Internalization of Oncolytic Reovirus by Human Dendritic Cell Carriers Protects the Virus from Neutralization

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Abstract

Purpose: Dendritic cells (DC) may be the most effective way of delivering oncolytic viruses to patients. Reovirus, a naturally occurring oncolytic virus, is currently undergoing early clinical trials; however, intravenous delivery of the virus is hampered by pre-existing antiviral immunity. Systemic delivery via cell carriage is a novel approach currently under investigation and initial studies have indicated its feasibility by using a variety of cell types and viruses. This study addressed the efficacy of human DC to transport virus in the presence of human neutralizing serum.

Experimental Design: Following reovirus-loading, DC or T cells were cocultured with melanoma cells with or without neutralizing serum; the melanoma cells were then analyzed for cell death. Following reovirus loading, cells were examined by electron microscopy to identify mechanisms of delivery. The phagocytic function of reovirus-loaded DC was investigated by using labeled tumor cells and the ability of reovirus-loaded DC to prime T cells was also investigated.

Results: In the presence of human neutralizing serum DC, but not T cells, were able to deliver reovirus for melanoma cell killing in vitro. Electron microscopy suggested that DC protected the virus by internalization, whereas with T cells it remained bound to the surface and hence accessible to neutralizing antibodies. Furthermore, DC loaded with reovirus were fully functional with regard to phagocytosis and priming of specific antitumor immune responses.

Conclusions: The delivery of reovirus via DC could be a promising new approach offering the possibility of combining systemic viral therapy for metastatic disease with induction of an antitumor immune response. Clin Cancer Res; 17(9): 2767–76. ©2011 AACR.

Introduction

Spontaneous cancer regression following natural viral infection has been reported since the early 1900s. Recently there has been renewed interest in viral therapy for cancer and several viruses have been evaluated in clinical trials (1–3). Oncolytic viruses preferentially replicate in and kill tumor cells, tumor selectivity being dependent on differences in the cellular processes of normal versus tumor cells; furthermore, genetic modification of the viruses can improve their tumor selectivity, safety, and efficacy (4).

However, delivery of oncolytic viruses is severely restricted by the immune response to systemically administered virus particles.

Reovirus is a nonenveloped, double-stranded RNA virus not normally associated with overt disease in humans. It preferentially replicates in and kills transformed cells with activated ras signaling (5), an aberration frequently seen in human cancers (6–10). Reoviruses are currently undergoing evaluation as oncolytic agents in clinical trial (2). For oncolytic viral therapy to achieve its full potential in the treatment of disseminated disease, effective systemic delivery will most likely be required. However, as reovirus is ubiquitous in the environment (11), most adults possess anti-reoviral antibodies due to prior exposure (12, 13). Moreover, antibody titer increases dramatically following intravenous (i.v.) therapy (14), so that neutralization by specific antibodies (15, 16) hampers efficient i.v. delivery. The use of cell carriers to transport viral particles within the circulation and deliver them to tumors is currently under investigation as a novel approach to systemic delivery. Preclinical studies have shown that various carrier cells including T cells (17), monocytes, endothelial cells (18), and irradiated tumor cells (19) can carry virus within the circulation.
Translational Relevance

Oncolytic viruses are under clinical trial but their efficacy may be seriously curtailed by an immune response if they are given systemically. It has been shown that dendritic cells (DC) are effective for reovirus delivery to tumors in mice in the presence of neutralizing antibodies. This article shows that this also seems to be true for human DC and that the biological basis for this is internalization of the virus by the DC thus protecting it from neutralization. Furthermore, reovirus-loaded DC are fully functional with regard to phagocytosis and immune priming. Thus, the use of DC to transport virus offers the possibility of combining viral delivery with induction of an antitumor immune response, as oncysis by the viral payload will release tumor antigens for uptake by DC.

In addition to direct tumor cell lysis, it is becoming increasingly clear that oncolytic viruses may have a role in modifying the immune response to tumors. Oncolytic viruses have the potential to break immune tolerance to the tumor by releasing tumor antigens for uptake by DC and providing the danger signal required for immune activation (20), thus providing a rationale for the therapeutic combination of DC and oncolytic viruses.

