Antitumoral Immune Response by Recruitment and Expansion of Dendritic Cells in Tumors Infected with Telomerase-Dependent Oncolytic Viruses

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Abstract

Virotherapy can potentially be used to induce tumor-specific immune responses and to overcome tumor-mediated tolerance mechanisms because apoptotic tumor cells are exposed together with viral danger signals during oncolysis. However, insufficient numbers of dendritic cells (DC) present at the site of oncolysis can limit a tumor-specific immune response and the resulting therapeutic benefit. We investigated MHC class I peptide–specific immune responses against model antigens ovalbumin (OVA) and hemagglutinin (HA) in mouse tumor models that support efficient replication of the oncolytic adenovirus hTERT-Ad. Virotherapy resulted in peptide-specific cytotoxic T-cell responses against intracellular tumor antigens. Triggering of DC and T-cell infiltration to the oncolytic tumors by macrophage inflammatory protein 1α (MIP-1α, CCL3) and Fms-like tyrosine kinase-3 ligand (Flt3L) enhanced both antitumoral and antiviral immune responses. Although immune-mediated clearance of the virus can restrict therapeutic efficacy of virotherapy, MIP-1α/Flt3L–augmented hTERT-Ad virotherapy inhibited local tumor growth more effectively than virotherapy alone. In agreement with the hypothesis that immune-mediated mechanisms account for improved outcome in MIP-1α/Flt3L virotherapy, we observed systemic antitumoral effects by MIP-1α/Flt3L virotherapy on uninfected lung metastasis in immunocompetent mice but not in nude mice. Furthermore, MIP-1α/Flt3L virotherapy of primary tumors was strongly synergistic with tumor DC vaccination in inhibition of established lung metastasis. Combined viroimmunotherapy resulted in long-term survival of 50% of treated animals. In summary, improvement of cross-presentation of tumor antigens by triggering of DC and T-cell infiltration during virotherapy enhances antitumoral immune response that facilitates an effective viroimmunotherapy of primary tumors and established metastases. [Cancer Res 2009;69(4):1448–58]

Introduction

Tumor-specific oncolytic viruses are a class of anticancer agents that have the unique property of tumor-dependent self-propagation with the ability to specifically kill tumor but not normal cells. They are capable to destroy human tumors (1) and probably also lymph node metastases (2) in immunodeficient nude mice by intratumoral spreading of infection and tumor-wide oncolysis. Recently, attention has been paid to the role of virotherapy in promoting antitumor immune response (3–8). Although cytotoxic T cells directed against tumor antigens have been frequently observed in animal tumor models and cancer patients, immunotherapy with tumor antigens is usually ineffective against established tumors in animal experiments or clinical studies. Most likely, limited efficacy of cancer immunotherapy can be attributed to the strong tumor-specific immunotolerance that is induced by growing tumors (9–11). Tumor-specific replicating viruses can be used to overcome tumor-mediated tolerance mechanisms, as their replication provides danger signals for the immune system at the oncolytic site and simultaneously generates an immunogenic environment. Usually, the tumor bed produces various factors that inhibit maturation of dendritic cells (DC) and leads to tumor-specific immunotolerance. In virally infected tissue, antigen uptake by DCs is associated with Toll-like receptor (TLR) stimulation, which is essential for final maturation of DCs, allowing them to prime and boost T cells by effective cross-priming of cellular antigens (12, 13). Studies with murine tumor models confirmed the induction of cytotoxic CD8+ T cells against tumor antigens due to oncolytic virotherapy (6, 7). There is also evidence that virus-induced killing of human tumors directly promotes tumor immune recognition by DCs in vitro (14, 15).

Virotherapy results in exposure of viral antigens in draining lymph nodes and peripheral tissue by dissemination of viral particles, and also to a strong local production of both virus- and tumor-associated antigens by oncolysis. Thus, induction of a tumor-specific immune response could be compromised by an inadequate number of DCs at the site of oncolysis. Triggering of DC migration to infected tumor tissue and subsequent expansion of mature DCs can be useful for efficient uptake of apoptotic cells by DCs and improved cross-presentation of tumor antigens during oncolytic virotherapy. It has been shown that immature DCs are attracted by different chemokines such as RANTES (CCL5), MIP-1α (CCL3), MIP-1β (CCL4), and MIP-3α (CCL20; refs. 16–20), whereas granulocyte macrophage colony-stimulating factor (GM-CSF) and Flt3L are potent growth factors that have been reported to expand DCs in vivo (21–25).

In this study, we established syngeneic mouse tumor models and generated MIP-1α– and Flt3L–expressing adenoviral vectors to investigate the contribution of DC attraction and expansion on the induction of virus- and tumor-specific immune responses during
oncolytic virotherapy. We observed a strong synergistic effect of MIP-1α and FLT3L in infiltration of tumors with DCs and T cells. Compared with gene therapy with nonreplicating adenoviruses, Ad-MIP-1α/Ad-FLT3L–supported oncolytic virotherapy resulted in enhanced maturation of infiltrated DCs and improved immune response against tumor-specific antigens. Although expression of MIP-1α/FLT3L did not only cause an enhanced tumor-specific T-cell response but also increased antiviral immune responses, virotherapy with MIP-1α/FLT3L inhibited local tumor growth more effectively compared with virotherapy alone. Additionally, MIP-1α/FLT3L–augmented virotherapy of s.c. tumor nodules significantly inhibited growth of uninjected lung metastasis and acts strongly synergistic with tumor DC vaccination, demonstrating the systemic efficacy of the viroimmunotherapy-mediated antitumor immune response.

