Pharmacological Inhibition of β3 Integrin Reduces the Inflammatory Toxicities Caused by Oncolytic Adenovirus without Compromising Anticancer Activity

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Abstract

Adenoviruses have been clinically tested as anticancer therapies but their utility has been severely limited by rapid, systemic cytokine release and consequent inflammatory toxicity. Here, we describe a new approach to tackling these dangerous side effects. Using human ovarian cancer cell lines as well as malignant epithelial cells harvested from the ascites of women with ovarian cancer, we show that tumor cells do not produce cytokines in the first 24 hours following in vitro infection with the oncolytic adenovirus Δ922-947. In contrast, Δ922-947 does induce inflammatory cytokines at early time points following intraperitoneal delivery in mice with human ovarian cancer intraperitoneal xenografts. In these animals, cytokines originate predominantly in murine tissues, especially in macrophage-rich organs such as the spleen. We use a nonreplicating adenovirus to confirm that early cytokine production is independent of adenoviral replication. Using β3 integrin knockout mice injected intraperitoneally with Δ922-947 and β3 null murine peritoneal macrophages, we confirm a role for macrophage cell surface β3 integrin in this Δ922-947–induced inflammation. We present new evidence that co-administration of a cyclic RGD-mimetic–specific inhibitor of β3 integrin significantly attenuates the cytokine release and inflammatory hepatic toxicity induced by Δ922-947 in an intraperitoneal murine model of ovarian cancer. Importantly, we find no evidence that β3 inhibition compromises viral infectivity and oncolysis in vitro or anticancer efficacy in vivo. By enabling safe, systemic delivery of replicating adenoviruses, this novel approach could have a major impact on the future development of these effective anticancer agents. Cancer Res; 75(14). 2811–21. ©2015 AACR.

Introduction

Oncolytic adenoviruses replicate selectively within infected cancer cells, causing cell death. Viruses target immunity to infected malignant cells, whereas oncolysis activates T cells by releasing tumor-specific antigens (reviewed in refs. 1 and 2). The potential of oncolytic adenoviruses as an anticancer therapy has repeatedly been demonstrated and one virus, H101 (3), has been licensed in China. A consistent and worrying feature of systemic viral therapy is the cytokine release that occurs rapidly after viral administration. These cytokines cause dose-limiting inflammatory toxicities, which can be severe (4–8) and have hindered further investigation and clinical development of these promising anticancer agents.

During adenoviral infection, the viral fiber terminal knob protein anchors cell surface coxsackie adenovirus receptor (CAR). Arg-Gly-Asp (RGD) motifs in penton proteins at the base of the viral fiber also bind cell surface integrins, predominantly αvβ3 and αvβ5. Virions are internalized and transported along microtubules to the nucleus where viral replication takes place. Oncolytic viruses replicate selectively within cells with matched genetic defects. E1A-CR2–deleted adenoviruses, including Δ922-947 (9) and Δ24 (10), target cells with Rb checkpoint abnormalities, a defining feature of ovarian cancer (11, 12). In a recent phase I trial, an E1A-CR2–deleted adenovirus induced stable disease in 14 of 19 patients with advanced gynecological cancers (13).

Ovarian cancer, in particular the high-grade serous subtype, spreads by seeding across peritoneal surfaces, producing ascites that is rich in macrophages and inflammatory cytokines. This local inflammation has been implicated in tumor promotion (14), and clinical trials have suggested that inhibition of the cytokine TNFα may have therapeutic potential (15). We have shown that siluximab (CNT0328), an inhibitory monoclonal antibody to another cytokine, IL6, induces antitumor responses in patients with relapsed ovarian cancer (16). Because tumor spread outside the peritoneum is rare, ovarian cancer is amenable to therapies delivered by the intraperitoneal route. In oncolytic virotherapy, intraperitoneal rather than intravenous delivery can reduce systemic, cytokine-induced toxicity but local inflammation still causes severe abdominal side effects (17). Although various mechanisms have been described by which viruses initiate inflammation, the interplay between viruses and the microenvironment of the peritoneal cavity in ovarian cancer is poorly understood and the extent to which inflammation helps or hinders viral oncolytic efficacy remains unclear.
We have previously shown that the replication-selective, E1A-CR2-deleted adenovirus, d922-947, has more potent anticancer activity in vitro than wild-type (WT) adenovirus (18). In female nude mice with intraperitoneal human ovarian cancer xenografts, which recapitulate the cytokine and macrophage-rich peritoneal environment of human ovarian cancer, survival was significantly prolonged by intraperitoneal d922-947 but similar to the human experience with these agents, high levels of ascitic cytokines (unpublished) and marked hepatic toxicity were observed (18). We previously attempted to modulate these inflammatory side effects by combining oncolytic adenovirus with inhibitors of TNFα. Although this improved viral efficacy, liver toxicity still occurred (19).

