C using a CellMa Quad system. Two × 10^7 vectors (XM6:antifos or XM6:sensefos) in a volume of 0.5 ml were administered daily five times 10 days after inoculation of MCF-7 cells. By day 45, nine of nine mice treated with
ANTIFOS INHIBITS ESTABLISHED BREAST TUMORS

Table 1 Number of nude mice tumors from XM6:antifos and control-treated MCF-7 cells

<table>
<thead>
<tr>
<th>Retroviral vector</th>
<th>2 weeks</th>
<th>4 weeks</th>
<th>Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>XM6:sensefos (control)</td>
<td>10/12</td>
<td>12/12</td>
<td>11/12</td>
</tr>
<tr>
<td>XM6:antifos</td>
<td>0/12</td>
<td>10/12</td>
<td>2/12</td>
</tr>
<tr>
<td>LXSN control</td>
<td>12/12</td>
<td>12/12</td>
<td>10/12</td>
</tr>
</tbody>
</table>

* Microscopic analysis of invasion. Control tumors had vascular, perineural, and skeletal muscle invasion, while two of the antifos tumors showed only skeletal muscle invasion.

Table 2 Tumor volumes of XM6:antifos-treated mice

<table>
<thead>
<tr>
<th>Retroviral vector</th>
<th>Days postinoculum</th>
<th>Volume (mm³)</th>
<th>SE</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>XM6:sensefos</td>
<td>11</td>
<td>52</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>102</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>188</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>XM6:antifos</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0.0045 (0.0013)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>18</td>
<td>5</td>
<td>0.015 (0.0067)</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>30</td>
<td>7</td>
<td>0.0032 (0.0010)</td>
</tr>
</tbody>
</table>

* P was determined by t test and is listed as paired or independent (shown in parenthesis).

DISCUSSION

We have tested the tissue specificity and antitumor efficacy of XM6:antifos, a retroviral vector expressing an antisense RNA complementary to the c-fos oncogene, in a nude mouse model. When given systemically, only mouse mammary gland tissue expressed the antisense mRNA by RT-PCR (Fig. 1). By this same method, vector DNA was present in all blood samples 4 h after i.p. administration, suggesting that the result with mouse mammary tissue follows the systemic dissemination of transduction-capable vector via the blood stream. This mammary tissue selectivity could be explained by the MMTV-LTR promoter enhancer, which would activate the antisense fos only in MMTV-regulated breast cells, as supported by studies in transgenic mice overexpressing constructs under the control of the same promoter (19—22, 26).

In addition, XM6:antifos was able to transduce with high efficiency MCF-7 human breast cancer cells, both in tissue culture and in an established tumor in estrogen-primed nude mice. This resulted in down-regulation of the endogenous c-fos mRNA, reduction in tumorigenicity and invasiveness, detectable histological differentiation, elimination of established tumors in some cases, and marked prolongation of survival of tumor-bearing animals in the absence of any detectable clinical toxicity.

A single injection of $5 \times 10^6$ retroviral particles inhibited established tumors while transducing 25—40% of cells within the tumor specimens. This transduction rate might be surprisingly high if we consider that $5 \times 10^6$ MCF-7 cells had been injected previously. However, it is conceivable that many of the injected cells do not survive and, hence, the virus:tumor cell ratio is much closer to 1:1 at the time of vector administration. A second possibility (not exclusive of the former) is that endogenous c-fos signaling is critical for estrogen-stimulated autocrine/paracrine growth effects early in breast cancer progression and that its disruption affects transduced and adjacent nontransduced cells. This is supported by multiple reports of estrogenic induction of c-fos and c-jun in vivo as well as the presence of estrogen response elements in the c-fos gene (2—5, 27, 28). In addition,

the control vector had died secondary to tumor burden (renal obstruction, bowel obstruction, and extrahepatic obstruction), whereas all nine animals in the antisense-treated cohort were alive and well (Fig. 5A). One of nine animals in the latter group remained tumor-free 200 days after MCF-7 cell inoculation. Other than intraabdominal tumor, there were no histological abnormalities or tumor cell metastases in any of the antisense-treated animals. Consistent with the marked inhibition of tumor growth, 40—70% of tumor cells contained XM6:antifos DNA (Fig. 5B). Of note, there was a low level of vector transduction into mouse peritoneum but in the absence of antisense fos RNA expression (Fig. 5B and data not shown).