Materials and Methods

Mice. Eight- to 10-wk NMRI-nu/nu mice and C57BL/6 and DBA/2 female mice were obtained from Charles River, Germany. All in vivo experiments were conducted according to the German legal requirements with approval of Medical School of Hannover animal facility.

Cells. The human cell lines HepG2, HEK293 cells and the murine cell lines JC and KLN-205 were obtained from the American Type Culture Collection. The lung adenocarcinoma cell line CMT-64 was a kind gift from Wilfred Jefferies (Vancouver, Canada). To obtain cell lines with stable expression of model antigens, KLN-205 and CMT-64 cells were transduced by MoMLV retrovirus containing an HA-IRESEGFP or OVA-IRESEGFP expression cassette and sorted for green fluorescence. Cells were maintained in growth medium (DMEM + Glutamax, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 100 units/mL penicillin, and 100 μg/mL streptomycin (Seromed) at 37°C in 5% CO₂.

Antibodies and peptides. The peptides HA (IYSTVASSL, H₂-Kᵈ), LacZ (TPHPARIGL, H₂-L⁵), OVA (SIINEFKL, H₂-K⁷), and LacZ (DAPIYTNV, H₂-K⁷) were obtained from Proimmune. The following antibodies were obtained from eBioscience, except otherwise indicated: CD16/CD32 (mouse Fc Block, BD Pharmingen), hamster IgG1, κ isotype control (BD Pharmingen), IFN-γ clone AN-18, bovine IFN-γ clone R4-62, purified hamster CD3ε clone 145-2C11 and CD11c clone N418 (BD Pharmingen), mouse anti-hamster cocktail (BD Pharmingen), and CD40 clone HM40-3.

Construction and production of adenovirus. The Ad-Ad virus is a conditionally replicating adenovirus that was previously shown to be capable of replicating in all human tumor cells but not in primary somatic cells (26).

Adenoviral plasmids were constructed using the Ad-Easy system (BD Bioscience-Ad-1 from Stratagene as backbone vector). Plasmids containing mouse MIP-1α and mouse FLT3L were a kind gift of Dan H. Barouch (Harvard Medical School, Boston, MA). Plasmids were digested and the isolated fragments containing MIP1-α and Fli3L, respectively, were ligated into pShuttle-CMV vector before recombination into Ad-Easy 1 vector. Ad-luciferase and Ad-GFP were likewise constructed. Ad-LacZ was kindly provided by Dr. D. Brenner (Chapel Hill, NC). Adenoviral particles were obtained as described previously (26). Infectious titers of adenovirus preparations were determined by Rapid Titer Kit (BD Biosciences) according to the manufacturer's protocol. In all adenoviral preparations, endotoxin contaminations were excluded by the LAL test kit (Chromogenix). Virus preparations were stored at −20°C in 25% glycerol, 10 mM/L Tris-HCl (pH 7.4), and 1 mM/L MgCl₂, and dialysed against physiologic medium before application.

Luciferase assays and bioluminescence imaging for assessment of viral replication. KLN-205-HA or CMT-64-OVA cells (6 × 10⁴) were seeded in 60-mm dishes 24 h before transfection. The cells were transduced with multiplicity of infection (MOI) 20 of Ad-LacZ, hTert-Ad, and Ad-luciferase. Cell layers were harvested at the indicated time points following infection. Cellular extracts were obtained according to standard procedures and measured for luciferase activity in a Berthold Lumat LB9501. Luciferase activity was normalized against the protein concentration determined by the Bio-Rad protein assay.

For in vivo investigations of viral replication, subcutaneous tumors of KLN205-HA and CMT64-OVA were established. When tumors reached an approximate size of 10 mm in diameter, tumors were injected with 1 × 10⁷ plaque-forming unit (pfu) hTERT-Ad or control virus in combination with 3 × 10⁷ pfu Ad-luciferase. For in vivo imaging 4.5 mg of t-luciferin (Applichem) in 100 μL of RPMI medium were co-injected with ketamine and Rompun i.p. After 20 min, images were captured with the IVIS imaging system series 200 by using living image2.5 software (Xenogen). Photon emission values were calculated as recommended by the manufacturer.

Histologic methods. Tissues were fixed in paraffin and sections were stained with H&E. For immunohistochemistry, acetone-fixed 5-μm sections were air dried for 10 min then washed in PBS for 5 min. Sections were treated with Triton-X and glycine for 5 min and blocked with FCS for 30 min. Sections were then incubated with the primary antibodies (hamster anti-mouse CD3 and CD11c) at 4°C overnight. Subsequently, the slides were washed thrice with PBS and then incubated with secondary antibody (biotin anti-hamster) for 1 h. Finally, sections were incubated with streptavidin-conjugated Alexa 488 and analyzed by fluorescence microscopy.

For immunohistochemical analyses of adenoviral hexon protein, 7-μm slices were prepared from cryopreserved tissue specimen and fixed in 4% paraformaldehyde in PBS. As the primary antibody, a goat anti-hexon antibody ( Fitzgerald) was used. A secondary antibody (Cy3-conjugated anti goat) was used to detect hexon expression by fluorescence microscopy.