In non–tumor-bearing mice, wild-type adenovirus 5 (Ad5-WT) has been shown to promote inflammation via viral RGD motifs binding β3 integrins on macrophages, including hepatic kupffer cells and splenic marginal zone macrophages (20). Cytokine expression was attenuated with an RGD-deleted adenovirus (20). We now describe a novel, clinically applicable approach to control the inflammatory side effects of oncolytic adenoviruses through pharmacological inhibition of β3 integrins in ovarian cancer. In line with the accumulated evidence from clinical trials, we confirm that oncolytic adenovirus causes rapid, systemic cytokine induction in vivo. In agreement with previously published data, we observed that cytokines originated predominantly in macrophage-rich supporting tissues rather than malignant epithelial cells and that cytokine expression and release was dependent on β3 integrins.

In a murine ovarian cancer model, we show for the first time that virus-induced systemic cytokines and hepatic toxicity can be reduced by pharmacological inhibition of β3 with the cyclic RGD-mimetic peptide, H2574, without compromising antitumor activity. H2574, also described as EMD 66203, c(RGDfV), is widely used preclinically as a specific inhibitor of β3 integrins. H2574 inhibits binding of vitronectin to αvβ3 integrin with an IC50 of 0.0025, whereas inhibition of αvβ5 is minimal (reviewed in ref. 21). We used H2574 to maximize the clinical applicability of our findings because its N-methylated analogue, cilengitide [EMD 121974, c(RGDfV)], is already in clinical use. In contrast to H2574, cilengitide does have inhibitory activity for αvβ5 but this is still more than 100-fold lower than that for αvβ3 (IC50 = 140 nmol/L compared with 1 nmol/L for αvβ3; ref. 22).

The novel pharmacological approach we describe here should enable the safe delivery of many viral anticancer agents and thus facilitate further clinical testing of many more of these promising therapies.

Materials and Methods

Cell lines, viruses, and chemicals

SKOV3ip1 cells (23) were obtained from Cancer Research UK (Clare Hall, UK). OVCAR4 (24) were obtained from Dr. R. Camalier (NCI-Frederick, MD). Murine WT, β3−/−, and β3+/+ endothelial cells were transformed with Polyoma Middle T Antigen (obtained from Dr. S. Robinson, University of East Anglia, Norwich, UK; ref. 25). SKOV3ip1 and OVCAR4 cells were cultured in DMEM plus 1% penicillin and streptomycin and 10% FCS and underwent 16 locus STR verification (DNA Diagnostics Centre, London, UK; June 10, 2014). Immortalized endothelial cells were grown in 1:1 DMEM (low glucose) and Ham’s F12 containing 20% FCS, 50 mg Heparin (Sigma-Aldrich), 100 mL Glutamax (Gibco), 1% penicillin and streptomycin, and 100 mg endothelial mitogen (AbD-Serotec).

d922-947 (9) and dCR2-pIX-dsRed (26) are already described. Ad-GFP is an E1 deleted, nonreplicating, type-5 adenovirus, expressing GFP under the CMV promoter. H2574 was purchased from Bachem. Cell survival was assessed by MTT assay (18).

RNA extraction, reverse transcription, and quantitative-PCR

Cultured cells and 30 mg murine tissue were lyzed with TRIzol (Invitrogen) and homogenized by pipetting (cultured cells) or with a T25 Ultrotissue homogenizer (tissue). After 5-minute incubation at room temperature, 200 μL chloroform was added per 1 mL of TRIzol. Samples were mixed, incubated (2 minutes, room temperature), and centrifuged (15 minutes, 1,400 rpm, 4°C). RNA was precipitated from the aqueous upper phase with 500 μL isopropanol at −20°C for 20 minutes. The sample was then applied to a RNeasy mini column (Qiagen) according to the manufacturer’s instructions. One microgram of RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies; 25°C, 10 minutes; 37°C, 120 minutes; 85°C, 5 minutes). cDNA was subjected to qPCR using Taqman Gene Expression Assay primers (Life Technologies). Expression was normalized to 18s RNA.

