

*Advances in Brief***CD40 Is Expressed on Ovarian Cancer Cells and Can Be Utilized for Targeting Adenoviruses¹**

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Abstract

Purpose: CD40, a member of the tumor necrosis factor receptor superfamily, is widely expressed on various cell types in addition to hematopoietic cells. Recent studies show that CD40 expression is related to several carcinomas, although its role in cancer pathobiology is unknown. In this study, we demonstrate the expression of CD40 on several ovarian carcinoma cell lines and the ability of CD40 to mediate targeted adenoviral infection *in vitro*.

Experimental Design: CD40 expression on ovarian cancer cell lines and clinical patient samples was examined by reverse transcription-PCR and flow cytometry. To study the utilization of CD40 for gene delivery, we precomplexed a luciferase coding adenovirus (Ad), Ad5luc1, with a CD40-targeting molecule (CAR/G28).

Results: According to our studies, all of the examined ovarian cancer cell lines are expressing CD40. In addition, mRNA for CD40 was detected in every primary tumor sample, suggesting that CD40 is also expressed *in vivo*. Compared with nontargeted Ad, gene transfer was increased up to 40-fold in CD40+ cells when CD40-targeted Ad was used. Supporting the relation of targeted system to CD40, increasing the amount of targeting fusion protein results in dose response. Furthermore, blockade of CD40 receptors on cell

surface decreases the infectability of CD40+ cells with CD40-targeted virus, indicating the specificity of the targeting system for CD40.

Conclusions: These results suggest that CD40 is present in ovarian cancer cells and can be used for targeted gene delivery in a CAR-independent manner, circumventing the problem of the low expression levels of CAR in various cancer cells.

Introduction

Ovarian cancer causes more deaths than any other cancer of the female reproductive system. In 2001, more than 23,000 new cases were diagnosed, and nearly 14,000 deaths were reported (1). On this basis, it is clear that novel therapeutic strategies are desperately needed. In this regard, cancer gene therapy represents a promising intervention, which embodies the capacity for correction of disorders at a molecular level. All published ovarian cancer gene therapy interventions have exploited adenoviral vectors for *in situ* transduction of tumor cells (2). Specifically, for ovarian cancer, *i.p.* administration of recombinant Ad³ has been used for genetic modification of target tumor cells. Although these trials have demonstrated the relative safety of Ad-based delivery of genes for ovarian cancer therapy, they have also demonstrated a low rate of tumor cell transduction. This problem has been attributed to tumor cell deficiency of the primary Ad receptor, CAR (3, 4). Lack of CAR has also been reported for other tumor types and could be a ubiquitous phenomenon (2). As an approach to address this issue, tropism modifications of Ad have been made to allow CAR-independent gene delivery. Such maneuvers have allowed enhancement of gene delivery to otherwise Ad-refractory tumor cells (5). In addition, such modifications may improve the target:nontarget transduction ratio, an important determinant of the therapeutic index. Reported Ad targeting approaches have involved rerouting Ad to cell surface receptors highly expressed in ovarian cancers, including integrins (6), the Ad serotype 3 receptor (7), EpCAM (8), and epidermal growth factor receptor (9). These retargeting approaches embody the concept that tropism modifications of Ad may allow an improved outcome via Ad-based ovarian cancer gene therapy approaches. Such vector engineering efforts will be fostered by the definition of relevant ovarian cancer markers as potential selective targets. In this regard, one potential novel target is CD40, a type I transmembrane protein that belongs to the tumor necrosis factor receptor superfamily (10). It was first identified and characterized on B lymphocytes, and its central role in regulating T-cell-dependent B-cell activation is widely known. However, in recent years, it has been

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³The abbreviations used are: Ad, adenovirus; CAR, coxsackie adenovirus receptor; pfu, plaque-forming unit(s); RT-PCR, reverse transcription-PCR; CD40L, CD40 ligand.