DNA extraction and quantitative real-time PCR. Subcutaneous tumors and lung metastases were excised 48 h after viral injection of primary tumors and DNA was isolated using QiAamp DNA Mini Kit (Qiagen). Quantitative real-time PCR was performed on an ABI 7300 using qPCR Mastermix Plus (Eurogentec). All values were normalized to 18S; therefore, 18S Genomic Control Kit FAM-TAMRA (Eurogentec) was used according to the manufacturer's protocol. Late hexon gene was detected as described (27).

In vivo CTL assay. Cytotoxic activity and specificity of CTLs were determined by using a carboxy fluorescein succinimidyl ester (CFSE)–based in vivo killing assay. Target cells were prepared by using syngeneic splenocytes. After BRC lysis, splenocytes were pulsed with the SIINEFKL peptide and incubated for 30 min at 37°C. OVA peptide-pulsed splenocytes were then labeled with an equivalent concentration of the LacZ peptide and subsequently stained with 0.2 μmol/L CFSE. Both populations were then mixed 1:1 and 2 × 10⁵ cells per mouse were injected i.v. Untreated mice served as control. Specific in vivo cytotoxicity was determined by flow cytometric analysis of CFSE-positive splenocytes 18 h after target cell injection. The ratio between CFSElow and CFSEhigh cells was calculated to determine effector cell cytotoxicity.

Isolation of intratumoral DCs. To obtain intratumoral DCs, ~500 mm³ of freshly isolated tumor tissue was collagenase digested for 1 h in PBS. The digested material was then diluted with PBS, pipetted up and down vigorously to release lymphocytes, and then filtered through a 40-μm cell mesh. The suspension was subjected to Ficoll gradient centrifugation and lymphocytes were isolated and stained for CD11c expression.

Preparation of dendritic cells from bone marrow cultures. Bone marrow was prepared from femur and tibia of syngeneic donors according to standard procedures. After erythrocyte lysis, cells were resuspended in RPMI medium containing 10% FBS, sodium pyruvate, nonessential amino acids (both from Life Technologies), and 50 μmol/L β-mercaptoethanol (Sigma). Cells (3 × 10⁶ per well) were seeded in a 6-well plate and were supplemented with 750 units/mL rGM-CSF (R&D Systems). Every 3 d, half of the culture medium was replaced. On day 6, cells were loaded with peptide antigen (0.3 μg/mL final concentration) and activated using 2 μg/mL polyC. About 18 h later, all cells were harvested and DCs were isolated by using magnetic CD11c microbeads (Miltenyi). For DC vaccination in mice,
$5 \times 10^5$ DCs were placed between tumor and inguinal lymph nodes by s.c. injection.

**Fluorescence-activated cell sorting analysis.** For fluorescence-activated cell sorting (FACS) analysis, cells were washed with PBS containing 0.1% bovine serum albumin and incubated with FC-block at 4°C for 10 min. Antibodies were then added to the cells and incubated at 4°C for 30 min. Before analysis, cells were washed twice and acquired by using a FACSCalibur or FACSComp II. Data were analyzed by using the software CellQuest and FlowJo, respectively.

**ELISpot.** High-temperature superconductor polyvinylidene difluoride plates (Millipore) were activated with 35% ethanol for 5 min, washed thrice with PBS, and incubated with 100 μL/well anti-mouse IFN-γ antibody (7.5 μg/mL overnight. After another washing step, plates were blocked with RPMI medium containing 10% FCS for 2 h. splenocytes were plated as indicated and incubated with 2 μg/mL of the corresponding peptide or with hTert-Ad. After 48 h, plates were developed by using biotinylated anti-mouse IFN-γ and streptavidin-horseradish peroxidase (HRP; eBioscience). The plates were developed with the AEC substrate (Sigma). Each value was calculated from eight wells using an ELISpot reader.

**ELISA.** Ninety-six–well plates (Nunc, Polysorb) were incubated with 108 adenoviral particles per well in 100 μL PBS overnight. Plates were then UV irradiated to inactivate viral particles and blocked with PBS containing 4% skim milk powder for 2 h. Serum was taken from each group, pooled, and prepared in serial dilutions of 1/250 to 1/32,000 in PBS containing 2% skim milk powder and added in triplicates of 100 μL onto the plates. After 2-h incubation, plates were washed and HRP-conjugated anti-mouse IgG/IgM antibody (Invitrogen) was added for another 2 h. Finally, plates were washed thrice and developed by using TMB substrate (BD). The reaction was stopped with 1 mol/L H3PO4 and measured using an ELISA reader. The dilution of the best ratio/noise value was used in the depicted graphs.

**Oncolysis assays.** For determination of oncolysis, cells were seeded in 24-well plates at a density of $2.5 \times 10^5$ per well. The next day, the medium was changed to 2% FCS. Eight hours later, infection was performed by adding adenovirus at increasing MOI as indicated. After 5 d, cell layer destruction was visualized by crystal violet staining. For this purpose, the plates were rinsed with PBS to remove dead cells and were then fixed for 30 min with 10% formalin in PBS. The solution was replaced by PBS followed by incubation for several hours and then stained for 2 h with 0.1% crystal violet in 10% ethanol.