Protein extraction and cytokine ELISA

Cell supernatants were collected over time after adenovirus. Tissues were lyzed in 600 μL lysis buffer (150 mmol/L NaCl, 20 mmol/L TrisBase, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100) with Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics) and homogenized with a T25 Ultra tissue homogenizer. After 30-minute incubation on ice, samples were centrifuged (15 minutes, 13,000 rpm, 4°C) and protein in the lower liquid phase diluted to 5 mg/mL. Cytokines were measured using the Mesoscale Discovery System multiplex plates (Mesoscale Discovery).

Immunofluorescence

Cells were grown on poly-L-lysine-coated coverslips and fixed in 3.7% formaldehyde (10 minutes, room temperature). Cells were washed, permeabilized in 0.5% Triton for 10 minutes, and then blocked in 10% goat serum (1 hour, room temperature). Cells were incubated with primary antibody [EpCam 1:500 (AbCam ab20160)] for 1 hour at room temperature in 0.2% PBS-Tween + 3% BSA, washed and then incubated with secondary antibody [Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes; Invitrogen)] for 1 hour at room temperature. Coverslips were stained with DAPI (Invitrogen) and mounted onto glass slides.

Viral internalization in murine peritoneal macrophages: PCR

Peritoneal macrophages were harvested from WT and β3−/− mice as described in animal models. A total of 5 × 10⁵ murine peritoneal cells were seeded in 6-cm plates. After overnight incubation, cells were washed in cold RPMI + 1% BSA and infected with d922-947 (MOI 10) on ice for 1 hour. Unbound virus was removed by washing in cold RPMI. Cells were then incubated at 37°C for 1 hour. DNA was harvested from cell pellets using a QiapDNA Extraction Kit (Qiagen). Hexon was quantified by qPCR against a standard curve of viral particles (5'-GTTGGCCATTACCCITTGACTTC-3', 3'-GGTTAAGCCGGCGTGATT-CATT-5', probe: [6FAM]CTGTACGGCTCGTGG [TAM]).
Viral internalization in murine peritoneal macrophages:
confocal microscopy

Peritoneal macrophages were harvested from WT and β2RI-/- mice as described in Animal Models. A total of 5 × 10⁶ murine peritoneal cells were seeded overnight on poly-λ-lysine coated coverslips. After overnight incubation, cells were washed in cold RPMI + 1% BSA and infected with dC2R2-pIX-dsRed (50,000 vp/cell) on ice for 1 hour. Unbound virus was removed by washing in cold RPMI. Cells were fixed in cold 95% ethanol and 5% acetic acid (10 minutes, room temperature) or the infection was continued at 37°C for a further 1 hour prior to washing and fixing. Fixed cells were blocked with 3% BSA for 30 minutes and then incubated at room temperature for 60 minutes in anti-CD11b-AlexaFluor488 (1:100; eBioscience). Cells were stained with DAPI (1:10,000; 1 mg/mL; Invitrogen), washed, and mounted onto glass slides.

Confocal microscopy

Confocal was performed with an inverted Zeiss LSM 510 META laser-scanning microscope with a Plan Apochromat 63×/1.4 Oil objective. DAPI was detected with a 405 nm laser. Alexa Fluor 488 with a 488 nm laser and dsRed with a 543 nm laser. Images were acquired in the x-, y-, and z-direction with a line average of 4. Z-sections were acquired at optimum interval levels with sections of 0.43 μm. maximal intensity Z-projections were assembled with the LSM5 image browser software.

Flow cytometry in murine peritoneal cells

Peritoneal macrophages were harvested from WT and β2RI-/- mice as described in Animal Models. For adenoviral receptors, cells were cultured with F, fragments for 15 minutes before addition of conjugated antibodies [CAR-PE (Millipore), β2RI-PE (eBioscience), β3RI-PE (eBioscience), and IgG-PE (eBioscience)] in PBS + 1% BSA for 1 hour on ice in the dark. Samples were analyzed on a BD FACSCalibur (BectonDickinson).