**Fig. 3.** Demonstration that xenografts became established prior to vector therapy. A, magnetic resonance imaging of a nude mouse 10 days after MCF-7 cell injection using a 4.7 Tesla magnet used to produce T2 weighted images. Arrow with the filled head, a ventral peritoneal implant corresponding to the histology in B; arrow with the open head, tumor in the porta hepatis. B, histology of tumor showing invasion of subperitoneal fat by tumor.
Fig. 4. Single-dose vector treatment. A, mass of tumor present 3 weeks after injection of tumor cells and 2 weeks after injection of a single i.p. dose of vector. Data show the means (n = 8); bars, SE. B, Southern blot of EcoR'I-digested DNAs from transduced mouse tumors probed with a human B globin probe. Probe was a 900-bp fragment from human B-globin gene that is present in vectors and human cells; EcoR'I digests produced the following expected sizes: 2.8 kb for XM6:sensefos and XM6:antifos with a human genomic band of 5.0. The percentage transduction was calculated by quantitating hybridization with the phosphorimager and then comparing hybridization of the presumed haploid vector lower band to that of the diploid globin upper band (percent transduction = 2 x vector signal/globin signal). Mouse globin would not be detected with this probe. Samples DNAs: Pro is the XM6:antifos PA317 producer cell line; all other samples are from single-dose treatment (left samples, P1-A2, Lanes 2–7). P1 and P2 are samples taken from the peritoneum of animals treated with XM6:sensefos; S1 and S2 are samples from XM6:sensefos-transduced tumors. A1 and A2 are samples from XM6:antifos-transduced tumors. C, RT-PCR analysis of RNAs obtained from tumors transduced with XM6:sensefos (S1, S2, and S3) or XM6:antifos (A1, A2, and A3) for TGF-B, TGF-3, c-fos, MMTV transcript, or glyceraldehyde-3-phosphate dehydrogenase (GADP) expression. The primers and conditions for RT-PCR are presented in “Materials and Methods,” and the expected size products are: c-fos, 150 bp; TGF-a, 190 bp; TGF-B, 229 bp; MMTV transcript, 286 bp; and GADP, 340 bp. PCR was performed on RNA samples following reverse transcription and on samples without adding reverse transcriptase as a control for DNA contamination. Lane 1, from an untransduced MCF-7 tumor. Even Lanes 2–12 show RT-PCR from samples 51, 52, 53, AS1, AS2, and AS3, respectively, while odd Lanes 3–11 are control samples showing PCR results without RT.

Fig. 5. Multiple dose vector treatment. A, survival curve following treatment of established i.p. tumors treated with XM6:sense fos (sense) or XM6:antifos (antifos) vectors. B, Southern blot of EcoR'I-digested DNAs from transduced mouse tumors probed with a human B globin probe. Probes, expected bands, and quantitation as described in the legend to Fig. 4B. Samples P1-A2, Lanes 1–6. P1 and P2 are samples taken from the peritoneum of animals treated with XM6:sensefos; S1 and S2 are samples from XM6:sensefos-transduced tumors. A1 and A2 are samples from XM6:antifos-transduced tumors.
vector-mediated down-regulation of c-fos was associated with down-regulation of the fos-dependent TGF-α (29) and TGF-β1 genes (16). These encode important autocrine/paracrine growth-regulatory molecules for breast carcinoma cells. TGF-α is markedly induced by estradiol in hormone-responsive MCF-7 cells (1, 6). Stable expression of an antisense TGF-α mRNA in breast cancer cells abrogates estrogen-induced proliferation (30, 31), supporting a critical role for TGF-α in the hormonal control of mammary tumorigenesis. On the other hand, overexpression of TGF-β1 in breast cancer cells can accelerate their tumorigenicity (32), whereas blockade of TGF-β1 with neutralizing antibodies prevents breast tumor formation in nude mice (7). These data support the notion that tumor cell TGF-β1 might be critical for the progression of some breast cancer cells.

More prolonged administrations of XM6:antisios increased tumor cell transduction rate and markedly lengthened the survival of tumor-bearing animals. The absence of host tissue toxicity from antisense fos therapy was shown with possible higher doses and/or prolonged infusions to enhance the therapeutic window of XM6:antisios. Obviously, this also raises the possibility of inappropriate expression in host tissues in which fos might be physiologically important. However, we reported recently that despite genomic integration of XM6: antisios in mouse kidney, the antisense fos was not expressed, and clinical nephrotoxicity was absent (8). In the present study, similar findings were encountered with mouse peritoneal specimens (Fig. 5B and data not shown).

Further experimental work is needed to assess how critical the down-regulation of c-fos target genes is for the observed antitumor effect. To determine whether other fos-associated invasion genes (33) are potentially involved will also require additional research. Despite these unanswered questions, these data indicate that fos might be a reasonable therapeutic target in some human breast cancer cases. Based on these results, a Phase I study of XM6:antisios in which patients with metastatic breast cancer receive intrapleural or i.p. vector injections is currently in progress.

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