**Animal experiments.** Primary tumors or lung metastases were established by s.c. injection of $1 \times 10^7$ cells into the flanks of mice or by i.v. injection of $1 \times 10^5$ cells. Subcutaneous tumor nodules were grown to a size of ~6 to 7 mm (~200 mm3) before infection. The viruses were dialysed twice against a solution containing 10 mmol/L Tris (pH 8.0), 1 mmol/L

![Figure 1](image-url). Construction of murine MIP-1α and murine FLT3L-expressing adenoviral vectors. A, construction of the nonreplicating adenoviruses Ad-MIP-1α and Ad-FLT3L. The genetic setup of the oncolytic virus hTert-Ad is additionally displayed at the bottom of the figure. B, C57/Bl6 mice were injected i.v. with $1 \times 10^9$ pfu of the indicated adenoviral vectors. After 5 d, the serum concentrations of MIP-1α and FLT3L were determined to confirm expression of Ad-MIP-1α and Ad-FLT3L. C, spleens of mice were harvested 5 d after treatment with $1 \times 10^9$ pfu of hTert-Ad, $5 \times 10^8$ pfu hTert-Ad/5 $\times 10^8$ pfu Ad-MIP-1α, $5 \times 10^8$ pfu hTert-Ad/5 $\times 10^8$ pfu Ad-FLT3L, $3 \times 10^8$ pfu hTert-Ad/3 $\times 10^8$ pfu Ad-MIP-1α/3 $\times 10^8$ pfu Ad-FLT3L, $5 \times 10^8$ pfu Ad-GFP/5 $\times 10^8$ pfu Ad-MIP-1α, $5 \times 10^8$ pfu Ad-GFP/5 $\times 10^8$ pfu Ad-FLT3L, and $3 \times 10^8$ pfu Ad-GFP/3 $\times 10^8$ pfu Ad-MIP-1α/3 $\times 10^8$ pfu Ad-FLT3L. Average spleen weight was compared using three animals per group.
MgCl₂, and 140 mmol/L NaCl at 4°C. Subsequently, s.c. tumors of KLN-205-HA in DBA/2 or CMT-64-OVA in C57Bl6 mice were treated with intratumoral injection of 3×10⁸ pfu of single adenoviruses (Ad-LacZ, Ad-GFP, Ad-MIP-1α, Ad-FLT3L, hTert-Ad) or with combined injections of 3×10⁸ pfu Ad-MIP-1α + 3×10⁸ pfu Ad-FLT3L (indicated as MF), 3×10⁸ pfu hTert-Ad + 3×10⁸ pfu Ad-MIP-1α, 3×10⁸ pfu hTert-Ad + 3×10⁶ pfu Ad-FLT3L or 3×10⁸ pfu hTert-Ad/3×10⁸ pfu Ad-MIP-1α/3×10⁸ pfu Ad-FLT3L (indicated as hTert-Ad-MF or MFT) at the indicated time points. The total amount of virus was equalized in each group to 9×10⁸ pfu by adding Ad-GFP.

Tumor sizes were measured as indicated using a digital caliper. The volume of the primary tumor was calculated using the following equation: V(tumor) = (length x width²)/2. The difference in mean tumor volume or thenumber of lung metastasis between treatment groups was compared for statistical significance using the unpaired, two-tailed t test with \( P < 0.05 \) accepted as denoting statistical significance.

Statistical analysis. Survival curves were statistically analyzed by SPSS version 11.0 with the Kaplan-Meier log-rank test. Unpaired \( t \) test was performed using GraphPad Prism V3.02 software.

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Results

Generation of MIP-1α– and FLT3L-expressing adenoviruses. For oncolytic virotherapy, we used a conditionally replicating E3-deleted adenovirus (hTert-Ad) expressing E1A genes under the control of the hTert promoter (26, 28). To investigate the role of murine MIP-1α and FLT3L in induction of a tumor-specific immune response, we constructed two E1- and E3-deleted adenoviral vectors that express MIP-1α or FLT3L under the control of a CMV promoter (Fig. 1A). Systemic application of Ad-MIP-1α and Ad-FLT3L increases serum MIP-1α and FLT3L concentration in mice and significantly enhances the spleen weight of the animals, confirming expression and functional activity in vivo (Fig. 1B and C).

Establishment of two syngeneic tumor mouse models for investigation of MHC class I–restricted immune responses against intracellular model antigens HA and OVA during hTert-Ad oncolytic therapy. Several syngeneic mouse tumor models supporting efficient adenoviral DNA replication and

