Abstract

Purpose: The growth-inhibitory activity of recombinant CD40 ligand (CD40L) is well documented in human multiple myeloma (MM). We examined MM-targeted delivery of CD40L by a conditional replicative oncolytic adenovirus, AdEHCD40L.

Experimental Design: The growth-regulatory activity of AdEHCD40L was determined in vitro and in vivo. Differential analysis with AdEHCD40L and parental virus (AdEHNull)–infected cultures allowed the identification of cellular and molecular pathways modulated by the CD40L transgene.

Results: Conditional expression of viral E1A and CD40L transgene was shown in human MM lines RPMI 8226 (interleukin (IL)-6 independent) and Kas-6/1 (IL-6 dependent) under hypoxic conditions commonly found in MM in situ. AdEHCD40L inhibited MM cell growth more effectively than AdEHNull. This enhanced growth-inhibitory activity was abrogated by cotreatment with a CD40L antibody. Chemoresistant MM lines (MR20 and LR5) were similarly susceptible to AdEHCD40L treatment. AdEHCD40L induced apoptosis and S-phase cell cycle blockade while uniquely up-regulating the previously described proapoptotic elements tumor necrosis factor–related apoptosis-inducing ligand, Fas, and IL-8. Intratumoral injections of AdEHCD40L reduced the growth of severe combined immunodeficient/hu RPMI 8226 xenografts by >50% compared with 28% reduction by AdEHNull. Adenoviral hexon and CD40L were detected in AdEHCD40L-treated tumors at day 35 after infection primarily in necrotic areas, suggesting viral replicative activity.

Conclusions: These findings show that CD40L acts in concert with viral oncolysis to produce MM growth inhibition through activation of cellular apoptosis. The direct growth-inhibitory activity of AdEHCD40L, together with the well-known immune-potentiating features of CD40L, may be clinically applicable for the experimental treatment of MM or plasma cell leukemia.

Multiple myeloma (MM) is a B-cell malignancy characterized by the clonal expansion and accumulation of malignant plasma cells in the bone marrow (1). Despite recent advances in induction therapy, the long-term outlook for patients with this disease is poor. Promising new agents, such as thalidomide-like immunomodulatory drugs (lenalidomide) and proteasome inhibitors (bortezomib), have improved prognosis and survival (2), but MM is still incurable.

CD40 belongs to the tumor necrosis factor (TNF) receptor superfamily and is commonly expressed on human MM cells but not normal, terminally differentiated plasma cells (4). This differential pattern of expression presents an opportunity for exploiting the CD40 growth-regulatory pathway for growth inhibition of MM. We and others have found that binding of the CD40 receptor by its natural ligand (CD40L, CD154) produced
Translational Relevance

Despite recent advances in induction therapy, the long-term outlook for patients with multiple myeloma (MM) remains poor. New adjuvant biotherapeutics (thalidomide-like immunomodulatory drugs and proteasome inhibitors) have improved prognosis and survival, but MM is still incurable. In this article, we have characterized the anti-MM properties of a novel, conditional replicative, oncolytic adenoviral construct (AdEHCD40L) for targeted delivery of the multifunctional molecule CD40L as an integrated transgene. The immune-potentiating features of CD40L are well known. The recombinant CD40L protein has been previously shown by us and others to directly modulate MM cell growth. This current study shows that AdEHCD40L induced growth inhibition in vitro and in vivo through viral oncolysis and CD40L-mediated growth modulation. This activity was restricted to MM cells that expressed the hypoxia-inducible factor-1α, a common feature of human MM in situ. Chemoresistant MM lines were similarly susceptible to AdEHCD40L treatment. High-throughput gene expression array analyses suggested that AdEHCD40L affected multiple signaling pathways (interleukin-8, RANTES, Fas, and tumor necrosis factor–related apoptosis-inducing ligand) previously shown to be involved in MM pathophysiology. Our findings show that CD40L acts in concert with viral oncolysis to produce MM growth inhibition through activation of cellular apoptosis. AdEHCD40L represents a novel therapeutic agent that may be clinically applicable for the experimental treatment of MM or plasma cell leukemia.

Materials and Methods

Cell lines. MM lines RPMI 8226 (CCL-155) and U266B1 (TIB-196) and the normal lung fibroblast line IMR-90 (CCL-186) were obtained from the American Type Culture Collection; Kas-6/1 MM cells were kindly provided by Dr. Diane Jelinek (Mayo Clinic College of Medicine, Rochester, MN). The chemoresistant MM lines RPMI 8226/MR20 and RPMI 8226/LRS, together with parental RPMI 8226/S lines, were obtained from Dr. William Dalton (Moffitt Cancer Center, University of South Florida, Tampa, FL). All studies were done with cell lines suspended in phenol red–free RPMI 1640 (Invitrogen) and 10% charcoal dextran–treated fetal bovine serum (HyClone). Viral infection was carried out at a multiplicity of infection (MOI) of 1, unless stated otherwise.

