Antitumor Activity of an Oncolytic Adenoviral-CD40 Ligand (CD154) Transgene Construct in Human Breast Cancer Cells

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Abstract

Purpose: CD40 ligand (CD40L, CD154) plays a central role in immunoregulation and also directly modulates epithelial cell growth and differentiation. We previously showed that the CD40 receptor is commonly expressed in primary breast cancer tissues. In this proof-of-principle study, we examined the breast cancer growth–regulatory activities of an oncolytic adenoviral construct carrying the CD40L transgene (AdEHCD40L).

Experimental Design: In vitro and in vivo evaluations were carried out on AdEHCD40L to validate selective viral replication and CD40L transgene activity in hypoxia inducing factor-1α and estrogen receptor–expressing human breast cancer cells.

Results: AdEHCD40L inhibited the in vitro growth of CD40+ human breast cancer lines (T-47D, MDA-MB-231, and BT-20) by up to 80% at a low multiplicity of infection of 1. Incorporation of the CD40L transgene reduced the effective dose needed to achieve 50% growth inhibition (ED50) by ~10-fold. In contrast, viral and transgene expression of AdEHCD40L, as well its cytotoxicity, was markedly attenuated in nonmalignant cells. Intratumoral injections with AdEHCD40L reduced preexisting MDA-MB-231 xenograft growth in severe combined immunodeficient mice by >99% and was significantly more effective (P < 0.003) than parental virus AdEH (69%) or the recombinant CD40L protein (49%). This enhanced antitumor activity correlated with cell cycle blockade and increased apoptosis in AdEHCD40L-infected tumor cells.

Conclusions: These novel findings, together with the previously known immune-activating features of CD40L, support the potential applicability of AdEHCD40L for experimental treatment of human breast cancer.

Breast cancer is a major health problem worldwide. An estimated 40,480 women are expected to die from breast cancer in the United States in 2008 (1). The outcome for patients with metastatic disease has improved significantly but these patients remain incurable (2).

Cancer-selective biotherapy or gene therapy approaches have been considered to be the next horizon toward developing a cure for breast cancer. Small targeted molecules in an adjuvant setting have shown to extend relapse-free survival of patients with early-stage disease (3). However, therapeutic options for advanced, metastatic malignancies remain limited (4). Adenoviruses have been used as delivery platforms to deliver cytotoxic agents to cancers of the head and neck, multiple myeloma, ovarian, prostate, and breast (5). Breast cancer primary tumors express high levels of the Coxackie virus and adenovirus receptor, the primary cell surface receptor for adenovirus attachment during the viral infection process (6). Coxackie virus and adenovirus receptor expression has been shown to increase with tumor grade (7), supporting the strategy of adenoviral-based gene transfer for experimental therapy of human breast cancer. The capacity of the adenoviral early gene E1A to down-regulate Her-2/neu activity has led to phase I clinical trial for breast cancers in this regard (8). Adenoviral delivery of p53 or other novel tumor-suppressive transgenes (9, 10) has been shown to be safe and potentially applicable for the treatment of breast cancer patients with advanced disease (5).

CD40L (CD154) is a member of the tumor necrosis factor superfamily (TNFSF5) with multiple antitumor activities. CD40L is best known for its immunopotentiating repertoire, spanning from B-cell and dendritic cell activation (11) to the heightening of immune rejection against epithelial tumors (12). In addition, we and others (13) have shown that CD40L (CD40 ligand, also known as CD154), when given in the form of a trimeric recombinant protein (rCD40L), directly inhibited the
Translational Relevance

Breast cancer is a major health problem worldwide. Patients with metastatic disease remain incurable. Cancer-selective biotherapy or gene therapy has been considered to be the next horizon toward developing a cure for breast cancer. In particular, adenoviral delivery of p53 or other novel tumor-suppressive transgenes has been shown to be safe and potentially applicable for the treatment of breast cancer patients with advanced disease. In this article, we have characterized the antitumor properties of a novel, conditional replicative, oncolytic adenoviral construct (AdEHCD40L) carrying the multifunctional molecule CD40L in the form of an integrated transgene. CD40L has been shown by us and others to exhibit direct tumor growth-modulatory activities in epithelial cancers. In the current study, we have shown selective oncolytic and CD40L transgene activities of AdEHCD40L in hypoxia inducing factor (HIF)-1α–expressing human breast cancer cells. The direct tumor growth–inhibitory efficacy of AdEHCD40L was validated in vivo, where intratumoral injections led to >99% reduction in preexisting MDA-MB-231 xenograft growth in severe combined immunodeficient mice. AdEHCD40L was significantly more effective than the parental virus or the recombinant CD40L protein alone. These unique findings, together with the previously known immune-activating features of CD40L, support the applicability of AdEHCD40L for experimental treatment of human breast cancer.

growth of human breast cancer cells through its binding to the surface receptor CD40 (11). The direct cancer growth–inhibitory activity of CD40 ligation has been confirmed in multiple human solid cancer cell types, including bladder, ovarian, cervical, as well as breast carcinomas (11, 13). This feature is consistent with the previously described function of the CD40L-CD40 circuitry in modulating the growth and differentiation of normal, proliferative, and basal epithelial cells (14).