Figure 2. Replication and oncolysis of hTert-Ad in mouse cell lines expressing model antigens HA or OVA. A, KLN-205-HA and CMT-64-OVA cells were infected with the indicated adenoviruses in combination with Ad-luciferase. After 24 and 48 h, cellular extracts were analyzed for luciferase activity. B, subcutaneous tumors of KLN-205 and CMT-64-OVA were infected with the indicated adenoviruses in combination with Ad-luciferase once tumor size reached a mean volume of 500 mm³. At the indicated time points, mice were analyzed by luminescence imaging. C, the human tumor cell line HepG2 and the murine tumor cells KLN-205-HA, CMT-64-OVA, and JC were adenovirally infected at different MOIs as indicated in the figure. Cell layer destruction due to viral replication was visualized by crystal violet staining. D, CMT-64-OVA and KLN-205-HA cells were infected with MOI 10 of Ad-wt, hTERT-Ad, and Ad-GFP, respectively. After the given time points, cells were subjected to three freeze-thaw cycles, centrifuged to remove the debris, and the supernatant was analyzed for the number of infectious particles by rapid titration assay. Human HepG2 hepatoma cells served as positive controls.
oncolysis have been described (3, 29, 30); however, the lack of immunocompetent tumor models expressing established model antigens has been limiting for investigating viroimmunotherapy with oncolytic adenoviruses. The lung adenocarcinoma cell line CMT-64, which is syngeneic for C57Bl6 mice, was already described to be susceptible for adenoviral replication and oncolysis (30). In addition to CMT-64, we identified the lung squamous cell carcinoma cell line KLN-205 that can be efficiently infected and killed by hTert-Ad. KLN-205 is syngeneic for DBA/2 mice, thus facilitating investigation of immune responses against HA in a H2-Kd-specific manner, respectively. We transduced cell lines with retroviruses expressing HA or OVA and selected the transduced cells by EGFP fluorescence. Replication of hTert-Ad in KLN-205-HA and CMT-64-OVA was investigated in cell culture and in s.c. tumors in vivo. By coinfection of an E1/E3–deleted, luciferase-expressing adenovirus together with the replication-competent adenovirus hTert-Ad, the E1 gene products will be provided in trans by the oncolytic virus in telomerase-positive tumor cells. Thus, both viruses will replicate if the cells are permissive for adenoviral replication (31). Compared with coinfection with another E1-deleted adenovirus (Ad-lacZ), co-infection with hTert-Ad resulted in enhanced photon emission, demonstrating viral DNA replication in hTert-Ad–infected KLN-205-HA and CMT-64-OVA cells (Fig. 2A and B). Although hTert-Ad–mediated oncolysis was comparable in both cell lines (Fig. 2C), photon emission in KLN-205-HA was significantly higher in cell culture and more sustained in vivo compared with CMT-64-OVA, suggesting a stronger viral replication in KLN-205-HA. In agreement with this result, we observed a higher generation of infectious progeny of adenovirus wild-type and hTert-Ad in KLN-205-HA cells compared with CMT-64-OVA cells by rapid titer assays during the time course of infection (Fig. 2D). However, oncolytic activity and viral replication of hTert-Ad was at least 10-fold lower in the murine tumor cells compared with the human cell line HepG2.

Expression of MIP1α and FLT3L recruits and expands DCs and T cells at the site of oncolysis. Next, we wanted to determine whether the expression of MIP-1α and FLT3L at the site of viral replication would lead to increased cellular infiltration of

![Figure 3](image_url)
Figure 4. Antitumoral and antiviral immune response after virotherapy. A, the treatment protocol. Subcutaneous tumors of KLN-205-HA in C57Bl6 or CMT-64-OVA in DBA/2 mice were treated with intratumoral injection of adenoviruses (Ad) as described. Subsequently, the animals were investigated for antitumoral and antiviral immune responses. B, immune response against intratumoral antigen was investigated by IFN-γ release of splenocytes after incubation with MHC class I–restricted HA or OVA peptides compared with MHC class I–restricted control peptides from LacZ in ELISPOT assays. C, antiviral cellular immune response was investigated by ELISPOT assays (left) following incubation of $1 \times 10^5$ splenocytes per well with hTert-Ad at a MOI of 50 and antiviral humoral immune response was assessed by ELISA (right). MF, Ad-MIP1α + Ad-FLT3L. D, tumor growth measurements ($n = 10$ per group) showed therapeutic efficacy of virotherapy in the syngeneic animal models and improved local tumor control of virotherapy by intratumoral expression of MIP-1α and FLT3L. *, $P < 0.05$, between untreated mice and mice treated with hTert-Ad. **, $P < 0.05$, between mice treated with hTert-Ad and mice treated with hTert-Ad+MF (MF, Ad-MIP-1α+Ad-FLT3L).
virotherapy-treated tumors. Compared with the E1/E3-deleted adenovirus Ad-LacZ, intratumoral application of hTert-Ad in KLN205-HA (Fig. 3) and CMT-64-OVA (data not shown) resulted in oncolysis of tumor cells, but only a slightly enhanced infiltration of the tumors by inflammatory cells could be observed. In contrast, infection of tumors with Ad-MIP1α and Ad-FLT3L or coinfection of hTert-Ad together with Ad-MIP1α and Ad-FLT3L caused a strong cellular infiltration (Fig. 3A). To identify the components of these cell infiltrates, we performed immunohistochemical analyses using antibodies directed against the DC cell marker CD11c and the T-cell marker CD3. Injection of Ad-MIP1α or Ad-FLT3L in KLN-205-HA tumors resulted only in slightly enhanced infiltration of tumor tissue with DCs and T cells, but coinfection of both adenoviruses showed a strong synergistic effect on DC and T-cell infiltration (Fig. 3B). Interestingly, there was no difference between the MIP-1α/FLT3L–mediated cellular infiltrations in tumors treated with nonreplicating adenoviruses compared with tumors with hTert-Ad–dependent oncolysis. To further investigate the activation state of the infiltrating DCs, the tumor tissue was homogenized and collagenase/trypsin digested, and the obtained cells were analyzed by FACS staining. Application of Ad-MIP1α/ Ad-FLT3L resulted in up-regulation of CD40 expression of the infiltrating DCs, which was further significantly enhanced by co-application of the oncolytic virus hTert-Ad. However, compared with hTert-Ad treatment alone, the co-application of hTert-Ad with Ad-MIP1α/Ad-FLT3L also resulted in significant enhanced up-regulation of CD40 expression, which suggests an improved priming of a cytotoxic T-cell response in MIP-1α/FLT3L–supported oncolytic virotherapy (MFT) compared with hTert-Ad virotherapy or Ad-MIP1α/FLT3L gene therapy (Fig. 3C).