AdEHCD40L configuration. Genomic modifications of the previously described (15), conditionally replicating adenovector AdEH include (a) substitution of the endogenous E1A promoter with the 5XEH3 promoter, (b) replacement of the endogenous E4 promoter with the E2F-1 promoter, and (c) insertion of the CD40L open reading frame cDNA by standard cloning techniques as previously described (16) into the deleted E3, gp19K/6.7K region under the control of an endogenous adenoaviral late promoter (Fig. 1).

Flow cytometric immunophenotype analysis. CD40, CD40L, CAR, and viral E1A expression of MM cells was characterized before and after infection with AdEHCD40L or a control virus. Cells (5 × 10^5 per well) were infected with virus suspended in 2 mL of culture medium in six-well plates for 90 min (37°C, 5% CO_2) in the presence or absence of 25 μmol/L CoCl_2 for the induction of hypoxia-inducible factor-1α (HIF-1α). Determination of CD40L expression was carried out following incubation with a phycoerythrin-conjugated mouse anti-human CD40L antibody (23°C, 30 min in the dark; BD Pharmingen; ref. 3). Viral E1A expression was quantified after membrane permeabilization (Fix and Perm cell permeabilization kit, Invitrogen Diagnostics) and treatment with either a mouse anti-E1A primary antibody (10 μg/mL) or an IgG2a isotypic control antibody (23°C, 30 min in the dark) followed by incubation with a goat anti-mouse, FITC-conjugated secondary antibody (10 μg/mL, 23°C, 20 min in the dark; all from BD Pharmingen). Cell surface CAR expression was quantified following incubation with a human CAR primary antibody (50 μL; Abcam, Inc.) and FITC-conjugated goat anti-mouse secondary antibody (10 μg/mL; ICN Pharmaceuticals-Cappel). All reagents were fixed in 500 μL of 1% paraformaldehyde and analyzed by flow cytometry (FACScan, Becton Dickinson) with an excitation at 488 nm. The frequency distribution of positive and negative staining cells was analyzed by the CellQuest software (Becton Dickinson) based on 5,000 events.

growth arrest and/or apoptosis in MM, B-cell lymphocytic leukemia (B-CLL), as well as B lymphoma cells (3). CD40-dependent growth inhibition, which can be augmented by concomitant activation of the Apo-1/Fas (CD95) death receptor (3, 5), forms the basis of recent MM clinical trials with CD40L or CD40 antagonistic antibodies.

A limitation of CD40L-based systemic therapy is the potential side effects that may result from nonspecific immune activation due to common expression of CD40 on a variety of normal cell types (6). We have examined the use of a conditionally replicative, oncolytic adenovirus (AdEH) for targeted delivery of the CD40L transgene to MM cells. Freshly isolated MM cells and cell lines (7, 8), but not normal hematopoietic stem cells (9), express high levels of cossackievirus and adenovirus receptor (CAR) and integrin, cell surface receptors that facilitate entry of infectious adenoviruses into mammalian cells (10). Thus, MM cells in situ are likely to be susceptible to adenoviral infection. Further, hypoxia, a reduction in the normal level of tissue oxygen tension and a key regulatory factor in tumor growth, has been observed in the affected bone marrow milieu of MM animal models and in MM patients (11, 12). Additionally, MM cells have been reported to express estrogen receptors (13). These phenotypic features may be exploited for the selective targeting of myeloma cells.

The conditional replicative adenovirus AdEH places the essential viral E1A and E4 transcriptional units under the control of a hybrid promoter containing the estrogen response element and hypoxia response element. The endogenous viral E4 promoter was also replaced by a human E2F-1 promoter, further limiting viral replication to E2F-1–overexpressing cells. The AdEH backbone has previously been shown to be an effective transgene delivery vehicle, as illustrated in a proof-of-principle study with the proapoptotic transgene hara-kiri (14). The growth-inhibitory effects of AdEH containing the CD40L gene have been shown recently in breast cancer cells. Our current findings showed that the AdEH-delivered CD40L transgene was expressed in MM cells under viral permissive conditions and acted in concert with viral oncolysis to produce MM growth inhibition by triggering endogenous regulatory pathways.
Bromodeoxyuridine proliferative assay. Bromodeoxyuridine (BrdUrd) incorporation was carried out to quantify proliferative activity of MM cells following viral infection, according to the manufacturer’s instructions (Roche Diagnostics). BrdUrd uptake by MM cells (7.5 × 10^4 per well) was measured at graded time points after infection by spectrophotometry (SpectraMax 340, Molecular Devices), following treatment with a peroxidase-conjugated detection antibody and substrate (15 min, 23°C). Background level of BrdUrd uptake (OD_{SDS}) was determined with culture wells that were treated with 1% SDS at the beginning of culture. The percentage growth inhibition by viral treatment was determined by the following formula: (OD_{untreated} - OD_{virus}/OD_{untreated} - OD_{untreated} × 100%). To determine the involvement of CD40L transgene-mediated growth inhibition, AdEHNull- and AdEHCD40L-infected cultures were co-incubated with the CD40L/CD154 antagonist MAb 24-31 (10 μg/mL; Ancell Corp.) under hypoxic conditions at the start of culture and maintained for 72 h.