We previously showed that >90% of primary breast cancers of various histologic types expressed the CD40 receptor (13). In contrast, most normal, nonproliferating epithelial tissues lack CD40 expression. The attractiveness of CD40-directed targeted therapy, however, may be hampered by the common expression of CD40 on nonepithelial tissues and immune cell types, thereby presenting the possible risk of systemic, proinflammatory, and/or autoimmune activation (11). A potential solution is to use a delivery platform that limits CD40L expression to within the tumor microenvironment.

The existence of a hypoxic microenvironment within the tumor lesion is a common feature of many solid tumors, including breast cancer (15). Up to 40% of breast cancers have been shown to be hypoxic, with a median oxygen concentration that is well below that of normal breast tissues (16). The recent studies by Hernandez-Alcocea and coworkers showed that incorporation of the hypoxia responsive element (HRE) and the estrogen response element (ERE) to regulate early viral gene expression was highly effective in limiting viral replication in human breast cancer cells (17, 18). As illustrated in a proof-of-principle study with the nonreplicating adenoviral construct AdEHhrk, hypoxic-dependent expression of the proapoptotic hara-kiri (19) transgene can be attained in breast cancer cells of the appropriate phenotype, resulting in tumor-selective, transgene-mediated apoptosis.

Accordingly, we have generated a novel conditional replicative oncolytic adenoviral construct (AdEHCD40L) that uses the ERE/HRE hybrid promoter in regulating the expression of the CD40L transgene (18). The premise that human breast cancer cells commonly express the estrogen receptor and HIF-1α (18, 19) and are expected to provide a permissive environment for AdEH-based constructs were validated with multiple human breast cancer cell lines. We have examined the in vitro applicability of AdEHCD40L in terms of conditional CD40L expression and concomitant enhancement of antitumor activity in CD40+ breast cancer cells. The direct growth-inhibitory activity of AdEHCD40L was validated in vivo using human breast cancer xenografts that were maintained in severe combined immunodeficient (SCID) mice lacking an intact immune repertoire. These novel findings support the potential applicability of AdEHCD40L for experimental treatment of human breast cancer.

Materials and Methods

Cell lines. The human breast carcinoma (T-47D, BT-20, MDA-MB-231, and ZR-75-1), nonmalignant lung fibroblast (IMR-90), and breast epithelial (MCF-10A) cell lines were obtained from the American Type Culture Collection and maintained according to American Type Culture Collection recommendations. For in vitro studies, cells were grown in phenol red–free RPMI 1640 (Invitrogen) to reduce estrogen content, which is present in phenol red. Charcoal dextran–treated fetal bovine serum (2% v/v; Hyclone) was used during culture to limit up-regulation of E2F-1 (18). All viral infections of cell lines were done at a multiplicity of infection (MOI) of 1 plaque-forming unit (pfu) per cell unless stated otherwise.

Construction of the CD40L carrying conditional replicative adenovirus. The conditional replicative AdEHCD40L adenovirus was constructed according to previously defined cloning strategies (18) and incorporates three key promoter and backbone sequence modifications: (a) substitution of the HRE (SXEH3) promoter and ERE promoter for viral EIA promoter; (b) replacement of the endogenous E4 promoter by an E2F-1 promoter; and (c) deletion of viral E3 19K/6.7K genes (28,555 and 293,555 bp of the adenoviral type 5 sequence) and substitution with the CD40L complementary DNA containing propidium iodide-SceI flanking sequences (Fig. 1A). These modifications place the expression of late viral genes and the CD40L transgene under the control of the endogenous adenoviral late gene promoter, which is activated by the conditional expression of early viral genes during the viral replicative process (20).

Hypoxia induction and immunoblot analysis for HIF-1α and E2F-1. The administration of CoCl2, a hypoxic mimetic agent (21), is commonly used to sustain a hypoxic state in vitro through prolongation of the half-life of transcription factor HIF-1α (22). Hence, treatment with CoCl2 was used to sustain and/or optimize infectivity of the AdEH constructs (AdEHCD40L and AdEHNull) in vitro. The lowest effective concentration of CoCl2 (25 μmol/L, 24 h) produced <15% cytotoxicity in all cell lines tested. HIF-1α expression was determined by immunoblot analysis as previously described (13). Briefly, total cell protein was extracted and quantified. Membranes were treated with blocking buffer, incubated for 1 h with a mouse anti-human E2F-1 or HIF-1α (both from BD PharMingen) reactive primary antibody, followed by incubation with a goat anti-mouse IgG horseradish
positive controls for CD40 and CD40L expression, respectively. Band intensities after normalization to β-actin were designated as follows: <0.1, + (0.1–<0.2), ++ (0.2–<0.5), +++ (0.5–<0.8), or ++++ (≥0.8 absorbance units). This is in reference to the HeLa-negative control cells that gave normalized values of <0.1 and HeLa-positive control cells maintained in CoCl2 (23, 24), which gave a maximal normalized value of >0.8. Cell lines were classified as constitutive when endogenous HIF-1α was detected (≥+) under normoxic conditions and inducible when HIF-1α became detectable or up-regulated after CoCl2 treatment.