Enhanced tumor infiltration by DCs and T cells during virotherapy leads to stronger immune responses and to improved local tumor control. To analyze MHC class I–restricted immune responses against the cellular tumor antigens, we infected subcutaneous tumors with different adenoviruses and subsequently harvested the spleen cells of the animals for ELISPOT assays (Fig. 4A and B). Viral infection of the tumors was efficient at generating HA- and OVA-specific IFNγ-producing CD8+ T cells. The combination of Ad-MIP1α with Ad-FLT3L resulted in a larger number of IFNγ-producing CD8+ cells compared with each of these adenoviruses alone. However, tumors with intratumoral viral replication and oncolysis elicited a stronger immune response compared with tumors infected with nonreplicating viruses. The number of HA- and OVA-specific IFNγ-producing CD8+ T in the hTert-Ad–treated tumors was comparable with Ad-MIP1α/Ad-FLT3L–treated tumors, but the strongest immune response was observed in the group of animals treated with hTert-Ad/Ad-MIP1α/Ad-FLT3L (Fig. 4B).

Early spreading of the small viral particles out of the tumor tissue would ensure effective exposure of viral antigens in draining lymph nodes and peripheral tissues. Therefore, we reasoned that local migration and expansion of DCs within virotherapy-treated tumors would predominantly enhance effective cross-priming of tumor antigens, whereas immune response against the virus would be less stimulated. However, the number of IFNγ-producing spleen cells after exposure of adenovirus showed a similar pattern compared with HA- and OVA-specific immune responses (Fig. 4C), indicating a parallel enhancement of antitumoral and antiviral immune responses by MIP-1α/FLT3L–mediated infiltration of tumors with inflammatory cells. In agreement with this result, MIP-1α/FLT3L also enhanced the humoral antiviral response in the course of oncolytic virotherapy (Fig. 4C).

Nevertheless, the stronger immune response against the viral vector can result in premature clearance of the oncolytic viruses and less therapeutic efficacy. We therefore studied the efficacy of MIP-1α/FLT3L–supported virotherapy on local tumor control. In both tumor models, virotherapy with MIP-1α/FLT3L was more effective in inhibiting local tumor growth compared with virotherapy alone, indicating that the antitumoral immune response had a stronger effect on the therapeutic outcome than the inhibitory effect of the antiviral immune response (Fig. 4D).

Interestingly, treatment of tumors with nonreplicating viruses expressing MIP1α and FLT3L or the combined application of Ad-MIP1α/Ad-FLT3L did not significantly inhibit tumor growth, emphasizing the essential contribution of the oncolytic virus for the therapeutic effect of the antitumoral immune response.

MIP-1α/FLT3L–augmented virotherapy acts synergistically with DC vaccination and resolves resistance of uninfected lung metastasis against DC vaccination. To investigate whether virotherapy-mediated antitumoral immune response is also effective against distant metastasis, we induced lung metastasis with CMT-64-OVA cells 2 days before tumor therapy. The experimental setup of virotherapy and/or DC vaccination is shown in Fig. 5A. In vivo CTL assays showed stronger OVA-specific CD8+ T-cell cytotoxicity in the group of animals treated with MIP-1α/FLT3L–supported virotherapy than with virotherapy alone. This observation confirms the improved antitumoral immune response by triggering of MIP-1α/FLT3L–mediated inflammatory cell infiltration during virotherapy. Compared with virotherapy or MIP-1α/FLT3L–supported virotherapy, vaccination with OVA-peptide–loaded DCs resulted in stronger OVA peptide–specific cell killing. However, the strongest OVA-specific cytotoxic T-cell response was observed in the group of animals treated with DC vaccination following MIP-1α/FLT3L–supported virotherapy, indicating a synergism of virotherapy-mediated cross-priming of tumor antigens with DC vaccination (Fig. 5B).

Two weeks after the last tumor therapy, the lungs of the animals were investigated. Although MIP-1α/FLT3L–supported virotherapy of the primary s.c. tumor resulted in less OVA peptide–specific cytotoxic T-cell killing compared with OVA peptide DC vaccination, we observed smaller tumor lesions and a lower number of lung metastasis in the group of animals treated with two courses of hTert-Ad/Ad-MIP1α/Ad-FLT3L, which was comparable with the combination of DC vaccination and hTert-Ad virotherapy without MIP1α/FLT3L. This finding may be explained by a broader antitumoral immune response due to cross-priming of whole tumor cell antigens in virotherapy (Fig. 5C and D).

However, in line with our results from in vivo CLT assays, the strongest therapeutic effect was observed in the group of animals treated with DC vaccination following MIP-1α/FLT3L–supported virotherapy. In this group, only a low number of small lung metastasis could be observed 2 weeks after tumor therapy (Fig. 5C and D). This resulted in significantly improved survival and even long-term survival of 50% of the animals (Fig. 5D).