To quantify macrophage cell surface markers using multichannel flow cytometry, cells were incubated with a viability dye (eBioscience) for 30 minutes in the dark on ice and then blocked with F, fragments for 15 minutes. Conjugated antibodies [CD45-FITC (eBioscience), CD11b-eFluor450 (eBiosience), mMR-PE/CD206 (BioLegend), Ly-6C-APC (eBioscience), Ly-6C-AlexaFluor700 (eBioscience), CsfR1/CD115 (eBioscience), and F4/80-PE-Cy5 (eBioscience)] were added for 15 minutes on ice in the dark. Cells were washed and fixed in 2% formaldehyde. Samples were analyzed on a BD LSRFortessa (BectonDickinson).

Animal models

Experiments complied with National Cancer Research Institute guidelines and were conducted under UK government Home Office personal (70/19,108) and project (70/7,263) licenses. Local approval was obtained from Queen Mary University of London (PAC60-3). β2RI-/- mice (B6.129S2-Igκbα<tm1Gwi>/Seml) and WT controls (obtained from Prof K. Hodivala-Dilke, Barts Cancer Institute, London, United Kingdom; ref. 27) were bred in-house and used between 6 and 8 weeks old. Intraperitoneal ovarian cancer xenografts were created by inoculating 5 × 10⁶ SKOV3ip1 cells intraperitoneally in 6-week-old CD1nu/nu female mice. For most experiments, 10¹⁰ particles of dl922-947 were injected intraperitoneally in 400 μL PBS. For virus efficacy, dL922-947 (5 × 10³ particles) was injected daily for 5 days in 400 μL PBS. Five milligrams of H2574 was dissolved in DMSO, diluted in H₂O, and then injected intraperitoneally (total volume 400 μL) or into an Alzet pump (Cupertino, total volume 210 μL). Alzet pumps were inserted subcutaneously and removed 2 weeks later under isofluorane inhalation anesthesia. Mice were assessed for weight, general health, and accumulation of ascites and were killed according to UK Home Office guidelines.

At necropsy, murine peritoneal macrophages were harvested by peritoneal lavage with 10 mL cold PBS. Samples were treated with Red Blood Cell Lysis Buffer (Sigma-Aldrich), washed, and cultured in RPMI, 1% penicillin and streptomycin, and 10% FCS. Murine blood was obtained by cardiac puncture. Serum liver function was analyzed at the Royal Veterinary College (Hertfordshire, UK) and cytokines were quantified with the Mesoscale Discovery System. Murine tissue was macrodissected at postmortem and either snap frozen in liquid nitrogen or fixed in 10% formaldehyde for the Western blot analysis. Four-micrometer sections were cut from formalin-fixed, paraffin-embedded tissues. All pathology and immunohistochemistry slides were interpreted and scored by a pathologist ‘blind’ to the experimental groups.

Statistical analysis

Prism 6.0 (GraphPad) was used for statistical analysis. Data are presented as mean ± SD throughout and the unpaired t test was used for all experiments except for analysis of changes over time for which two-way ANOVA was used. P < 0.05 is considered statistically significant.

Results

Oncolytic adenovirus does not induce early cytokine production in ovarian cancer cells

To explore the kinetics of early adenovirus-induced cytokine release, we used SKOV3ip1 cells (high β2RI, low CAR), because we had previously shown that these cells can produce high levels of TNFα and IL6 (19). Cytokine production by SKOV3ip1 cells was negligible for the first 24 hours after dl922-947 infection (Fig. 1A and B). Infecting cells with Ad5-WT or higher viral doses did not increase cytokine protein release compared with dl922-947 MOI 10 (Supplementary Fig. S1). To demonstrate that this observation was not unique, we evaluated another ovarian cancer cell line: OVCAR3 (high β2RI, moderate CAR). We again found no increase in cytokines in the first 24 hours (Fig. 1A and B). We also investigated tumor cells harvested from the ascites of two women with (high-grade serous) ovarian cancer (Fig. 1C). In these ascitic cells, transcription of IL6, IL8, and TNFα did not increase above baseline for the first 24 hours after ex vivo infection with dl922-947 (Fig. 1D).

Oncolytic adenovirus induces cytokines in murine tissues at early time points.