Flow cytometric analysis of CD40 and CD40L expression. Endogenous expression of the CD40 receptor and endogenous CD40L was determined by flow cytometric immunophenotyping (13) using a phycoerythrin-conjugated mouse anti-human CD40L antibody (BD PharMingen), mouse anti-human CD40 antibody (Beckman Coulter), or an isotypic antibody control (IgG1-PE; BD PharMingen). Similar analyses were done on virus-infected cultures (5 × 10⁴ cells per well) that were maintained with or without CoCl2 (25 μmol/L). Daudi and CD40L transfected mouse fibroblast L cells (13) were used as positive controls for CD40 and CD40L expression, respectively.

Viability assays. Breast cancer cells at logarithmic growth phase were infected with either AdEHCD40L or control vectors (AdEHNull or Adv-WT) in the presence or absence of hypoxia-inducing CoCl2 (25 μmol/L). Mitochondrial metabolism was quantified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (R&D Systems) as per manufacturer’s protocol. Viability was calculated as a function of absorbance readings and expressed as percent activity of the untreated culture. Propidium iodide–stained cells were analyzed by flow cytometry and used to confirm viral treatment outcome by the MTT assay.

Determinations of viral cytopathic effects on nonmalignant cells were determined by crystal violet staining in view of low baseline readouts by the MTT assay from low metabolic activity of the target cells. Dye uptake was quantified spectrophotometrically (540 nm) and compared against a concomitantly generated standard curve.

Viral replication by quantitative PCR. Total DNA was extracted from infected culture supernatant fluids collected over time (QiAmp DNA blood kit, Qiagen) to determine the viral replicative activity. Real-time quantitative PCR (qPCR) were performed according to Gu et al. (25) using Taq polymerase and the TaqMan Universal PCR Master Mix (Applied Biosystems) and sense (5′-GCTGGCGCAGAAGTATTCCA-3′) and antisense (5′-GTCCGGCTCTCAGTGATTTCC-3′) primers (Sigma-Aldrich). Viral copy number was determined by a standard curve generated with a serially diluted adenovirus reference standard (American Type Culture Collection). The minimal detectable limit was 50 viral copies per reaction.

Apoptotic analysis. The frequency of apoptotic cells was determined by flow cytometric analysis according to Annexin V (ANN V) binding per manufacturer’s protocol (BD PharMingen; ref. 13). Apoptotic activity was determined as a function of binding to the fluorescein probe (Becton Dickinson, FACScan) and by CELLQuest software analysis (Becton Dickinson). Fas antibody–treated T-47D cells were used as positive control for apoptotic determinations.

Cell cycle analysis. Cell cycle distribution analysis was done after propidium iodide staining of cell nuclei, as described previously (26). Cell cycle distribution was analyzed with the Verity cell cycle analysis software (Verity, Inc.) with tamoxifen-treated cells as a positive control.