Growth inhibition of MM-severe combined immunodeficient xenografts. MM-severe combined immunodeficient (SCID) xenografts were induced in immunodeficient CB.17/scid/scid mice (Harlan Sprague Dawley) by s.c. inoculation of RPMI 8226 cells (3 × 10^3) mixed with Matrigel (1:1 v/v; total volume of 0.1 mL; Becton Dickinson). On tumor emergence at approximately 3 to 4 wk after inoculation, each mouse received five daily intratumoral injections of either AdEHCD40L, AdEHNull, or PBS only. Tumor size was measured as a function of tumor diameter by Vernier caliper measurements perpendicular to each other. Animals were sacrificed by cervical dislocation when tumors reached >12 mm in diameter. Both tumors and major organs were harvested and snap frozen for archive storage before immunohistochemical analysis of CD40L and adenviral hexon expression. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Immunohistochemical analysis of MM xenografts. Immunohistochemical analysis of snap-frozen tumor xenografts was carried out as described previously (17). Briefly, tissues were fixed in acetone (10 min, 23°C), rehydrated, and quenched in a 1% H_2O_2/methanol mixture for 10 min. After blocking, the reagents were treated with a CD40L antibody (Ansell) or an adenviral hexon antibody (10 μg/mL, 1 h, 23°C, Novus Biologicals), washed (PBS and 1% horse serum), and developed with the Vectastain Universal ABC kit and the 3,3′-diaminobenzidine substrate (both from Vector Laboratories). The reaction was examined by light microscopy and compared with isotype-matched, control primary antibody–treated samples. Reactions were quantified following examination of two fields at ×100 magnification and graded in a blind fashion by two independent observers as ++ (5-25% reactive cells), +++ (25-50% reactive cells, low intensity), ++++ (50-75% reactive cells, moderate intensity), and ++++++ (75% reactive cells, high intensity).

Apoptosis and cell cycle analyses. Apoptotic activity was characterized by an Annexin V-binding assay, which quantifies early apoptotic cells with loss of membrane phospholipid asymmetry (3). Briefly, cells (1 × 10^5) were harvested at 48, 72, and 96 h after treatment and incubated with FITC-conjugated Annexin V-FITC and the vital dye propidium iodide (PI; 10 min, 23°C). The reaction was quenched by diluting the reagents with binding buffer (2 × v/v) and analyzed immediately by two-color immunofluorescence flow cytometry (FACScan). Cells were classified as early apoptotic (Annexin V positive), late apoptotic/necrotic cells (Annexin V and PI positive), necrotic/dead cells (PI positive), and live cells (Annexin and PI negative).

Cell cycle distribution analysis was carried out with MM cells in logarithmic growth phase following cold ethanol fixation (70%, v/v, overnight) and PI staining (40 μg/mL in PBS; with 10 μg/mL RNase A, 100 μg/mL 30 min, 37°C). Cell cycle distribution was determined within 30 min of staining by flow cytometry (BD FACS) and analyzed with the Verity cell cycle analysis software (Verity, Inc.) as a function of fluorescence emission at 600 nm following excitation at 488 nm. Concurrent analysis of cyclohexamide-treated cells (10 μg/mL) was used as a reference control.

Gene expression array and proteomic analysis. High-throughput gene expression array analysis was carried out to compare the differential gene expression profile of virus-treated MM cells (Human U133 Plus 2.0 array, Affymetrix, Inc.). Total RNA from treated cultures was extracted (RNeasy Mini kit, Qiagen) as per the manufacturer’s protocol. Gene expression array analysis was carried out at the Microarray Facility Core at University of Texas Southwestern Medical Center (Dallas, TX) using a standardized sample RNA input. mRNA expression in treated samples was compared with that of the untreated control following normalization to GADPH expression and analyzed by the GeneSpring software (Agilent Technologies). Significant differential responses were determined by an altered expression of >2-fold. Cytokine-specific ELISAs (Quantikine, R&D Systems) were carried out as per the manufacturer’s protocol to validate altered transcriptional activity at the protein level.

