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 and invasiveness. In vivo transduction of established i.p. MCF-7 tumors induced expression of antifos RNA, decreased expression of the c-fos target mRNA, induction of differentiation, and inhibition of s.c. tumor growth and invasiveness. 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 and invasiveness. In vivo transduction of established i.p. MCF-7 tumors increased expression of antifos RNA, decreased expression of the c-fos target mRNA, induction of differentiation, and inhibition of s.c. tumor growth and invasiveness. In vivo transduction of established i.p. MCF-7 tumors increased expression of antifos RNA, decreased expression of the c-fos target mRNA, induction of differentiation, and inhibition of s.c. tumor growth and invasiveness. In vivo transduction of established i.p. MCF-7 tumors increased expression of antifos RNA, decreased expression of the c-fos target mRNA, induction of differentiation, and inhibition of s.c. tumor growth and invasiveness.

INTRODUCTION

Retroviruses have evolved as effective agents that can introduce DNA into specific cells and use molecular mimicry to cause cancer in animals. The MMTV 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 antisense vector could lead to disruption of paracrine factors and an antitumor effect, providing a strategy for cancer gene therapy.

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 pBlII β globin was cloned to XhoI-digested XM6, and we selected for the orientation that retained the 3’ XhoI site; and (c) the XhoI-BamHI fragment from our MMTV antisense plasmid (13) was cloned to Xhol-BamHI-digested XM6:β globin.

The PA317 master cell bank was produced by transfecting PA317 cells with transgenic and 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.

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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.

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Tissue Specificity of Antisense fos RNA. We first tested whether the MMTV-based XM6:antisfos 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 Lanes 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 10⁶ particles of either XM6:antisfos or XM6:sensefos (Fig. 2A). The XM6:antisfos transduced tumors, which consistently invaded skeletal muscle or s.c. lymphatics (Fig. 2C). The XM6:antisfos-transduced tumors appeared more differentiated by morphological criteria and stained with alcian blue, a marker for mammary differentiation (Fig. 2C).

RESULTS

In Vivo Transduction of Established Tumors Inhibits Tumor Growth and Gene Expression. We next tested the effect of XM6:antisfos 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:antisfos 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 × 10⁶ particles of XM6:antisfos 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 RNA were detected in both the experimental and control tumors, whereas the antisense RNA was only found in the experimental tumors (Fig. 4).

[Image of RT-PCR of mouse tissue RNAs 4 h after i.p. injection of XM6:antisfos vector. Lane 1, RNA from transuntranscribed MCF-7 cells; Lane 2, RNA from untransuntranscribed MCF-7 tumors. Lanes 3–8, from tissues that contain detectable vector DNA by PCR. Lane 3, pancreas; Lane 4, spleen; Lane 5, liver; Lane 6, ovary; Lane 7, lung; Lane 8, brain; Lane 9, mammary tissue; Lane 10, PCR of RNA sample without prior reverse transcription as a control for DNA contamination. The upper band is the 340 bp expected product for glyceraldehyde-3-phosphate dehydrogenase (GAPD), and the lower band is the 286-bp expected product for the MMTV-regulated transcript. The primers and conditions for RT-PCR are presented in "Materials and Methods."]

4 Unpublished data.
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 × 10⁷ 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
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).

**Table 2 Tumor volumes of XM6:antifos-treated mice**

Tumors were measured with calipers to determine volume based on the formula $V = \frac{1}{2} \times L \times W^2$. Each data point represents the mean of eight.

<table>
<thead>
<tr>
<th>Retroviral vector</th>
<th>Days postinoculum</th>
<th>Volume (mm$^3$)</th>
<th>SE</th>
<th>$P^a$</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>

$^a$ $P$ 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 $5 \times 10^5$ 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 on 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,
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 EcoRI-digested DNAs from transduced mouse tumors probed with a human β globin probe. Probe was a 900-bp fragment from human β-globin gene that is present in vectors and human cells; EcoRI 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 phosphoimager and then comparing hybridization of the presumed haploid vector lower band to that of the diploid globin upper band (percent transduction = 2 × 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-β1, TGF-α, 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-α, 190 bp; TGF-β1, 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 EcoRI-digested DNAs from transduced mouse tumors probed with a human β 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:antifos increased tumor cell transduction rate and markedly lengthened the survival of tumor-bearing animals. The absence of host tissue toxicity from antisense fos might be physiologically important. However, we reported recently that despite genomic integration of XM6:antifos in mouse kidney, the antisense fos was not expressed, and clinical nephrotoxicity was absent.5 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 carcinomas. Based on these results, a Phase I study of XM6:antifos in which patients with metastatic breast cancer receive intrapleural or i.p. vector injections is currently in progress.

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