![Schematic of the AdEHCD40L and cytotoxicity of CD40L transgene in human breast cancer cells. A, cloning strategy of the AdEHCD40L construct. The viral E1A promotor was deleted and substituted with a hybrid promotor containing the ERE and the HRE. The gpt19K/6.7K E3 region of the adenoviral genome was deleted and replaced by an 800-bp CD40L transgene cassette. This allows for the transcripational regulation of the CD40L transgene by the endogenous late promotor, which, in turn, is governed by the early expression of viral early genes. The wild type E4 promotor region has been substituted by an E2F-1 promotor to reduce viral replication in normal cells. B and C, enhanced growth-inhibitory activity of AdEHCD40L. The growth-inhibitory effect of AdEHCD40L (●) was compared with that by AdEHNull (○; MOI = 1 pfu/cell) by MTT assay in the CD40+ cell line T-47D (B) and the CD40- cell line ZR-75-1 (C) over 144 h. Values were normalized and expressed as percent of untreated culture. Points, mean (n = 4); bars, SE. *, P < 0.05. D, relative cytotoxicity of AdEHCD40L, AdEHNull, and Adv-WT. Cytotoxicity to viral infected T-47D cells (MOI = 1 pfu/cell) was quantified as a function of vital dye (propidium iodide, PI) incorporation by flow cytometric analysis under viral permissive conditions. Values are average cell counts from three separate experiments.](www.aacrjournals.org)
In vivo activity of AdEHCD40L. AdEHCD40L activity against preexisting tumors was evaluated in a s.c. xenograft model. Xenograft induction was carried out by injecting $3 \times 10^7$ MDA-MB-231 breast cancer cells in Matrigel (1:2; v/v) into the left flank of CB17/IcrHsd SCID mice. Each mouse received five daily intratumoral injections of medium only, the AdEHNull or AdEHNull virus ($6 \times 10^8$ pfu in 100 μL), or rCD40L (preoptimized concentration of 1 μg/mL; ref. 13; Alexis Biochemicals) when tumors reached a size of >4 mm in diameter. Tumor diameter measurements were carried out twice daily, and mean tumor diameter was determined by the geometric mean of two perpendicular measurements. The animals were observed until the mean tumor diameter of the mock-treated group exceeded 12 mm, at which time all animals were sacrificed. Necropsy was done and primary tumors from individual mice were excised and weighed. Histopathologic evaluation was carried out by a trained medical pathologist in a blinded fashion to determine the frequency and cytologic features of tumor-infiltrating leukocytes in H&E stained, cryopreserved tumor biopsy sections. Immunohistochemical analysis of HIF-1α expression was determined with tumor xenografts that were excised at the end of the observation period by an automated biotin-avidin immunohistochemical staining technique after incubation with the HIF-1α primary antibody (clone ESE1122, Novus Biologicals; 8 μg/mL) or an isotypic control (BD PhatMingen; ref. 9). The percent increase in tumor size at day x was determined for each individual tumor bearing mouse by comparing the mean tumor diameter ($T_x$) to that before first treatment injection ($T_0$) using the formula: $\left\{\left(\frac{T_x}{T_0}\right)^{1.00}\right\} \times 100\%$. Growth inhibition was calculated by determining the reduced increase in tumor size after normalization to the mock-treated group. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Baylor Research Institute.

Results

Generation of AdEHCD40L, a CD40L gene therapy oncolytic vector. The novel AdEHCD40L is constructed from the second-generation AdEH oncolytic adenovirus platform (18) and carries the entire 261-amino-acid CD40L in the viral E3A region (Fig. 1A). These modifications place the late viral genes, as well as the CD40L transgene, under the control of the endogenous adenoviral late gene promoter. The late gene promoter in turn is activated by the conditional expression of early viral genes under the control of the hybrid promoter containing the HRE and the ERE (Fig. 1A). Validation studies by us and others (18, 19) indicated that HIF-1α was markedly more effective in modulating AdEH viral gene expression than estrogen (data not shown). AdEHNull, a similar construct without the CD40L transgene, was created as a reference control for delineating CD40L transgene activity. The modified E1A, E3, and E4 region primary sequences, as well as the wild-type CD40L cDNA transgene sequence, were verified by DNA cycle sequencing reactions.

Phenotypic validation of human breast cancer models. We previously showed that >90% of primary breast cancer tumors expressed the CD40 receptor (13) and hence are susceptible to CD40L-mediated growth modulation. The antitumor activity of AdEHCD40L was examined in breast cancer lines that expressed a high frequency (>95%) of CD40+ cells (T-47D, BT-20, and MDA-MB-231) and the ZR-75-1 cell line that lacked CD40 expression (<1% CD40+ cells; Table 1). All breast cancer lines contained <5% of CD40L-positive cells, although significant levels of CD40L expression was observed on the nonmalignant MCF-10A mammary epithelial line and the IMR-90 lung fibroblast line (Table 1).

Of the four breast cancer lines tested, CD40+ T-47D, MDA-MB-231, and CD40- ZR-75-1 constitutively expressed endogenous HIF-1α (Table 1), whereas the CD40+ BT-20 cells did not. However, all these breast cancer lines, as well as the nonmalignant lines MCF-10A and IMR-90, displayed up-regulated HIF-1α expression through in vitro induction of the hypoxic state (CoCl2, 25 μmol/L), thereby serving as appropriate, constitutive, and inducible models for viral replication and transgene expression.