To model the adenovirus-induced inflammatory response in more clinically relevant models, we created intraperitoneal...
SKOV3ip1 xenografts in female nude mice. Mice with established tumors received a single intraperitoneal injection of $d_{922-947}$ ($10^{10}$ particles). Cytokines were quantified in blood, intraperitoneal tumors, spleen, and liver (Fig. 2). All cytokines tested increased in the blood within 1 hour (Fig. 2A). Using species-specific reagents, we found that circulating cytokines originated predominantly in murine tissues rather than the human xenografted SKOV3ip1 cells. This was true for all cytokines (Fig. 2A). To determine the origin of these cytokines, mRNA was quantified in excised tissues. Although adenovirus did not induce early cytokine release in SKOV3ip1 cells in vitro, human cytokine mRNA was identified in intraperitoneal SKOV3ip1 tumors (Fig. 2B). In a separate experiment, cytokine mRNA correlated closely with cytokine protein isolated from the same intraperitoneal tumors (Supplementary Fig. S2). This suggests that cytokine production by tumor cells is enhanced by interaction with the tumor stroma or in this case the murine component of the xenograft. Tumor stroma appears also to be an important source of inflammatory cytokines since murine cytokine mRNA in excised tumors exceeded human mRNA for several cytokines (IL6, IL10, and TNFα; Fig. 2B). Murine spleen and liver were also rich sources of inflammatory cytokines. Although the profiles of individual cytokines differed in the two organs, cytokines peaked in both spleen and liver at 3 hours and had largely returned to baseline 24 hours after adenovirus (Fig. 2C and D).

Cytokine induction correlates with viral presence

The adenoviral replication cycle takes at least 24 hours and is severely attenuated in murine cells (28, 29). Our observations that in vivo cytokine release originates in murine tissue and occurs at early time points following intraperitoneal injection imply that adenovirus-induced cytokine release is independent of viral...
Adenovirus causes rapid cytokine induction in murine tissues. Female CD1nu/nu mice with intraperitoneal SKOV3ip1 tumors were injected intraperitoneally with d922-947 (10^10 particles). Samples were analyzed in triplicate and data are presented as mean ± SD (n = 3–4).

A, cytokine protein in murine serum using mouse and human-specific plates shown relative to 0 hours. Mouse and human cytokines are compared (*, P < 0.05; **, P < 0.01; ***, P < 0.001). hpi, hours postinfection. B, qRT-PCR analysis of tumor. Expression at 1, 3, 6, and 24 hours is shown relative to expression at 0 hours. Mouse and human cytokines are compared (*, P < 0.05; **, P < 0.01; ***, P < 0.001). hpi, hours postinfection. C and D, qRT-PCR analysis of spleen (C) and liver (D). Cytokines at 1, 3, 6, and 24 hours are shown relative to 0 hours after intraperitoneal d922-947.

To explore this further, spleen and tumor were analyzed by immunohistochemistry following a single dose of d922-947 in mice bearing SKOV3ip1 xenografts (Fig. 3Ci). E1A was the first adenoviral protein to be expressed. As expected, we only found E1A expression in human tumor tissues and never in murine tissues (Fig. 3Ci). In contrast to cytokines, which were first seen at 1 hour and had resolved by 24 hours, E1A was first identified at 6 hours (Fig. 3Ciii) and persisted for up to a week (Fig. 3Ciiii and Supplementary Fig. S3). Hexon, the major structural protein of the adenoviral coat is indicative of viral presence rather than active replication. In the spleen, discrete hexon staining was first seen 1 hour postadenoviral injection (Fig. 3Di). By 3 hours, the staining was more diffuse and widespread (Fig. 3Di ii) and by 12 hours it had resolved (Fig. 3Di iii) mirroring the kinetics of cytokine release.

These findings suggest that cytokines are induced by adenoviral presence rather than replication.