**Fig. 1.** Schematic representation of genetic modifications in the conditional-replicative adenviral vector AdEHCD40L. The E1A promoter is replaced by an estrogen response element (ERE) and hypoxia response element (HRE). The wild-type E4 promoter region has been substituted by a promoter for human E2F-1. The CD40L transgene cassette is cloned into the viral E3 region. Conditional promoters limit AdEHCD40L replication to cancer cells that overexpress HIF-1α and/or ER.
Although AdEHCD40L exerted a more profound growth-inhibitory effect (93.7 ± 0.35%, n = 3) compared with AdEHNull (61.2 ± 3.9%, n = 3; P = 0.0001) at 96 hours after infection. Similarly enhanced growth inhibition by AdEHCD40L was evident with the IL-6–dependent Kas-6/1 cells (89.1 ± 5.7% versus 77.9 ± 3.3% by AdEHNull; P = 0.04).

To identify the contributory role of the CD40L transgene, blocking studies were done by coincubation with the antagonistic CD154 MAb 24-31 (19). Treatment with MAb 24-31 alone did not significantly alter BrdUrd uptake of RPMI 8226 cells (P = 0.67, two-tailed Student’s t test), indicating that endogenous CD40L was not involved in the cell-to-cell growth interaction. In contrast, MAb 24-31 significantly reduced MM growth inhibition by AdEHCD40L from 90.3 ± 2.1% to 42.8 ± 9.9% (P = 0.002; n = 3). This reduced level was at par with the growth-inhibitory activity of AdEHNull (66 ± 5.7%; P = 0.08), indicating that AdEHCD40L-mediated growth reduction was attained in part by CD40L transgene triggering of the CD40 pathway.

The effective dose required to reach 50% growth inhibition (ED 50) was determined in virus-infected RPMI 8226 and Kas-6/1 cultures under CoCl 2-induced hypoxic condition to compare the relative efficiency of AdEHCD40L and AdEHNull treatments (Table 1). The ED 50 of AdEHCD40L was 3.5-fold lower than AdEHNull for RPMI 8226 cells and 2-fold lower for Kas-6/1 cells (Table 1). In contrast, no marked gain in anti-MM efficacy by AdEHCD40L was observed in U266 MM cultures that expressed low levels of CD40 (<5% Table 1).

Parallel evaluations with chemoresistant cell lines indicated that the AdEH constructs exhibited similar growth-inhibitory activities on MM cells that had developed melphalan (RPMI 8226/MR20) and mitoxantrone (RPMI 8226/LR5) resistance (Table 1). Growth reductions ranged from 99.0 ± 1.0% for RPMI 8226/MR20 to 76.2 ± 5.0% for RPMI 8226/LR5 at 96 hours after infection compared with 96.5 ± 1.5% of RPMI 8226/S. As with chemosensitive RPMI 8226 cells, AdEHCD40L treatment was more effective than AdEHNull in inhibiting RPMI 8226/LR5 (4.4-fold) and RPMI 8226/MR20 cells (2-fold; P = 0.002).

To validate the selective activity of AdEHCD40L, we examined its growth-inhibitory effects on the normal human lung fibroblast line IMR-90 with low (<5%) CD40 expression and normal peripheral blood mononuclear cells that contained 2% CD40+ B cells but lacked CAR expression (20).

### Results

**Transgene expression in AdEHCD40L-infected MM cells.** Pre-established, chemosensitive, and chemoresistant MM cell lines were used to characterize the growth-regulatory effects of AdEHCD40L. Confirmatory analysis showed that the wild-type CD40 receptor was expressed in >50% of the interleukin (IL)-6–independent RPMI 8226 line and the IL-6–dependent Kas-6/1 line (Table 1). Both lines also expressed CAR and hence are susceptible to adenoviral infection. Further, CD40 and CAR expression was maintained in RPMI 8226 sublines that have been selected for melphalan (RPMI 8226/LR5, 5-fold resistant to melphalan) and mitoxantrone resistance (RPMI 8226/MR20, 20-fold resistant to mitoxantrone). None of the tested cell lines expressed endogenous CD40L (Table 1).

Transgene expression was characterized following infection of MM cell lines at the MOI of 1. The AdEH backbone incorporates hybrid promoters that limit viral E1A expression to cells that overexpress HIF-1α and/or estrogen. Previous studies have established hypoxia response element to be a stronger inducer of AdEH activity (15), which was confirmed in this study (data not shown). To simulate in vivo hypoxic growth and HIF-1α production (18), RPMI 8226 cultures were maintained with 25 μmol/L CoCl 2 following viral infection. Viral E1A and the CD40L transgene were first detected at 24 hours after infection and only in the hypoxic state (49% and 22%, respectively) compared with low levels (<10%) of expression under normoxic conditions. Viral E1A and transgene expression peaked at 48 hours after infection (62% and 67%, respectively). Similarly, 43% of the IL-6–dependent Kas-6/1 cells expressed the CD40L transgene under hypoxia following AdEHCD40L treatment. These findings indicate that human MM cells are susceptible hosts to AdEH infection and expressed the CD40L transgene under hypoxic conditions.