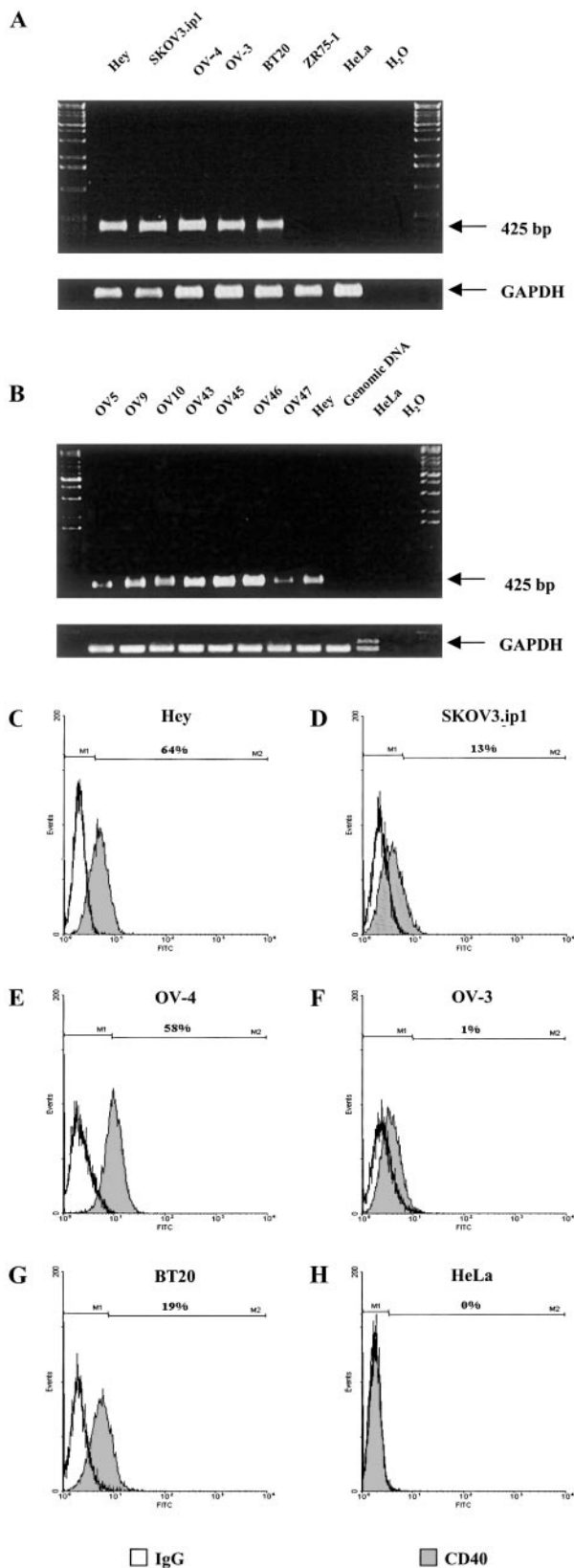


Fig. 1 CD40 expression on ovarian cancer cells. **A**, detection of CD40 mRNA by RT-PCR from Hey, SKOV3.ip1, OV-4, and OV-3 ovarian carcinoma cell lines. BT-20 (CD40+), HeLa (CD40-), and ZR-75-1

shown that CD40 is also widely expressed in monocytes, dendritic cells, endothelial cells, epithelial cells, and several types of carcinoma (*e.g.*, breast, lung, colon, and bladder carcinoma), suggesting a role beyond the lymphoid system (10–12). In contrast, the mediator of CD40 activation, CD40L (CD154), is exclusively expressed on activated CD4+ T cells (13). Cellular responses to CD40-CD40L interaction differ based on cell type and range from cell proliferation and differentiation to growth inhibition and apoptotic signaling (12). Even though the presence of CD40 has been suggested on some epithelial malignancies, its exact role in carcinomas still remains unclear (11, 14). Despite this uncertainty, several recent studies show therapeutic implications related to CD40-CD40L interaction. It has been demonstrated that CD40 ligation on carcinoma cells results in growth inhibition and sensitizes cells to apoptosis induced by a variety of agents *e.g.*, chemotherapeutic drugs (14, 15). These contrasting effects of CD40 ligation on normal *versus* malignant cells suggest that CD40 may be an important therapeutic target for antitumoral effect. In the present study, we demonstrate the expression of CD40 in ovarian carcinoma tumor cells. Furthermore, we describe the utilization of CD40 as a candidate pathway for targeted gene transfer via tropism-modified Ad for ovarian cancer gene therapy applications.