Cytokine induction by oncolytic adenovirus is β3 integrin–dependent

In non–tumor-bearing mice, WT adenovirus induces cytokines via β3 integrin–expressing macrophages (20). Having shown that marked cytokine production occurs in macrophage-rich tissues such as liver and spleen, we explored the role of β3 integrins following oncolytic adenovirus in the malignant setting. First, we injected WT and β3−/− mice with a single dose of d922-947 intraperitoneally (10^10 particles). Immunohistochemistry identified hexon in the spleens of WT mice 3 hours postinjection (Fig. 4A). In contrast, no hexon was identified in the spleens of β3−/− mice (Fig. 4A and Supplementary Fig. S4). Cytokine transcription was significantly higher in the spleens of WT compared with β3−/− mice (Fig. 4B), indicating that β3-mediated viral uptake induces splenic cytokine transcription. This was most marked for IL1β (P < 0.05), IL6 (P < 0.05), and TNFα (P < 0.001). There was no difference in baseline circulating cytokines in the two genotypes, but adenovirus-induced cytokines were significantly higher in the circulation of WT compared with β3−/− mice (P < 0.05 IL1β and IL6; P < 0.01 TNFα; Fig. 4C). Although cytokine induction was attenuated in β3−/− compared with WT animals, some
cytokines were induced in $\beta_3^{-/-}$ mice, most notably keratinocyte chemoattractant (KC; murine homologue of IL8), confirming that $\beta_3$ is not the only mediator of the virus-induced inflammatory response.

Because ovarian cancer is predominantly a disease of the peritoneum, we investigated peritoneal cells in WT and $\beta_3^{-/-}$ mice. In both genotypes, these cells showed strong expression of CD11b (WT: 70.1 ± 13.1% of live cells, $\beta_3^{-/-}$: 76.1 ± 12.6%) and F4/80 (WT: 51.3 ± 14.9% of live cells, $\beta_3^{-/-}$: 51.9 ± 6.8%), indicating that they were predominantly macrophages (Fig. 5A). As expected, expression of $\beta_3$ was significantly higher in peritoneal cells from WT compared with $\beta_3^{-/-}$ mice ($P < 0.001$) but $\beta_3$ and CAR were negligible in both genotypes (Fig. 5B). Peritoneal cells were infected ex vivo with $\Delta B_{22}$-947 or vehicle and cytokine production measured 6 hours later (Fig. 5C). With the exception of KC, in both genotypes the addition of virus significantly increased cytokine production compared with control (WT: IL6, $P < 0.01$; IL1β, IL-10, TNFα, $P < 0.001$; and $\beta_3^{-/-}$: IL10, $P < 0.01$; IL1β, IL-6, TNFα, $P < 0.001$). However, release of all cytokines over time following adenovirus was significantly attenuated in $\beta_3^{-/-}$ compared with WT cells (IL6, $P < 0.01$; IL1β, KC, IL10, TNFα, $P < 0.001$).

We then tested whether cytokine release could be reduced by pharmacological inhibition of $\beta_3$ integrin. Peritoneal cells from WT mice were treated ex vivo with $\Delta B_{22}$-947 together with the cyclic RDG-mimetic inhibitor of $\beta_3$ integrin, H2574. The addition of H2574 caused a significant reduction in IL1β ($P < 0.05$), IL10 ($P < 0.01$), and TNFα ($P < 0.001$) but $\beta_3$ integrin is not essential for adenoviral cytotoxicity

$\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins are known to act as secondary receptors during adenoviral infection. Pharmacological inhibition of these integrins might therefore have the undesirable effect of reducing adenoviral infectivity and thus anticancer activity. In murine peritoneal cells infected ex vivo with $\Delta B_{22}$-947, PCR for adenoviral hexon showed that, whereas adenovirus was internalized by $\beta_3^{-/-}$ cells, this was lower than in WT cells (Fig. 6A). To explore this, we used a fluorescent capsid-labeled adenovirus, $\Delta C R 2$-pIX-dsRed (26) and demonstrated adenovirus at the cell surface after infection at 4°C followed by viral internalization when cells were warmed to 37°C in both genotypes (Fig. 6B).