**CD40L-dependent growth inhibition by AdEHCD40L.** MM cell growth was quantified by the BrdUrd uptake assay to characterize the growth-regulatory effect of the CD40L transgene under CoCl 2–induced hypoxia. Both AdEHCD40L and the parental construct (AdEHNull) inhibited RPMI 8226 cell growth, although AdEHCD40L exerted a more profound growth-inhibitory effect (93.7 ± 0.35%, n = 3) compared with AdEHNull (61.2 ± 3.9%, n = 3; P = 0.0001) at 96 hours after infection. Similarly enhanced growth inhibition by AdEHCD40L was evident with the IL-6–dependent Kas-6/1 cells (89.1 ± 5.7% versus 77.9 ± 3.3% by AdEHNull; P = 0.04).

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### Table 1. Phenotypic characterization of MM lines tested and their ED 50 values to viral treatment

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% Expression*</th>
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<tr>
<td></td>
<td>CD40</td>
<td>CD40L</td>
<td>CAR</td>
<td>AdEHCD40L</td>
<td>AdEHNull</td>
<td>Reduction in ED 50 (fold)</td>
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<td>RPMI 8226/S</td>
<td>49.8</td>
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<td>&lt;0.1</td>
<td>33.3</td>
<td>0.11</td>
<td>0.26</td>
<td>2.4</td>
</tr>
<tr>
<td>RPMI 8226/LR5</td>
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<td>&lt;0.1</td>
<td>30.0</td>
<td>0.66</td>
<td>2.89</td>
<td>4.4</td>
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<tr>
<td>Kas-6/1</td>
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<td>&lt;0.1</td>
<td>43.8</td>
<td>0.26</td>
<td>0.5</td>
<td>1.9</td>
</tr>
<tr>
<td>U266</td>
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<td>&lt;0.1</td>
<td>48.3</td>
<td>5.57</td>
<td>6.23</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*CD40, CD40L, and CAR expression was determined by the direct or indirect immunofluorescence assay and analyzed by flow cytometry.

†Cells were infected with AdEHCD40L and AdEHNull (MOI of 0.1-10) under CoCl 2–induced hypoxic condition. Proliferative activity at 96 h after infection was calculated as % untreated (mean ± SD; n = 3). ED 50, or the effective dose needed to achieve 50% growth reduction, was calculated by linear regression analysis of the dose-response curve. For RPMI 8226/MR20, ED 25 (effective dose for 25% growth reduction) was calculated because ED 50 for this cell line was <0.1 MOI.

### Statistical analysis

Parametric analysis (Student’s t test, regression analysis) was done using the GraphPad Prism software (version 4; Hologram Publishing).
AdEHCD40L did not inhibit peripheral blood mononuclear cell growth and generated only minimal cytotoxicity (<2%) on MM-90 cells under normoxia. By comparison, wild-type adenovirus (AdWT) reduced IMR-90 cell viability by 67%, as determined by the crystal violet assay at 96 hours after infection (data not shown).

**Growth inhibition of MM xenografts.** Growth inhibition of MM xenografts by AdEHCD40L was examined with a s.c. model previously established in our laboratory (21). RPMI 8226 xenografts exhibited exponential growth after 15 days, with correspondingly increased M protein in serum ($r^2 = 0.82; P = 0.097$) and urine ($r^2 = 0.96; P = 0.019$). Five daily intratumoral injections of AdEHCD40L significantly reduced xenograft growth by 53% at day 35 ($4.8 \pm 0.9$ mm versus $10.5 \pm 1.2$ mm in untreated tumors; $P = 0.002$; Fig. 2A). This treatment was more effective than AdEHNull ($P = 0.03$), which reduced tumor growth by 27% within the same time period ($7.6 \pm 1.1$ mm). Approximately 70% of tumor volume in the AdEHCD40L-treated tumor xenografts exhibited necrotic features at the time of autopsy compared with 40% in AdEHNull-treated specimens. Thus, intratumoral treatment with AdEHCD40L effectively reduced MM cell growth in vivo, where optimal effect was dependent on expression of the CD40L transgene.

MM growth inhibition likely constituted the combined outcome of viral oncolysis and CD40L-mediated growth-inhibitory effects. To characterize AdEHCD40L replicative activity in the injected xenograft, viral hexon expression was determined by immunohistochemical analysis. Untreated xenografts were uniformly unreactive to the adenoviral hexon antibody, whereas >90% of AdEHCD40L-treated tumors displayed the viral hexon protein at 35 days after treatment (Fig. 2B). Viral capsid expression was concentrated in the necrotic areas of the tumor and in scattered foci in other areas of the tumor xenograft, which constituted approximately 10% to 15% of the residual, nonnecrotic regions.