Materials and Methods

Cell Culture and Ovarian Tumor Samples. Hey, SKOV3.ip1, and OV-4 ovarian adenocarcinoma cell lines were kind gifts from Dr. Judy Wolf, Dr. Janet Price (both from M. D. Anderson Cancer Center, Houston, TX), and Dr. Timothy J. Eberlein (Harvard Medical School, Boston, MA), respectively. The other cell lines, OV-3 (ovarian adenocarcinoma), HeLa (cervical cancer), and BT-20 and ZR-75-1 (breast cancers), were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were cultured as recommended. Ovarian tumor samples were collected from patients undergoing surgical evaluation at the University of Alabama at Birmingham for suspected epithelial ovarian carcinoma or primary peritoneal carcinoma. Permission to obtain specimens was reviewed and approved by the University of Alabama at Birmingham Institutional Review Board for Human Experimentation, and informed consent was given before surgery. Specimens were collected sterily at the time of surgery and immediately frozen at -70°C .

RT-PCR. Total RNA was isolated from cultured cells using the RNeasy Kit (Qiagen, Valencia, CA) and treated with DNase before RT-PCR. Amplification (35 cycles,

(CD40-) were used as controls. **B**, detection of CD40 mRNA by RT-PCR from primary ovarian cancer patient samples. Hey (CD40+), HeLa (CD40-), and genomic DNA were included as controls. CD40-specific primers were used to generate a 425-bp product from isolated total RNA. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers were used to ensure the quality of RNA. **C–H**, CD40 expression levels on ovarian carcinoma cell lines determined by flow cytometry. The *white curve* represents staining with FITC-labeled isotype control antibody, whereas the *gray curve* represents staining with FITC-labeled monoclonal antihuman CD40 antibody. HeLa cells were used as a negative control, and BT-20 cells were used as a positive control. Percentages above the markers indicate proportions of shifted cells.