Hypoxic regulation of viral E1A and CD40L expression. Evaluations with wild-type adenovirus (Adv-WT)–infected cultures showed that all of the breast cancer cell lines tested were

### Table 1. Phenotype of human cell lines tested

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>CD40 %</th>
<th>CD40L %</th>
<th>HIF-1α % at 48 h</th>
<th>E1A at 48 h</th>
<th>CD40L at 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
<td>Expression status</td>
<td>Normoxia Hypoxia</td>
<td>Normoxia Hypoxia</td>
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<tr>
<td>Cancer lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-47D</td>
<td>97%</td>
<td>&lt;2%</td>
<td>+++</td>
<td>29.4 ± 7.8</td>
<td>38.5 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+++</td>
<td>47.1 ± 8.6</td>
<td>65.5 ± 2.3</td>
</tr>
<tr>
<td>BT-20</td>
<td>97.90%</td>
<td>&lt;1%</td>
<td>-</td>
<td>8.9 ± 1.8</td>
<td>10.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>33.6 ± 14.4</td>
<td>43.2 ± 8.6</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>93.80%</td>
<td>&lt;1%</td>
<td>+++</td>
<td>74.0 ± 8.9</td>
<td>69.4 ± 3.9</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+++</td>
<td>73.7 ± 8.6</td>
<td>65.5 ± 3.9</td>
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<tr>
<td>ZR-75-1</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
<td>+++</td>
<td>23.4 ± 8.1</td>
<td>50.2 ± 2.5</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+++</td>
<td>34.5 ± 1.2</td>
<td>74.6 ± 1.5</td>
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<tr>
<td>Nonmalignant lines</td>
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<td></td>
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<tr>
<td>MCF-10A (mammary epithelial)</td>
<td>14.3 ± 9.6</td>
<td>44.90%</td>
<td>-</td>
<td>13.2 ± 0.5</td>
<td>1.9 ± 1.5</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>72.9 ± 0.8</td>
<td>50.3 ± 2.1</td>
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<tr>
<td>IMR-90 (lung fibroblast)</td>
<td>5.0 ± 1.1</td>
<td>12.0 ± 12.8</td>
<td>+++</td>
<td>1.5 ± 0.5</td>
<td>3.6 ± 1.6</td>
</tr>
</tbody>
</table>

*Values are mean ± SD.

† CD40 and CD40L expression was determined by direct immunofluorescence and flow cytometric analysis.

‡ HIF-1α expression was determined by immunoblot analysis. Band intensities were normalized to -actin and designated as - (<0.1), + (0.1-<0.2), ++ (0.2-<0.5), +++ (0.5-<0.8), or ++++ (>0.8). Cells lines were classified as constitutive when endogenous HIF-1α was detected under normoxic condition and inducible when HIF-1α becomes detectable or up-regulated following CoCl2 treatment.
susceptible to adenoviral infection, as determined by viral E1A expression. At a MOI of 1 pfu/cell, E1A expression ranged from 44.7 ± 7.7% for T-47D, 92.0 ± 0.4% for MDA-MB-231, 38.8 ± 4.9% for ZR-75-1, and 23.9 ± 12.5% for BT-20 (mean ± SD). For breast cancer cell lines that constitutively expressed HIF-1α (T-47D, MDZ-MB-231, and ZR-75-1), 24% to 74% were viral E1A positive after AdEHCD40L infection (MOI of 1), compared with <10% of the HIF-1α–negative BT-20 cells. Hypoxic induction significantly enhanced AdEHCD40L infectivity in T-47D, ZR-75-1, and BT-20 cells, as manifested by elevated E1A expression (Table 1). However, this phenomenon was not observed in MDA-MB-231 cells, likely due to its high endogenous HIF-1α expression and its susceptibility to adenoviral infection at normoxia.

There was general concordance in the frequency of E1A and CD40L expression after AdEHCD40L infection at normoxic and hypoxic states (P = 0.04). Transgene expression increased with time and was detected for up to 4 days posttreatment (data not shown). Our data with these constitutive (MDA-MB-231, T-47D, and ZR-75-1) and inducible (BT-20, T-47D, and ZR-75-1) lines serve as proof-of-principle findings regarding the dependence of viral and transgene expression on HIF-1α status in human breast cancer cells.

**Cytotoxic activity of AdEHCD40L.** The direct growth-inhibitory activity of AdEHCD40L was assessed as a function of metabolic activity by the MTT assay using CD40+ T-47D and CD40− ZR-75-1 cells. Significantly decreased T-47D viability was observed as early as 48 hours (32.2 ± 3.5%; Fig. 1B) after AdEHCD40L infection (MOI of 1 pfu/cell) and was evident through the observation period of 144 hours, when T-47D viability was reduced by 80.5 ± 1.1% compared with untreated culture (Fig. 1B). This cytotoxic effect was significantly more profound than the parental virus (AdEHNull; 48.7 ± 8.8% at 144 hours, P < 0.05). Enhanced cytotoxicity of AdEHCD40L was confirmed after vital dye staining with propidium iodide (Fig. 1D). Cell viability in T-47D viral permissive breast cancer cell lines was reduced by 82.3% in T-47D cells at 144 hours (Fig. 1D) compared with a reduction of 34.7% in AdEHNull-infected cultures.