Viral capsid expression was similarly distributed in AdEHNull-treated xenografts (data not shown). The positive expression of adenoviral hexon at this extended time point is indicative of viral replication. Its localization at necrotic areas suggests a contributory role for viral oncolysis in xenograft growth inhibition.

**AdEHCD40L-induced apoptosis and cell cycle arrest.** We examined the apoptotic activity of AdEHCD40L-infected RPMI 8226 cells by phenotypic analysis under CoCl$_2$-induced hypoxic condition. PI uptake and/or the surface binding of Annexin V are common indicators of cellular necrosis and apoptosis, respectively. Cells were identified as viable (Annexin V$^-$PI$^-$), necrotic (Annexin V$^-$PI$^+$), early apoptotic (Annexin V$^+$PI$^+$), and late apoptotic (Annexin V$^+$PI$^+$) by flow cytometric analysis at 72 hours after AdEHCD40L infection. We observed a significant decrease in the frequency of viable cells (Annexin V$^-$PI$^-$) following AdEHCD40L infection ($57.2 \pm 2.1\%$, compared with $81.1 \pm 1.7\%$ in untreated culture; $P < 0.0001$; $n = 4$) that was accompanied by an elevated Annexin V$^+$ subset ($18.8 \pm 1.7\%$ versus $7.7 \pm 1.0\%$ in untreated; $P = 0.0007$; Fig. 3A). Markedly lower apoptotic activity (Annexin V$^+$PI$^+$) was observed in the AdEHNull-treated culture ($17\%$ versus $29\%$ by AdEHCD40L). Similarly, flow cytometric analysis of cell cycle revealed the presence of a markedly increased sub-G0 fraction (<2N DNA) in AdWT-infected (23.7%), AdEHNull-infected (12.8%), as well as AdEHCD40L-infected (23.0%) cultures that was consistent with elevated apoptosis. AdEHCD40L-treated cells also displayed an increased S-phase blockade over time ($67.5 \pm 2.6\%$ versus $47.0 \pm 3.0\%$ in untreated at 72 hours after treatment; $P = 0.0004$) that was not observed with parental AdEHNull (Fig. 3B).

**Candidate mediators for AdEHCD40L-induced growth inhibition.** To elucidate the molecular mechanism of AdEHCD40L-mediated growth inhibition as related to MM pathophysiology, high-throughput gene expression array screening was carried out at 48 hours after viral infection. Differential analysis of
AdEHNull-treated cultures (versus untreated) yielded 842 genes whose altered expression was likely attributable to the viral infectious process, including 325 genes that were up-regulated and 517 genes that were down-regulated by >2-fold. Further, differential subtraction of gene expression of the AdEHCD40L treatment from AdEHNull during this initial screening process yielded 1,005 genes that were uniquely altered, likely representing transcriptional modulation by the CD40L transgene. The 267 up-regulated genes and 738 down-regulated genes altered ≥2-fold included genes from chemokine and cytokine families (CXCL9, CXCL10, CXCL11, IL-12B, and the apoptotic mediator inhibitor-β-A). In particular, the cytokines IFN-α, IL-8, and RANTES (regulated on activation, normal T-cell expressed and secreted) were up-regulated by 2.3-, 2.7-, and 2.5-fold, respectively. In contrast, mRNA transcripts of other cytokines previously described to affect MM pathophysiology (IL-6, IL-1β, TNF-α, macrophage inflammatory protein-1α, fibroblast growth factor-2, stromal cell-derived factor-1, and insulin-like growth factor-I; ref. 22) were not altered significantly.

Consistent with previous findings of collateral activation of other TNF receptor superfamily members via CD40 binding (23), an increased transcriptional expression of APRIL (a proliferation inducing ligand), BAFF (B lymphocyte activating factor belonging to the TNF family), osteoprotegerin, as well as two key members best known for the induction of apoptosis [Fas/TNFRSF6 and TNF-related apoptosis-inducing ligand (TRAIL)/TNFSF10] was observed (Fig. 4A). The complete data set of gene expression array analysis is provided as Supplementary Table S1.