Having recently shown that DC could deliver reovirus for the clearance of metastases from lymph nodes in mice with preexisting immunity to the virus (21), this study sought to evaluate the potential of human DC to act as cell carriers for reovirus. We show that both human DC and T cells were effective carriers of reovirus in vitro in the absence of human serum, but that only DC were able to deliver the virus for tumor cell killing when neutralizing serum was present. Electron microscopy suggested that this was because of the different localization of the virus on the 2 cell types, DC being able to protect the virus by internalization, whereas on T cells it remained surface-bound and accessible to neutralization by serum components. Furthermore, DC loaded with reovirus were fully functional with regard to phagocytosis of tumor cells and priming of adaptive antitumor immune responses. Thus, in addition to viral protection and delivery, human DC may be particularly effective for enhancing therapy via induction of antitumor immunity in patients even in the presence of neutralizing antibodies.

Materials and Methods

Cells and virus

Reovirus type 3 Dearing was provided by Oncolytics Biotech Inc. Viral titers were measured by standard plaque assay on L929 cells. The human melanoma cell lines Mel-888 and MeWo were obtained from the Cancer Research UK cell bank and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) plus 10% (v/v) fetal calf serum (FCS; Biosera) and 2 mmol/L L-glutamine (Sigma-Aldrich). Cells were passaged for less than 6 months from thawing; they were routinely tested for mycoplasma and found to be free of infection. Human peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy donors by Ficoll-Hypaque density centrifugation. Immature DC (iDC) were derived from monocytes isolated by using anti-CD14 magnetic beads (Miltenyi Biotech) and cultured in RPMI 1640 (Invitrogen) plus 10% (v/v) FCS, 2 mmol/L L-glutamine, 800 U/mL granulocyte macrophage colony stimulating factor (Peprotech) and 500 U/mL IL-4 (R&D Systems) for 4 days. Mature DC (mDC) were generated by culture of 3.5-day iDC with 10 μg/mL OK432 (ref. 22; Chugai Pharmaceutical Co. Ltd.). T cells were isolated by negative selection of the CD14+ PBMC fraction by using Pan T selection beads (Miltenyi Biotech) and cultured in RPMI 1640 + 10% FCS + 2 mmol/L L-glutamine.

Flow cytometry

A FACS Calibur (Becton-Dickinson) was used for acquisition and Cell Quest Software (BD Biosciences) for analysis. Antibodies: anti–JAM-1 (Santa Cruz Biotechnology); anti-human HLA-DR-PE, CD11c-APC, CD80-PE, CD86-PE, CCR7-PE, IFN-γ-FITC, CD107a-FITC, CD107b-FITC, CD3-FITC, CD8-PerCP, anti-mouse Ig-FITC (BD Pharmingen); reovirus loading was detected by using anti-reovirus α3 capsid protein (DSHB, University of Iowa, Iowa City, IA) followed by anti-mouse IgG-FITC.

Reovirus loading of carrier cells

Aliquots of 5 × 10⁶ iDC, mDC, or T cells were loaded with reovirus at 0, 1, or 10 pfu/target in a total volume of 1 mL PBS, at 4°C for 3 hours, then washed twice in 13 mL PBS.

Reovirus retention

Estimation of surface reovirus retention was done by fluorescence-activated cell sorting (FACS) and plaque assay after loading cells with or without 10 pfu/target reovirus at 4°C. For FACS analysis, cells were labeled with anti-reovirus α3 followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG; BD Pharmingen). For plaque assay, cells were resuspended in 100 μL PBS and freeze thawed (3 cycles, 10 minutes freeze in methanol/dry ice followed by 10 minutes thaw at 37°C); this preparation was used in a standard plaque assay on L929 cells. Removal of sialic acid was by incubation with 5.5 mU/mL sialidase (Roche) at 37°C for 1 hour in serum-free medium.

In vitro delivery of reovirus via carrier cells

Target cells (Mel-888, MeWo) were seeded at 3 × 10⁵ cells/well in 6-well plates and allowed to adhere for 3 hours. Direct reovirus was added at 0, 1, or 10 pfu/target cell. For delivery via cell carriage, iDC, mDC, or T cells were loaded with reovirus at 0, 1, or 10 pfu/cell and added to melanoma targets at a 1:1 ratio. Human blocking serum was added to the wells at 0, 2, or 30% (v/v). After a further...
48 and 72 hours, wells were harvested, cells were labeled with FITC-conjugated anti-CD11c or anti-CD3 to allow gating out of carrier cells, stained with propidium iodide (Sigma), and analyzed for target cell death by flow cytometry. For junction adhesion molecule 1 (JAM-1) blocking, 10 μg/mL anti–JAM-1 was added to MeWo cell cultures and incubated for 30 minutes; reovirus or reovirus-loaded carrier cells were then added. After 48 hours the cells were harvested and cell death was analyzed as described earlier in the text.