Augmented cytotoxic activity by AdEHCD40L was similarly observed in CD40+ MDA-MB-231, and BT-20 cells under viral permissive conditions. At 96 hours postinfection, viability of AdEHCD40L-infected MDA-MB-231 cells was reduced by 49.1 ± 7.5% compared with 28.1 ± 4.6% in AdEHNull-infected cultures (P < 0.05). BT-20 viability was decreased by 41.5 ± 3.3% compared with 22.3 ± 3.3% in AdEHNull-infected cells (P < 0.05). In contrast, no apparent cytotoxic advantage was observed with the CD40-negative ZR-75-1 cells by AdEHCD40L (Fig. 1C).

To better quantify the effect of CD40L expression on breast cancer cell survival, ED₅₀ or the viral dose needed to achieve 50% loss in viability, was determined under viral permissive conditions. The ED₅₀ for AdEHCD40L was ~7- to 11-fold lower than that of AdEHNull in all CD40+ lines evaluated (Table 2). These findings indicate that incorporation of the CD40L transgene augments breast cancer cell cytotoxicity by AdEH under viral permissive conditions.

**Attenuated AdEHCD40L activity in nonmalignant lines.** Promoter modifications in the viral E1A (HRE) and E4 (E2F-1) regions are intended to limit viral gene and CD40L transgene expression to malignant cells expressing HIF-1α and exhibiting a high E2F-1 content. The selectivity of viral gene expression and cellular cytotoxicity of AdEHCD40L was further examined with the nonmalignant mammary epithelial line, MCF-10A. Although MCF-10A was susceptible to Adv-WT infection (E1A expression of 61.8 ± 4.0%, compared with 49.4 ± 4.5% of T-47D cells), these cells exhibited markedly reduced viral gene expression after AdEHCD40L treatment (<15%, compared with 44.7 ± 7.7% for T47D). AdEHCD40L cytotoxicity to MCF-10A was correspondingly reduced (25 ± 19.4%) compared with Adv-WT (65.8 ± 10.2%; Fig. 2A) according to crystal violet viability analyses. The observed moderate level of toxicity may be attributable to E2F-1 expression in MCF-10A cells when maintained in culture.

Manifestations of viral cytotoxicity was also examined in the normal lung fibroblast line IMR-90, as lung tissues are commonly affected by breast cancer metastasis and are highly susceptible to adenoviral infection. Similarly attenuated cytotoxicity by AdEHCD40L and AdEHNull was observed compared with Adv-WT (Fig. 2A and B). Viral yield in AdEHCD40L-infected IMR-90 cultures were reduced by ~100-fold compared with Adv-wt infection (2.5 × 10⁶ and 3.6 × 10⁵ vp, respectively). Hence, the integration of the CD40L transgene potentiated breast cancer cytotoxic activity without altering the attenuated features of the AdEH viral construct in nonmalignant cell types.

**Antitumor activity of AdEHCD40L in vivo.** To focus on the direct tumor growth–inhibitory (as opposed to immune-activating) features of AdEHCD40L, in vivo analyses were carried out with breast cancer heterotransplants in SCID mice that lack an intact adaptive immune repertoire. Pilot studies using viral-pretreated T-47D breast cancer cells showed that AdEHCD40L had an enhanced antitumorigenic activity compared with AdEHNull (data not shown). Subsequent studies were done against preexisting breast cancer xenografts of MDA-MB-231 heterotransplants (Fig. 3A). Five daily intratumoral injections of AdEHCD40L (6 × 10⁶ pfu), AdEHNull (6 × 10⁶ pfu), or rCD40L protein (1 μg/mL) were given into cohorts of 10 mice when tumor xenografts reached a mean tumor size of 100 mm³.

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**Table 2. ED₅₀ for AdEH constructs in CD40+ human breast cancer cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ED₅₀ (in MOIs)*</th>
<th>Reduction in ED₅₀ (in fold difference)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AdEHCD40L</td>
<td>AdEHNull</td>
</tr>
<tr>
<td>T-47D</td>
<td>0.66</td>
<td>5.89</td>
</tr>
<tr>
<td>BT-20</td>
<td>3.67</td>
<td>39.1</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>4.09</td>
<td>27.71</td>
</tr>
</tbody>
</table>

* Determined at 96 h posttreatment by the MTT bromide assay; ED₅₀: viral dose needed to achieve 50% loss in viability.
diameter of >4 mm. Tumor growth was followed until the mock-treated tumors reached a diameter of ≥12 mm.

Tumor growth was followed until the mock-treated tumors reached a diameter of ≥12 mm. Treatment-related growth reduction was determined by comparing the mean percent increase in tumor size of each treated group with that of the mock-treated animals at the same time point. We found that AdEHCD40L treatments were significantly more effective in reducing tumor xenograft growth, where tumor load was decreased by 99.9% compared with 69% reduction by AdEHNull and 49% by preoptimized concentration of rCD40L (Fig. 3A). Although rCD40L and AdEHNull significantly reduced tumor growth compared with mock-treated animals (P = 0.008 and 0.0006, respectively, Student’s two-tailed t test), treatment with AdEHCD40L produced a growth-inhibitory effect that was significantly more effective than AdEHNull (P = 0.0034) or rCD40L (P < 0.0001).