To confirm modulated cytokine production at the translational level, multiplex analysis was done to determine viral infection outcome on the secretion of fibroblast growth factor-2, IFN-α, IL-1β, IL-6, IL-12p40, IL-15, TNF-α, macrophage inflammatory protein-1α, IL-8, RANTES, vascular endothelial growth factor, and transforming growth factor-β1. Culture supernatants of rCD40L-treated MM cells showed up-regulated levels of IL-8, RANTES, and transforming growth factor-β1 but not IFN-α at 48 hours after infection (data not shown). Further, ELISA analyses showed that AdEHCD40L significantly up-regulated IL-8 (49.3 ± 3.4 pg/mL) compared with untreated (17.9 ± 3.0 pg/mL; \(P = 0.004\)) or AdEHNull-treated culture (16.1 ± 2.5 pg/mL; \(P = 0.004\)) (Fig. 4B). RANTES was significantly elevated by AdEHCD40L (132.0 ± 10.3 pg/mL versus 53.7 ± 4.6 pg/mL; \(P = 0.0003\)), although AdEHNull also induced a moderate increase of this cytokine (83.5 ± 3.2 pg/mL; \(P = 0.0015\)). Thus, AdEHCD40L is likely to up-regulate IL-8 in a unique manner, whereas both AdEHCD40L and AdEHNull stimulated RANTES production. Both AdEHCD40L and AdEHNull treatments also down-regulated transforming growth factor-β1 to undetectable levels (as compared with 55.9 ± 14 pg/mL in untreated cultures). This cytokine is often associated with immunosuppression and disease progression in MM (24).

**Discussion**

The present study extends our previous work on MM by considering the use of a selectively replicating, oncolytic adenovirus (AdEHCD40L) to deliver CD40L in the form of a transgene. We examined its applicability for restricted transgene expression as a means of limiting nonspecific immune activation as well as the feasibility of achieving enhanced MM growth inhibition through viral oncolysis and perturbation of the CD40L growth-regulatory pathway. The expression of HIF-1α, E2F-1 (25), and estrogen receptors (13) has been documented in human MM cells in situ. Normal hematopoietic cells (including normal BM cells) lack CAR expression (9) and are resistant to adenoviral infection, whereas freshly isolated, primary MM cells express high levels of CAR and integrin receptors (7, 8). Hence, the MM-involved BM microenvironment is likely to be permissive for HIF-1α/estrogen-dependent AdEHCD40L infection and CD40L transgene expression (8, 26). The activation of CD40-expressing T cells via CD40L binding is well documented as an indirect, immune-activating component of CD40L-based antmyeloma outcome. However, we anticipate these events to be manifested primarily in the myeloma microenvironment following AdEHCD40L treatment because CD40L transgene expression is limited by the hypoxia backdrop. By the same token, CD40L expression would be severely restricted in normoxic tissues, thereby limiting the likelihood of toxic events as related to CD40 ligation of epithelial cells. This premise is supported by...
our recent finding of minimal AdEHCD40L cytotoxicity in non-malignant mammary epithelial cells (27).

Consistent with our earlier findings with the recombinant CD40L protein, AdEHCD40L effectively inhibited the growth of the IL-6–independent RPMI 8226 line by >90% at a low infectivity dose (MOI of 1). A more profound growth-inhibitory effect by AdEHCD40L (compared with parental AdEHNull), which was abrogated by anti-CD154 treatment, is indicative of the contributive role of the CD40L transgene. The activation of CD40 pathway in normal B cells commonly leads to up-regulation of IL-6, a putative MM autocrine (28, 29). We did not detect any increased IL-6 transcripts or protein following AdEHCD40L treatment (data not shown). Further, AdEHCD40L infection of the IL-6–dependent Kas-6/1 MM line similarly yielded a growth-inhibitory effect (rather than growth stimulation). Hence, the CD40L transgene likely affected MM cell growth independent of the IL-6 growth modulatory loop. AdEHCD40L treatment led to significant growth

Fig. 4. Altered gene transcription following viral treatment. High-throughput microarray analysis was done in viral-treated RPMI 8226 cultures at 48 h after infection. A, change in the level of cytokines and TNF superfamily transcripts was normalized to that of the untreated culture. B, cytokine (IL-8 and RANTES) secretion in viral-treated cultures was quantified by specific ELISA assays.
reduction of MM cells that have developed resistance to melphalan (RPMI 8226/LR5) and mitoxantrone (RPMI 8226/MR20), suggesting that this experimental therapeutic approach may be potentially applicable for chemorefractory disease.

To our knowledge, this represents the first report that documents the in vivo anti-MM effect of a conditional replicative oncolytic adenovirus construct carrying the CD40L transgene. AdEHCD40L treatment led to tumor volume reduction of up to 54% compared with the untreated control and was markedly more effective than AdEHNull. Evidence of viral replicative activity was supported by the expression of late adenoviral hexon protein and CD40L proteins at 35 days after treatment. Together with the observed antitumor effect by AdEHNull (significantly reduced tumor size, 40% necrosis in residual tumor), the concentration of AdEHCD40L gene expression in the tumor necrotic region suggests the involvement of viral oncolysis in tumor cell kill. It is of interest that the in vivo inhibitory effect of AdEHCD40L fell short of the >90% inhibitory outcome observed in vitro. A potential explanation is that tumor size measurements may underrepresent the anti-MM outcome, given that the bulk (~70%) of the residual xenograft was composed of necrotic, nonviable tissue. Alternatively, encapsulation of the MM xenograft may limit the viral disseminative process within the tissue architecture (30). Confirmatory studies are planned to validate our proof-of-principle study with this SCID/hu myeloma xenograft model, where the anti-MM activity of i.v. injected AdEHCD40L and its replicative activity will be defined in an orthotopic myeloma model in an immunocompetent host (31). This model will also be used to identify any additive or synergistic antitumor effect that may be derived from the well-known immune-potentiating features of CD40L expression on transfected cancer cells (32).