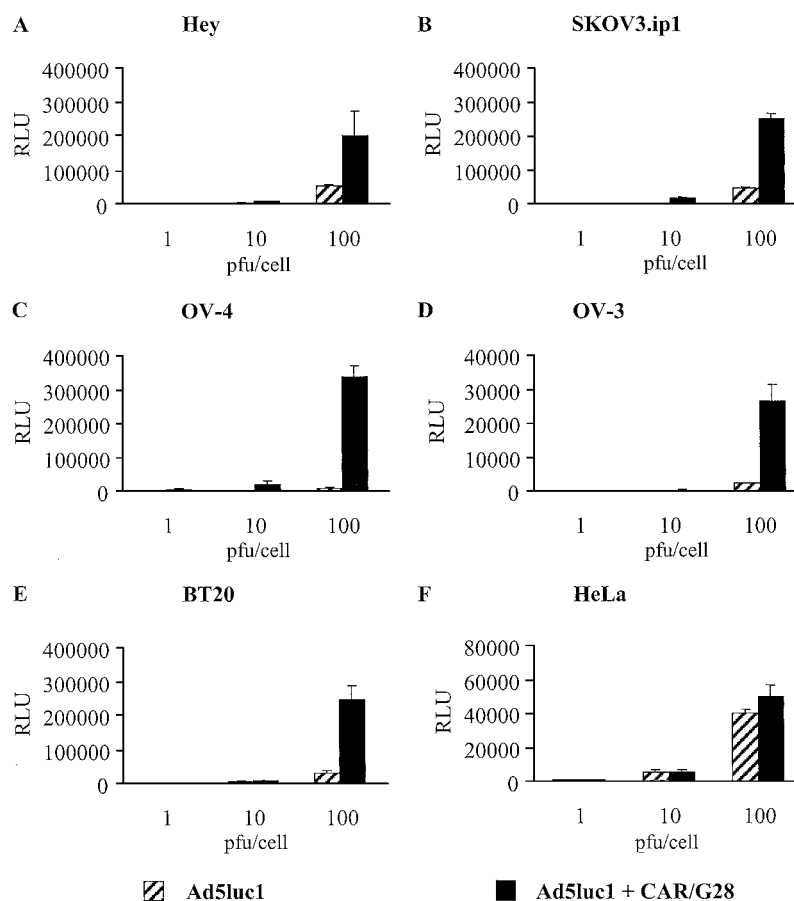


Fig. 2 Infectivity of CD40-expressing cell lines is enhanced when CD40 retargeted Ad is used. Cells were infected with Ad5luc1 (striped column) or with CAR/G28 retargeted Ad5luc1 (black column). Luciferase readings were measured 24 h after infection. BT-20 (CD40+) and HeLa (CD40-) were used as controls. The error bars indicate ± 1 SD.

annealing at 56°C) was carried out with the OneStep RT-PCR Kit (Qiagen) using CD40-specific primers (upstream, 5'-AGA-AGG-CTG-GCA-CTG-TAC-GA-3'; downstream, 5'-CAG-TGT-TGG-AGC-CAG-GAA-GA-3'). For amplification of glyceraldehyde-3-phosphate dehydrogenase (upstream, 5'-TCC-CAT-CAC-CAT-CTT-CCA-3'; downstream, 5'-CAT-CAC-GCC-ACA-GTT-TCC-3'), 30 amplification cycles were performed (annealing at 52°C). Total RNA from ovarian tumor samples was extracted using RNA STAT-60 (Tel-Test, Friendwood, TX) according to the manufacturer's instruction. RT-PCR was performed as described above.

Flow Cytometry. After trypsinization, 1×10^5 cells were resuspended in fluorescence-activated cell sorting buffer (PBS containing 2% fetal bovine serum) and stained either with FITC-conjugated mouse antihuman CD40 monoclonal antibody or FITC-conjugated IgG1 isotype control antibody (BD Biosciences, San Diego, CA). Cells were incubated at 4°C for 20 min and washed with fluorescence-activated cell sorting buffer before flow cytometric analysis (FACScan; Becton Dickinson, San Jose, CA).

Gene Transfer Assays. CAR/G28 fusion protein containing an anti-CD40 single chain Fv and the CAR ectodomain was made as described previously (16). Briefly, anti-CD40 single chain Fv cDNA generated from the G28-5 hybridoma cell line was linked to cDNA of the CAR ectodomain, resulting in

CAR/G28 fusion protein. CAR/G28 was produced using recombinant baculoviruses, purified, and characterized. Replication-incompetent luciferase-expressing Ad Ad5luc1 (3.9×10^{12} viral particles/ml, 5.5×10^{10} pfu/ml; Ref. 7) was incubated with CAR/G28 retargeting protein before infections for 45°C at room temperature. Cells were infected with either nontargeted or targeted virus for 1 h at 37°C, followed by washing. Cells (25,000 cells/well) were seeded in 96-well plates 1 day before infections and then infected with nontargeted or CAR/G28-targeted virus at a ratio of 100 ng/100 pfu. Luciferase assay was performed with the Luciferase Assay System (Promega, Madison, WI) 24 h after infections, following the manufacturer's instructions. In the dose-response assay, virus was preincubated with different amounts of CAR/G28 fusion protein (0, 3, 10, 30, 100, or 150 ng/100 pfu). Infections were carried out on 24-well plates using 50,000 cells/well. Luciferase assay was performed as described above.

Blocking Assay. Before infections, OV-4 and BT-20 cells were incubated with either growth media (containing 2% fetal bovine serum), supernatant from G28-5 hybridoma cells containing monoclonal antihuman CD40 antibody (blocking) (16), or supernatant from 1D11.16.8 hybridoma cells containing monoclonal anti-transforming growth factor $\beta 2$ antibody (irrelevant antibody). Infections were carried out on 24-well plates as described above.