Tumor xenografts have been reported to display the hypoxic features of human primary tumors, including HIF-1α overexpression (15). This finding was validated by immunohistochemical analysis, with 68 ± 2.5 and 77 ± 2.6% of nuclear HIF-1α–positive cells detected in representative mock- and AdEHCD40L-treated MDA-MB-231 xenografts, respectively (Fig. 3B.3 and B.6). A similar HIF-1α expression profile was evident in rCD40L-treated (62 ± 2.5% positive) and AdEHNull virus–treated (71 ± 3.6% positive) xenografts, demonstrating the hypoxic status in mock- or viral-treated breast cancer heterotransplants.

Further, histopathologic assessments revealed no significant alterations in the distribution or frequency of tumor-infiltrating leukocytes (ANOVA analysis, P = 0.69) after AdEHNull (18 ± 14.9% of total cells, n = 10), AdEHCD40L (22.4 ± 13.5%), or rCD40L protein (13 ± 9.4%) treatment compared with mock controls (16 ± 19.5%). These findings support the premise that immune activation contributes minimally to the tumor growth–inhibitory activity of AdEHCD40L.

Cellular mechanisms of AdEHCD40L cytotoxicity. According to Chu et al. (27) and Dotti et al. (28), increased tumor apoptosis seemed to be a contributory tumor cell kill mechanism for ectopically expressed CD40L. By comparison, elevated as well as reduced apoptosis has been described after oncolytic viral
treatment, possibly from the perturbation of host apoptotic pathways by viral E1A and E1B genes, respectively (5, 29, 30). The frequency of apoptotic cells in AdEHCD40L-infected T-47D cells was determined as a function of ANN V expression by flow cytometric analysis (Fig. 4A). ANN V+ cells can be detected as early as 48 hours in the AdEHCD40L-treated cultures and increased over time (33% at 144 hours). Further, consistently higher levels of ANN V+ cells were observed in AdEHCD40L-treated cultures between 48 and 144 hours postinfection compared with AdEHNull or Adv-WT–infected cells at the same time points (Fig. 4A), both of which contained a comparable level of ANN V+ cells. These findings indicate that elevated apoptotic activities were manifested by CD40L-mediated events.

For the T-47D breast cancer line, ~90% of untreated cells reside at the G0–G1 phase. This distribution represents the cell cycle steady state (89.5 ± 1.4% at G0–G1 after 144 h of culture) according to cell cycle distribution analysis (Fig. 4B). Cell cycle distribution analysis confirmed that viral treatment significantly increased the sub-G0 fraction (AdEHCD40L: 36.8 ± 16.5%; AdEHNull: 21.6 ± 8.0%; versus 1.5 ± 0.8% in untreated culture, P < 0.05), as consistent with the observed, increased ANN V binding described above. In addition, we observed an accumulation of cells in the S phase at 144 hours (AdEHCD40L: 32.8 ± 19.1%; AdEHNull: 58.6 ± 3.5%; untreated: 5.7 ± 1.4%; Fig. 4B). A significantly elevated G2-M fraction was seen after AdEHCD40L treatment (25.7 ± 12.2%, versus 2.6 ± 1.1% in untreated culture; P < 0.05) but not in AdEHNull-infected cells. These findings indicate that the incorporation of the CD40L transgene may induce unique, cell cycle perturbation events that were absent by AdEHNull treatment. These cell cycle blockade events, together with increased apoptosis, likely contribute to the augmented cytotoxic activity of AdEHCD40L (31).

**Discussion**

In this study, we have examined the direct cancer growth–inhibitory activities of a CD40L-integrated, conditional replicative oncolytic adenovirus that is constructed from the previously defined AdEH oncolytic virus backbone (18). As first shown by Bischoff (32), conditional replicative/oncolytic activity by adenoviruses can be attained through modification
of the viral promoter/enhancer elements to respond only to nuclear transcriptional factor(s) unique to the cancer cell. The incorporation of antitumor transgenes is a logical extension for augmenting the antitumor effects, given the history of predictable and successful transgene delivery by nonreplicative adenoviruses.