Next we tested whether integrins were required for adenoviral cytotoxicity in murine peritoneal cells from $\beta_3^{-/-}$ and WT mice (Fig. 6C). We also used immortalized endothelial cells from $\beta_3^{-/-}$

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**Figure 3.** Adenovirus-induced cytokine production correlates with viral presence. Female CD1nu/nu mice with intraperitoneal SKOV3ip1 tumors were injected intraperitoneally with the nonreplicating virus Ad-GFP (10$^{10}$ particles) or with vehicle (n = 3–5). hpi, hours postinfection. A, cytokine protein in murine serum relative to 0 hours. Ad-GFP and vehicle are compared. Samples were analyzed in triplicate and mean ± SD is shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). B, qRT-PCR analysis of spleen. Expression at 1, 3, and 6 hours is shown relative to 0 hours. Ad-GFP and vehicle are compared. Samples were analyzed in triplicate and mean ± SD is shown (*, $P < 0.05$; **, $P < 0.01$; ***., $P < 0.001$). C and D, female CD1nu/nu mice with intraperitoneal SKOV3ip1 tumors were injected intraperitoneally with $\Delta B_{22}$-947 (10$^{10}$ particles). Representative images are shown following immunohistochemistry for adenoviral EIA in intraperitoneal tumors (C; tumor; S, spleen; scale bar, 50 µm) and Hexon protein in murine spleen (D; scale bar, 50 µm (i and ii) or 100 µm (iii)).
and WT mice, as well as immortalized endothelial cells created from β3/C0/C0 mice, which overexpress human β3 (hβ3+; Fig. 6D). In all cells, cytotoxicity was not influenced by β3 integrin expression. We then investigated whether β3 inhibition reduced adenoviral cytotoxicity in human cancer cells. Two ovarian cancer cell lines with high β3 integrin expression, SKOV3ip1 (low CAR) and OVCAR4 (moderate CAR), were treated with dl922-947 together with H2574. The addition of H2574 did not impair viral cytotoxicity in either cell line (Fig. 6E). Together, these experiments confirm that inhibition of adenoviral uptake was not a concern, supporting further testing of the dl922-947 and H2574 combination in vivo.
**β3 inhibition reduces adenoviral inflammatory toxicity in a murine model of ovarian cancer**

Female CD1nu/nu mice with SKOV3ip1 xenografts were treated with a single intraperitoneal dose of dl922-947 (10^10 particles) and H2574 (5 mg). In a separate experiment, mice received our standard therapeutic viral dosing regimen of 10^9 particles intraperitoneally daily for 5 days (18), together with a subcutaneous pump infusing H2574 (5 mg/2 weeks). Vehicle controls were used for all experiments. In the single-dose experiment, mice were sacrificed 6 hours after treatment, whereas mice with subcutaneous pumps were sacrificed either at 4 weeks or when they reached UK Home Office endpoint. At 6 hours, cytokine levels were lower in mice that had received H2574, reaching statistical significance for all cytokines by 4 weeks (Fig. 7A).

To determine whether this H2574-induced reduction in circulating cytokines correlated with diminished inflammatory toxicities, livers were examined. As early as 6 hours postinjection, eosinophilic degeneration, indicative of viral hepatotoxicity, was apparent in dl922-947–treated mice but not in mice treated with dl922-947 and H2574 combined (Fig. 7B). Liver function tests upheld this observation as both bilirubin and alanine transaminase were reduced by co-administration of dl922-947 with H2574 (Fig. 7C), although this did not reach statistical significance. This experiment was not powered to detect differences in survival. However, as an indication of antitumor efficacy, we weighed all residual peritoneal tumor in the small number of mice that reached legal limits. In accordance with our in vitro findings, the combination of dl922-947 and H2574 did not reduce anticancer efficacy compared with dl922-947 alone (Fig. 7D).

**Discussion**

Systemic inflammation induced by oncolytic adenoviruses is a major limitation to the clinical development of these promising anticancer agents. Here we show in primary malignant cells, ovarian cancer cells lines, genetically modified mice and ovarian cancer xenografts that the oncolytic adenovirus, dl922-947, causes rapid induction of inflammatory cytokines that originate primarily in macrophage-rich host tissues. We confirm that cytokine release and the associated inflammatory hepatic toxicity is β3 integrin–dependent and show here for the first time that these
toxicities can be controlled by pharmacological inhibition of β3 integrins without obviously compromising anticancer efficacy.

Ovarian cancer is characterized by a tumor-promoting network of cytokines (14). We previously showed that oncolytic adenoviruses, including d922-947, induce TNFα in ovarian cancer cells between 24 and 72 hours (19). However, the clinical experience with systemic adenovirus suggests that cytokine release occurs much earlier (5, 6). Most notably, in one highly publicized death, the patient had already developed advanced symptoms of systemic adenovirus infection (5, 6). The existence of these varied mechanisms explains our observation that, although inhibition of β3 integrins reduces inflammatory cytokine release, it does not completely abrogate the cytokine response. Nonetheless, the interaction between adenoviral coat RGD and β3 integrins on the macrophage surface is likely to be a major source of the systemic inflammatory cytokines reported here.