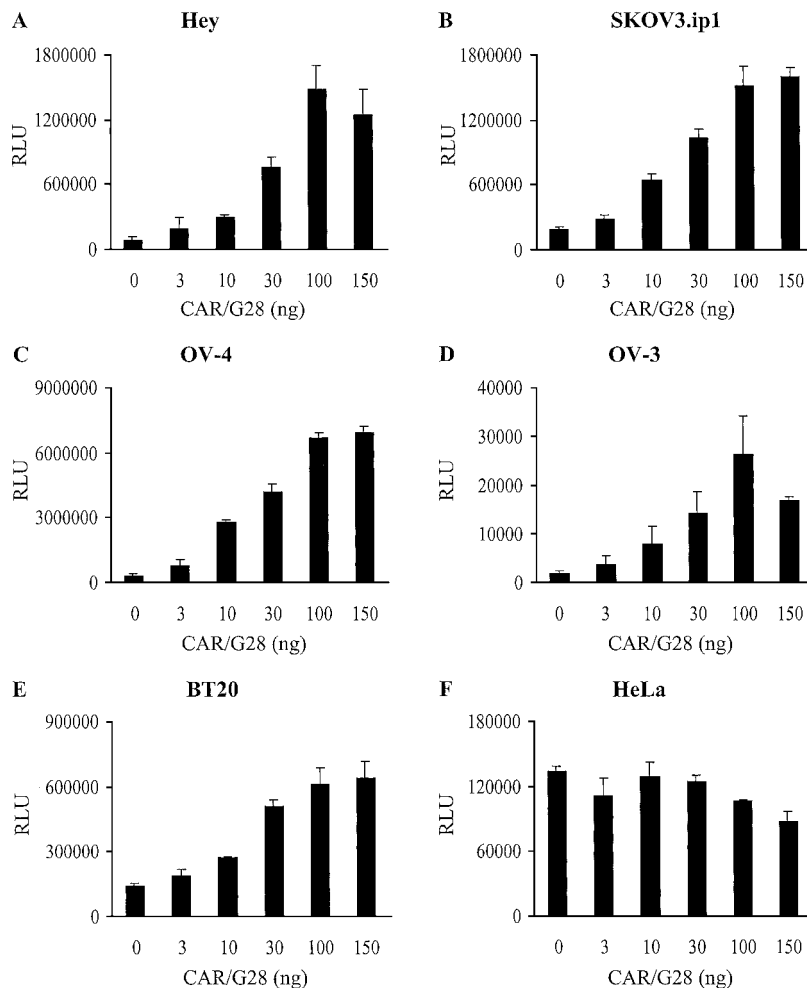


Fig. 3 Increasing the amount of retargeting protein (CAR/G28) results in dose response in CD40+ cells. Ad5luc1 was preincubated with 0–150 ng/100 pfu CAR/G28, and luciferase assay was performed after 24 h. BT-20 (CD40+) and HeLa (CD40–) cell lines were used as controls. The error bars indicate ± 1 SD.

Results and Discussion

Although the biological role of CD40 in carcinoma cells is mostly unknown, several studies have focused on CD40 expression in various cancer types (11, 15, 17, 18). In contrast to B cells, CD40-CD40L interaction in carcinoma cells has been shown to prevent cell growth and sensitize cells to apoptosis (11, 14, 15). Additionally, some studies have suggested CD40 as a potential marker to distinguish benign tumors from malignant tumors (17). On this basis, we wanted to study CD40 expression in the context of ovarian cancer. RT-PCR was performed with total RNA isolated from various ovarian carcinoma cell lines (Fig. 1A). All of the examined cell lines (Hey, SKOV3.ip1, OV-4, and OV-3) and the positive control cell line (BT-20) generated a 425-bp RT-PCR product, indicating that all of the above-mentioned cells contain CD40 mRNA. ZR-75-1 and HeLa have been reported as negative for CD40 expression (11, 14), and they did not produce any amplification product. To study the presence of CD40 on the cell surface, cells were analyzed by flow cytometry using a FITC-labeled anti-CD40 antibody with an appropriate isotype control (Fig. 1, C–H). Based on previous studies (11, 14) and results from the RT-PCR, BT-20 (Fig. 1G) was chosen as a positive control, and