AdEHCD40L places downstream viral and transgene expression under the control of HRE/ERE hybrid promoter–regulated viral E1A transcripts, where HRE exerted a potent effect in regulating viral gene expression (18). Our in vitro studies showed that CD40L transgene expression, as well as AdEHCD40L oncolytic activity, was largely dependent on constitutive or induced HIF-1α expression in the human breast cancer lines tested. The physiologic relevance of this system is supported by recent findings that HIF-1α expression may be critical for human breast cancer cell cycling and angiogenesis (33). The hypoxic state has also been suggested to be integral for the evolution of the malignant process toward increased growth aggressiveness and resistance to endocrine therapy in human breast cancers (16). Recently, Krishnamurthy (34) showed that hypoxic status is a key survival component of the putative breast cancer stem cell. Further studies are planned to better identify the effect of AdEHCD40L treatment on this HIF-1α–overexpressing breast cancer stem cell subset.

Our current findings confirmed prior observations that CD40L can deliver growth-modulatory signals to breast cancer cells, most of which express the cognate CD40 receptor (13). AdEHCD40L produced a more profound growth-inhibitory effect in CD40* breast cancer lines with a viral permissive phenotype (T-47D, BT-20, and MDA-MB-231) compared with parental AdEH, which lacked the CD40L transgene. As shown by in vitro ED50 comparative analysis on CD40+ breast cancer lines, incorporation of the CD40L transgene elevated cancer cytotoxic activity by ~10-fold. This augmented antitumor activity was confirmed in vivo by treatment of preexisting MDA-MB-231 xenografts, where AdEHCD40L reduced tumor growth by >2 logs compared with 60% to 76% of growth reduction by rCD40L or AdEHNull alone. Conversely, AdEHCD40L lacked an enhancing antitumor activity in the viral permissive but CD40* breast cancer specimens. In the recent study by Baxendale (35), CD40L reportedly was coexpressed in a limited number of CD40+ primary breast cancer specimens. However, the endogenous activity of CD40L or its relevance to modulating tumor growth was not shown. This finding of constitutive expression of CD40L by primary breast cancer cells was also contrary to previous findings by us and others (13).

Past studies have identified soluble and membrane-bound isoforms that result from alternative splicing during transcription (11). Although nanogram amounts of soluble CD40L (sCD40L) were detected in T-47D culture supernatants after AdEHCD40L infection (data not shown), it is unlikely that this soluble form contributes to the growth-inhibitory process. The detected level of sCD40L was several orders of magnitude below the threshold concentration for cancer growth modulation, which we previously determined to be ≥1 μg/mL (13). Hence, the observed CD40L-mediated growth inhibition likely represented the reciprocal binding of membrane-expressed CD40L with CD40-expressing neighboring cells.

Previously, Loskog (36) attributed the mobilization of proapoptotic elements and subsequently elevated apoptosis as hallmarks of membrane-bound CD40L-mediated cytotoxicity. We similarly observed that AdEHCD40L treatment corresponded to cell surface CD40L expression and increased apoptosis. In absence of the CD40L transgene, AdEHNull-infected cell cultures displayed S-phase accumulation, a phenomenon that is commonly manifested in cells infected with E1A-expressing adenoviruses (31). Although a similar accumulation of cells in the S phase was initially observed, AdEHCD40L-treated cultures additionally displayed a unique G2-M phase accumulation that likely constituted a CD40L-dependent outcome (37). Additional studies are planned to determine the relevance of cell cycle blockade and the involvement of molecular mediators, as exemplified by nuclear factor-κB and/or phosphatidylinositol-3 kinase inactivation in CD40-mediated growth inhibition of human cervical carcinoma cells, hepatocytes, and T-cell leukemias (38).

As seen in phase I/II clinical trials with over 500 patients, experimental therapy with conditional replicative oncolytic adenoviruses was safe and have produced limited clinical efficacy at the locoregional level, particularly in combination with chemotherapy (32, 33, 38, 39). Systemic viral replication and clinical outcome seemed to be independent of preexisting adenoviral neutralizing antibodies (5, 39). However, rapid clearance from circulation, transfection of nontargeted tissues, and viral immunogenicity remained to be key considerations for repeated, systemic adenoviral oncotherapy (40–42). Both out in vitro and in vivo findings revealed an augmented cancer cytotoxicity by the CD40L transgene at a low MOI of 1, thus favoring the applicability of this agent in spite of commonly encountered pharmacokinetic constraints (43). Of further consideration is the potential capacity of the CD40L to promote a durable, tumor cytotoxic immune response, favoring host survival. For the immunocompetent host, localized expression of CD40L within the tumor microenvironment and subsequent proapoptotic outcomes is likely to promote tumor antigen sensitization and processing (11, 44, 45). Loskog (46) recently showed that an adenovirus-delivered CD40L transgene can down-regulate immunosuppressive T regulatory cell, which led to cures from an aggressive, orthotopic bladder cancer in mice. The interplay of direct cancer growth inhibition of AdEHCD40L with its immune activating properties, together with the potential negative effect of viral antigen immunogenicity, will be examined in forthcoming studies, using a murine, syngeneic breast cancer model.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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