Both intratumoral vector administration and adenoviral oncolysis may result in a significant release of particles into the systemic blood flow. To rule out the possibility that inhibition of lung metastasis could be a result of oncolysis due to direct infection of lung metastasis by hTert-Ad, we determined viral infection of lung metastasis by immunohistochemistry against adenoviral hexon protein (Fig. 6A) and with real-time PCR for adenoviral DNA (Fig. 6B). The infection degree of the s.c. tumor was included as positive control. In contrast to the widespread, potent viral
Figure 5. Systemic antitumoral immune response elicited by MIP-1α/FLT3L virotherapy and DC vaccination. A, to investigate the systemic antitumoral immune response of MIP-1α/FLT3L-hTert-Ad virotherapy (MFT), a subcutaneous primary tumor and lung metastases of CMT-64-OVA were induced in syngeneic C57Bl6 mice. Subsequently, the primary subcutaneous tumor of the animals was treated by injection of adenoviruses and/or with s.c., paratumoral injection of OVA-loaded DCs at the indicated time points. The total amount of virus was equalized in each group to $9 \times 10^9$ pfu by adding Ad-GFP. B, 2 wk after the last treatment, in vivo CTL assays were performed using LacZ peptides as control (contr) and OVA peptides for investigation of cytotoxic T-cell responses against the intratumoral OVA antigen. Representative data of three independent experiments. C, lungs (n = 8 in each group) were harvested 14 d after final therapeutic application. H&E staining of the lungs showed a markedly reduced growth of lung metastasis (dark areas, predominantly located at the rims of the lungs) in the group of animals treated with two cycles of MIP-1α/FLT3L–supported hTert-Ad virotherapy (MFT) or with a combination of hTert-Ad or MFT and DC vaccination (T-DC or MFT-DC) compared with the untreated control. Representative data from each group. D, left, lung metastases were counted in H&E-stained sections, confirming both systemic therapeutic efficacy of MIP-1α/FLT3L–supported hTert-Ad virotherapy (MFT), and synergistic effects between hTert-Ad and DC vaccination (T-DC) as well as MFT and DC vaccination (MFT-DC), n = 8 in each group (*, P between mice treated with two cycles of MFT or with T-DC and untreated control mice; **, P between mice treated with MFT and mice treated with MFT in combination with DC vaccination). Right, survival curve (mice died because of lung metastases). DC vaccination alone did not result in significant prolonged survival compared with untreated animals (P = 0.06). Animals that received two treatments of MIP-1α/FLT3L–supported hTert-Ad virotherapy (MFT) survived longer compared with animals treated twice by DC vaccination or untreated animals (P < 0.001). Combined treatment with hTert-Ad together with DC vaccination (P = 0.01) or MFT together with DC vaccination (P < 0.001), resulted in improved survival compared with untreated animals. Animals that received the combined treatment of MFT together with DC vaccination survived significantly longer compared with animals treated with two applications of MFT (P < 0.001).
infection detected within the primary tumor, no viral infection could be observed in lung metastasis by immunohistochemistry. Consistently, only a very small number of adenoviral genomes (similar to background levels) could be detected by real-time PCR in the lung metastases of the treated animals. The comparison of the progressive infection within the primary tumor reveals that the number of viral particles detected in lung metastases does not reflect an efficient oncolysis.

To rule out a direct inhibitory effect of MIP-1α and/or FLT3L on tumor growth, the experiments were repeated in nude mice with the same conditions as described in Fig. 5. In this system, lung metastasis of CMT-64-OVA occurred predominantly at the rim of the lungs. However, we did not observe any difference in tumor volume of the lung metastasis in the different groups of animals (Fig. 6C). Neither the number of lung metastasis nor the local tumor control by hTert-Ad was influenced by MIP-1α/FLT3L expression and DC vaccination in nude mice (Fig. 6D). In agreement with these results, MIP-1α/FLT3L–supported virotherapy did not improve survival in these animals compared with virotherapy alone (Fig. 6D), confirming that the therapeutic improvements of MIP-1α/FLT3L–supported viroimmunotherapy are the result of the enhanced tumor-specific immune response.

Discussion

The concept of our study is based on the fact that viral infection does not only lead to immune response against viral antigens but also to a TLR- and MyD88-dependent cross-priming of cellular antigens (12, 13). However, viral inflammation does not automatically promote an immune response against cellular antigens. In contrast to acute cytopathic virus infection, chronic inflammation by noncytotoxic viruses seems to actively suppress antigen-specific responses by the adaptive immune system, thereby preventing dangerous tissue damage (32).

Until now, the lack of suitable animal tumor models with established model antigens was a limitation to investigate the peptide-specific cytotoxic T-cell response during oncolytic adenoviral virotherapy. Using virotherapy-susceptible mouse tumors with expression of the model antigens HA and OVA, we showed that oncolysis by the telomerase-dependent replicating adenovirus hTert-Ad induces a cytotoxic T-cell response against intracellular tumor model antigens. Following oncolysis, the dead tumor cells have to be captured by antigen-presenting cells to elicit a MHC class I–restricted immune response by cross-priming. Because the number of DCs can determine the nature and the magnitude of an immune response (33), we reasoned that inadequate DC tumor infiltration during oncolytic virotherapy may be a limitation for virotherapy-mediated induction of tumor-specific immune responses. Several studies showed enhanced antitumoral immune responses by triggering of DC migration to tumors due to MIP-1α or MIP-3α expression (17, 19, 34, 35). However, in some tumors, this approach also led to progressive growth, which is most likely explained by intratumoral accumulation of tolerance-inducing immature DCs (36).