HeLa (Fig. 1H) was chosen as a negative one. HeLa cells, as expected, did not show any expression when stained with CD40 antibody, whereas in the case of BT-20, almost 20% of cells were gated as CD40 positive, demonstrating moderate CD40 expression. The four examined ovarian carcinoma cell lines (Fig. 1, C–F) displayed variable but positive CD40 expression levels. Both Hey (Fig. 1C) and OV-4 (Fig. 1E) cells expressed high levels of CD40, with approximately 60% of cells being gated as CD40 positive. CD40 was also detected on SKOV3.ip1 (Fig. 1D) and OV-3 (Fig. 1F) cells, but to a lesser extent, suggesting moderate and low expression of CD40, respectively.

It has been suggested that cells negative for CD40 expression *in vivo* could revert to a CD40-positive phenotype when cultured *in vitro* (12). To examine the CD40 status in unpassaged human primary ovarian cancer cells, RT-PCR was done on primary tumor samples obtained from seven patients. RT-PCR resulted in the expected 425-bp band with all samples, suggesting CD40 expression (Fig. 1B). Genomic DNA did not yield amplification product, suggesting the absence of pseudogenes or other sources of false positives. These results provide the first evidence of CD40 expression in clinical ovarian tumor specimens.

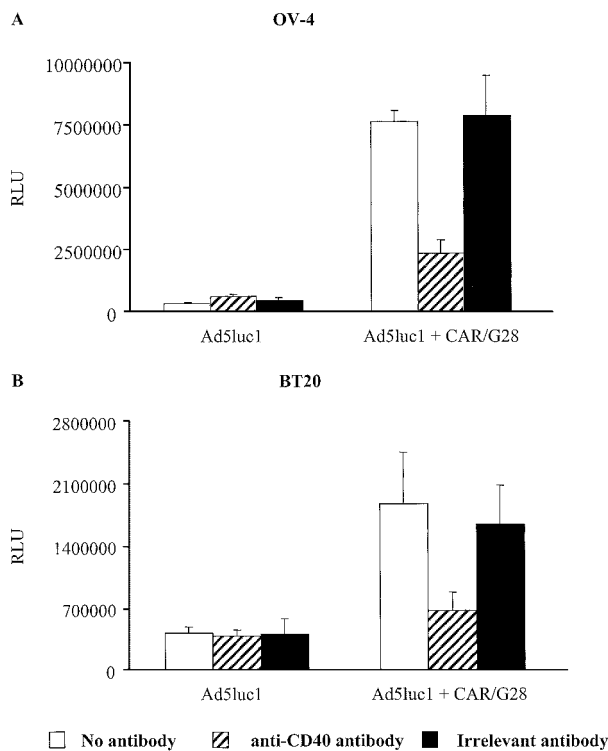


Fig. 4 Blockade of CD40 receptors decreases the infectivity of CD40 retargeted Ad in CD40+ cells. Cells were treated with growth media containing no antibodies (white columns), monoclonal anti-CD40 antibody (striped columns), or an irrelevant monoclonal antibody (black columns) before infections with Ad5Luc1 or Ad5Luc1 + CAR/G28. Luciferase activity was determined at 24 h after transduction, and error bars indicate ± 1 SD.