In ovarian cancer cell lines in vitro, we did not observe an increase in cytokines in the first 24 hours following adenovirus. In contrast, the very early cytokine production seen in murine xenografts more accurately reproduced the human experience and was associated with the inflammatory hepatotoxicity and elevation of liver enzymes that is well described following adenovirus infection (30–33). The differences we observed in ovarian cancer cells in vitro and in vivo implicate tumor/stroma interactions in the initiation of this inflammatory response. In support of this, adenoviral induction of proinflammatory cytokines has been shown to be augmented when malignant epithelial cells are cocultured with macrophages in vitro (34).

In tumor-bearing mice we found that virus-induced cytokines arose predominantly in murine tissues rather than the implanted human tumor cells. The cell types that are responsible for innate responses to adenovirus such as macrophages and dendritic cells (33, 35, 36) are present at high levels in liver, spleen, and peritoneum and are therefore likely to be a major source of the systemic inflammatory cytokines reported here.

Our finding that adenoviral cytokine induction is not dependent on viral replication and gene expression is also in keeping with a large body of evidence, acquired in non–tumor-bearing organisms, implicating a range of potential viral sensors as initiators of the innate immune response (20, 37, 38). In addition to the interaction of viral RGD with β3 integrins previously reported with Ad5–WT in the nonmalignant setting (20), internalized viral DNA is sensed by toll-like receptors, which stimulate dendritic cells and macrophages to release type I interferon. Viral DNA has also been shown to cause an inflammatory response in macrophages via maturation of IL1β within a cytosolic complex known as the inflammasome (38).

A, cytokines in murine serum were analyzed 6 hours and 4 weeks posttreatment by mesoscale. Mean ± SD (**, P < 0.01; *** P < 0.001; **** P < 0.0001). B, hematoxylin and eosin staining of murine livers 6 hours post-intraperitoneal d922-947 and H2574. C, liver function tests on murine serum 6 hours post-intraperitoneal treatment and when mice reached UK Home Office endpoint. BR, serum bilirubin; ALT, alanine transaminase (mean ± SD). D, weights of macrodissected intraperitoneal tumors when mice reached UK Home Office endpoint (mean ± SD).
particularly attractive for targeted therapeutic manipulation. Mutant RGD adenoviruses have been used to abrogate viral binding to β3 integrin (20), but the need to create RGD mutant versions of all clinically applicable oncolytic adenoviruses is problematic in terms of clinical development. Others have attempted to manipulate the inflammatory response with immunosuppressive agents such as cyclophosphamide (39) and cyclosporine (40). Both of these drugs have myriad other effects, including a paradoxical immunostimulatory effect of cyclophosphamide (41). Pharmacological inhibition of integrin binding to β3 is associated with a reduction in cytokine production by β3 null murine peritoneal cells (42). Several drugs targeting this pathway are currently undergoing testing as anti-cancer therapies (43) and cilengitide, the most widely studied inhibitor of β3 integrin (44), has shown some clinical activity in glioblastoma (45, 46). In ovarian cancer, a small case series identified β3 integrin expression in normal ovarian epithelium and low-grade tumors but in poorly differentiated cancers, expression was lost (47). In another series, β3 integrin was associated with a favorable prognosis in ovarian cancer (48) and because more than 80% of human ovarian tumors are high grade, poor prognostic malignancies, it is not surprising that cilengitide has not been clinically tested in ovarian cancer.

In this first report of combination therapy with an oncolytic adenovirus and H2574, a cilengitide-like inhibitor of β3 integrin, we significantly reduced the inflammatory cytokines and hepatotoxicity caused by systemic adenovirus. Importantly, in vitro and in the small numbers of mice tested, the anticancer activity of d1922-947 did not appear to be compromised by the addition of H2574. In summary, oncolytic adenoviruses have proven anticancer activity and combination with β3 integrin inhibitors could enable safer delivery and facilitate further clinical development of these promising agents.

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

Authors’ Contributions
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Wang, I.A. McNeish, M. Lockley
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