However, when MIP-1α and FLT3L were combined with DNA vaccination protocols that usually stimulate TLR signaling, dramatic synergistic effects in eliciting an immune responses were described (18). This indicates that in addition to DC infiltration, maturation of the DC following TLR stimulation at the side of antigen exposure is essential to induce a strong immune response (19, 37). Interestingly, we observed a stronger maturation of DCs and enhanced immune responses in MIP-1α/FLT3L virotherapy compared with MIP-1α/FLT3L gene therapy, which may be explained by stronger TLR stimulation by viral replication. In contrast to stable MIP-1α expression in tumor cells (17), the combination of MIP-1α and FLT3 in the context of a plasmid vaccination resulted in strong tissue infiltration by DCs, but not by T cells (18). However, in our experiments with adenoviral delivery of MIP-1α or FLT3L, migration and expansion of DCs were accompanied by a strong infiltration with T cells. The discrepancy between the studies may be explained by the different target tissues or the different effects of viruses compared with plasmid DNA on T-cell infiltration. In agreement with our results, it has recently been shown that naïve T cells up-regulate the receptor for MIP-1α (CCR5) after immunization but before antigen recognition, permitting these cells to be actively attracted to inflammatory tissue with MIP-1α expression. Because both DCs and CD4+ T cells produced MIP-1α upon activation, MIP-1α–mediated attraction of T cells may also enhance CD8+ T-cell contacts with DCs and/or DC/CD4+ T-cell clusters in virally infected tumors (38).

Strong inflammation can have opposing effects on virotherapy of tumors: It can augment virotherapy by enhancing tumor destruction; however, it can also lead to early clearance of the invading virus, thus preventing viral dissemination and oncolysis through the tumor tissue. It is important to note that the intratumoral expression of MIP-1α and FLT3 did not change the relation of antitumoral to antiviral immune responses in our experiments. There is evidence that unspecific immune suppression by cyclophosphamide chemotherapy or antibody-mediated depletion of natural killer cells results in increased tumor regression and improved survival in animal tumor models treated with oncolytic herpes simplex virus (39) or vesiculo stomatitis virus (40).

On the other hand, cytokine-mediated immunostimulation in combination with adenoviral virotherapy leads to improved therapeutic outcome in immunocompetent animals bearing susceptible tumors for adenoviral replication (3). Recently, it has been shown that oncolytic adenoviruses are more effective in immunocompetent than in athymic animals when they contain the E3 gene region that inhibits cytolistic T-cell recognition of infected tumor cells and protects cells from premature death receptor–mediated apoptosis (30). This observation supports the hypothesis that cross-presentation of tumor antigens rather than direct presentation accounts for the antitumoral immune response during adenoviral virotherapy. Consequently, it would be interesting to investigate whether the efficacy of MIP-1α/FLT3–supported virotherapy could be even more improved by using E3-expressing adenoviruses.

In addition to improved local tumor control, we detected a systemic antitumoral efficacy of MIP-1α/FLT3L virotherapy in virally uninfected lung metastasis in immunocompetent mice but not in nude mice. In contrast to vaccination against distinct tumor-specific antigens, uptake of infected apoptotic tumor cells by DCs may trigger T-cell reactivity against known and unknown tumor-associated antigens and may thus circumvent tumor immune escape mechanisms by eliciting a broadly based antitumoral immune response. In agreement with this hypothesis, MIP-1α/FLT3L virotherapy was more effective in inhibiting growth of virally uninfected lung metastasis compared with peptide-specific DC vaccination, although peptide-specific DC vaccination resulted in stronger peptide-specific cytotoxic T-cell responses in CTL assays in vivo.

In summary, our study shows that virotherapy of solid tumors leads to induction of peptide-specific cytotoxic T-cell responses...
against intracellular tumor antigens. Improvement of DC and T-cell infiltration of infected tumors mediated by MIP-1α and FLT3L elicits enhanced immune responses against the tumor, but also against the virus. Although a stronger virus-specific immune response may be a critical disadvantage for virotherapy with other oncolytic viruses, MIP-1α/FLT3L–augmented virotherapy (two courses of MFT) nor viroimmunotherapy (two courses of MFT + DC vaccination) inhibited growth of lung metastasis in nude mice. Comparable size and numbers of lung metastases were observed in all groups (n = 4 in each group). D, top, numbers of lung metastases observed in H&E-stained sections of treated animals were counted, confirming the lack of a therapeutic effect on distant metastasis in nude mice (n = 4 in each group). Middle, growth evaluation of s.c. tumors on nude mice showed that primary tumor growth was inhibited by intratumoral injection of two cycles of hTert-Ad. Combined viroimmunotherapy (two cycles of MFT + DC) did not result in additional therapeutic benefit in nude mice (n = 4 in each group). Bottom, survival of nude mice with CMT-64-OVA lung metastases and virotherapy-treated s.c. tumors (n = 6 in each group). Treatment with hTert-Ad alone or with MIP1α/FLT3L–supported hTert-Ad virotherapy in combination with DC vaccination does not result in significant prolonged survival in nude mice.

systemic efficacy of the antitumoral immune response due to adenoviral viroimmunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Correction: Antitumoral Immune Response by Recruitment and Expansion of Dendritic Cells in Tumors Infected with Telomerase-Dependent Oncolytic Viruses

In this article (Cancer Res 2009;69:1448–58), which was published in the February 15, 2009 issue of Cancer Research (1), there was a typographical error in the first author’s name. The correct name should be Ramakrishna Edukulla.

Reference

Antitumoral Immune Response by Recruitment and Expansion of Dendritic Cells in Tumors Infected with Telomerase-Dependent Oncolytic Viruses

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