There is increasing recent evidence suggesting lack of the primary Ad receptor, CAR, on ovarian (3, 4) and other types of cancer cells (2). Unfortunately, CAR expression is the major factor determining infection efficacy with Ad serotype 5 (2, 7). Therefore, several approaches have been tried to circumvent dependence on CAR (6–9). Having established CD40 expression on ovarian cancer cells, we investigated the potential of this phenomenon for targeting Ad. First, cells were infected with three doses of nontargeted (Ad5Luc1) or CD40-targeted (Ad5Luc1 + CAR/G28) Ads (Fig. 2). With the CD40-negative HeLa cells (Fig. 1), CAR/G28 conferred no advantage in gene expression. (Fig. 2F). In contrast, all CD40-positive cell lines (Fig. 2, A–E) demonstrated notable enhancement in gene transfer efficacy when CD40-targeted virus was used. In comparison with Ad5Luc1 alone, gene transfer was increased 4-, 6-, 42-, 13-, and 8-fold for Hey, SKOV3.ip1, OV-4, OV-3, and BT-20 cells, respectively, when 100 pfu/cell Ad5Luc1/CAR/G28 was used (Fig. 2).

To confirm that increased gene expression was due to the retargeting moiety, a second set of experiments was performed, in which the viral dose was kept constant, whereas the amount of fusion protein was increased (Fig. 3). In HeLa cells (negative control), the increase of retargeting protein did not cause a dose response (Fig. 3F). With the CD40-positive cell lines, increasing

CAR/G28 resulted in a dose-dependent increase in luciferase expression (Fig. 3, A–E). With some cell lines, the higher amounts of fusion protein (100 and 150 ng) may have saturated the Ad fibers available for binding the native receptor CAR, resulting in plateauing of the transgene expression. However, with cells expressing high levels of CD40, such as OV-4, the full retargeting potential was not reached. These results suggested that utilization of CD40 for increased transduction allowed enhanced transgene expression in cells low in CAR, such as these ovarian cancer cells (7)

Conceivably, the augmentation of transgene expression seen in the experiments described in Figs. 2 and 3 was due to CAR/G28-mediated binding, followed by internalization with the usual penton base RGD-cellular integrin-mediated mechanism. Because ovarian cancer cells typically express low levels of CAR (4, 7), including the cells used here, a higher frequency of CD40 receptors (Fig. 1) thus allowed increased binding, entry, and consequent marker gene expression. To confirm the dependence of transgene expression on CAR/G28-CD40 interaction, we preincubated cells with an anti-CD40 antibody. This blocked binding of the CAR/G28-targeted Ad and reverted luciferase expression close to levels achieved without targeting (Fig. 4). An irrelevant antibody, anti-transforming growth factor $\beta 2$, had no effect on targeted transgene expression. Furthermore, these experiments excluded the remote possibility that mere binding of CD40 would increase transgene expression to the degree seen here (Fig. 4, AdLuc1, striped bars).

The results of this study suggest that ovarian cancer cell lines and patient samples express CD40. Therefore, ovarian cancer is a potential target for CD40L therapy, which has been shown to cause apoptosis and suppress tumor growth *in vitro* and in animal models (11, 13–15). Moreover, we propose and demonstrate the feasibility of a strategy whereby CD40 is used for targeted gene delivery with adenoviral vectors. Although more experiments are required to confirm the feasibility of CD40 targeting *in vivo*, utilization of tumor associated receptors, such as CD40, could help to increase transduction of target tissues, resulting in augmentation of the clinical efficacy of adenoviral cancer gene therapy approaches (5). Furthermore, this could reduce the total dose required for antitumor efficacy. Importantly, targeting complexes have also demonstrated the potential for reducing transduction of nontarget tissues including the liver, which is the main organ responsible for Ad clearance and therefore a potential source of side effects (19, 20). Yet another application could be the use of CD40-targeted Ads in immunotherapeutic approaches, *e.g.*, modification of dendritic cells more efficient with reduced viral doses (16).

In conclusion, we have demonstrated high expression levels of CD40 on ovarian cancer cell lines and clinical primary tumor specimens. Then, we used a fusion protein for targeting Ad vectors to CD40 and, in comparison with untargeted Ad, we observed increased transgene expression, which has been shown to correlate with increased Ad transduction efficiency (16). Targeting to tumor-associated receptors could help improve the efficacy while reducing the side effects of cancer gene therapy approaches.

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