We recognize the limitation of the murine model in assessing adenoviral infectivity and toxicity, particularly when immunocompromised mice were used, which lacked an intact adaptive immune system (33). With respect to viral distribution, we have documented high-throughput gene array screening to identify perturbations in cytokine and TNF superfamily member gene expression of AdEHCD40L-infected MM cells. We did not observe altered expression of IL-6, fibroblast growth factor-2, stromal cell–derived factor-1, or macrophage inflammatory protein-1α that have been described previously to modulate MM cell growth (22). However, AdEHCD40L treatment up-regulated IL-8 and RANTES transcription and protein translation. IFN-α, previously used for maintenance therapy of MM, has shown proapoptotic activity in MM cell lines (40). Although IFN-α transcriptionally seemed to be increased, protein level was not elevated at 48 hours after infection. IL-8 is best known for its proangiogenic activity in MM pathophysiology (22), although a 77-amino acid isoform of this cytokine induced apoptosis in chronic myelogenous leukemia (41) and mediated CD40-dependent antitumor necrosis in acute myeloid leukemia (42). Further studies are under way to better define the molecular identity of AdEHCD40L up-regulated IL-8 and the proapoptotic activity of the 77-amino acid isoform of IL-8 in MM cells. The contribution of RANTES in CD40L-dependent MM growth inhibition also warrants further investigation, given its pro-osteoclastic activity in MM pathophysiology (43).

In normal B cells as well as MM cells, activation of the CD40 pathway is mediated by oligomerization of members of the TNF receptor–associated factor (TRAF) family of cytoplasmic signal transducers, which may in turn cross-activate other members of the TNF and TNF receptor family members (3). The observed up-regulation of TRAIL, a TNF family member, and Fas, a TNF receptor family member, is consistent with this premise. The synergistic, proapoptotic activity of Fas and TRAIL as an outcome of CD40L-mediated up-regulation has been previously shown in B-CLL (23, 36). Although the expression of DR4, DR5, and Fas was not determined in this study, their expression has been documented previously both in MM patients as well as in MM cell lines, where up to 85% of myeloma cells were found to express these cell surface receptors (44). These observations bring forth the intriguing possibility that these proapoptotic pathways may be used for CD40L-mediated growth modulation. To clearly define their mechanistic contribution in AdEHCD40L-mediated growth inhibition of MM cells, it will be necessary to further elucidate their expression at the protein level, together with outcome analysis by transfection with loss of function mutants.

Our findings of CD40L-induced apoptosis and cell cycle blockade were consistent with prior findings by Dotti and coworkers (3, 45). Several distinct structural motifs in the CD40 cytoplasmic domain regulate various CD40 signaling pathways, which involve both the TRAF and additional signaling proteins,
and lead to activation of kinases and transcription factors (46). Tai and coworkers (47) recently reported that blockade of the CD40 pathway by the antagonist antibody HCD122 (formerly CHIR12.12) was applicable for myeloma therapy in vitro. Dace-tuzumab (formerly known as SGN-40), a humanized, CD40 agonistic monoclonal antibody that is also in early-phase trials, induced apoptosis by up-regulating cytotxic lylidues of the TNF family (Fas/FasL, TRAIL, and TNFα) while blocking sCD40L-mediated phosphatidylinositol 3′-kinase/AKT and nuclear factor-κB activation (48). Our findings of the up-regulation of TNF family members paralleled the molecular outcome of SGN-40 treatment. Like CD40L, monoclonal antibody binding would also cross-link the CD40 receptor, the critical event for TRAF recruitment, and lead to multiple downstream pathway activation (32). The possibility exists that epitope binding by HCD122 and SGN-40 triggers overlapping as well as distinct signaling outcomes as those generated by the membrane-bound CD40L. Mutations in the CD40 receptor in MM cell lines and patient specimens have been described previously by us and others (3, 49). This consideration as well as the propensity of irreversible genomic instability through CD40 binding (50) are also potential explanations for CD40 agonism and antagonism converging to a common apoptotic outcome in myeloma cells. Clearly, further molecular studies that are based on our gene expression array findings may help resolve this conundrum.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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