Electron microscopy
DC and T cells were loaded with or without reovirus at approximately 170 pfu/cell, washed, resuspended in medium, and set at 37°C for 6 hours to allow internalization of the virus to take place, after which the cells were fixed with 2% paraformaldehyde (PFA) and 0.2% glutaraldehyde in 0.1 mol/L PHEM buffer (60 mmol/L PIPES, 25 mmol/L HEPES, 10 mmol/L EGTA, 2 mmol/L MgCl₂) for 2 hours at room temperature. Fixed cells were stored in 0.1 mol/L PHEM, 0.5% PFA at 4°C until they were pelleted and embedded in 12% gelatin. The pellet was cut into approximately 1 mm² cubes, which were cryoprotected in 2.3 mol/L sucrose and subsequently snap-frozen in liquid nitrogen. Ultrathin cryosections were incubated with anti-reovirus 63 (Developmental Studies Hybridoma Bank; 1:800), a bridging rabbit anti-mouse IgG antibody (Dako Cytomation; 1:200), and protein A-gold particles 10 nm. The specimens were viewed with a FEI Tecnai 12 Biotwin transmission electron microscope operating at 120 kV. Digital images were collected with a cooled charge-coupled device camera (4K Eagle, FEI company) at binning 2 to a final size of 2,048 × 2,048 pixels.

Phagocytosis assay
Mel-888 cells were labeled with 1 μmol/L Celltracker Green (Invitrogen) at 37°C for 30 minutes, then washed twice in medium and seeded in 6-well plates at 3 × 10⁶ cells/well. A total of 3 × 10⁶ aliquots of PFA-fixed or living iDC or mDC were loaded with or without reovirus at 10 pfu/cell and cultured with the labeled Mel-888 cells at a 1:1 ratio for 4 hours. Cells were then harvested, DC were labeled with anti-CD11c, and double-positive cells were identified by flow cytometry.

Generation of tumor-specific CTL
Reovirus at 0 or 10 pfu/cell, or iDC, mDC, or T cells loaded with reovirus at 0 or 10 pfu/cell were cultured with Mel-888 cells (1:1 ratio) for 24 hours. Nonadherent cells were harvested and cocultured with autologous isolated T cells or whole PBMC at a ratio of 1:10 to 1:30 in CTL medium [RPMI 1640 plus 7.5% (v/v) human AB serum (Sigma), 2 mmol/L L-glutamine, 1% (v/v) sodium pyruvate (Sigma), 1% (v/v) nonessential amino acids (Sigma), 1% (v/v) Hepes (Sigma), 20 μmol/L β-mercaptoethanol (Sigma), and 5 ng/mL IL-7 (R&D Systems)]. Cultures were restimulated by using the same protocol after 1 week. Cells were harvested at day 14.

CD107 lymphocyte degranulation assay
As a measure of cytotoxicity a lymphocyte degranulation assay was used as previously described (23). CTL and tumor targets were cultured at a 1:1 ratio in CTL medium; anti-CD107a and anti-CD107b antibodies + 10 μg/mL brefeldin A (Invitrogen) were added after 1 hour. After a further 4-hour culture, CTL were labeled with anti-CD8 and analyzed by flow cytometry.

ELISA
IFN-γ ELISA assays were carried out by using matched antibody pairs (BD Pharmingen) according to the manufacturer’s instructions.

Statistics
A paired Student’s t test was used to determine statistical significance.

Results
Human DC and T cells can be loaded with reovirus
Delivery of reovirus type 3 Dealing to tumor cells, via cell carriage on DC and T cells, has been previously shown in mouse models. As carriage of reovirus by human immune cells has not yet been shown, human iDC, mDC and T cells were investigated for their potential to deliver reovirus to tumors. Reovirus was detected on loaded iDC, mDC, and T cells by both FACS for surface-bound virus (Fig. 1A) and plaque assay. FACS analysis indicated more surface-detectable reovirus on T cells than DC. Plaque assay indicated that low levels of virus (approximately 4% of the loading dose) could be retrieved from the carrier cells, with no significant difference between iDC, mDC, and T cells (Fig. 1B). Replication of the virus within cells, which would serve to increase the dose of virus delivered to the tumor, was not detected within human DC or T cells (data not shown). JAM-1 has been identified as a cellular receptor for reovirus type 3 (24) and was found to be expressed at broadly similar levels by iDC, mDC, and T cells (Fig. 1C). However, JAM-1 was not required for loading, as blocking JAM-1 did not inhibit reovirus-loading of DC or T cells (Fig. 1D). Reovirus can also bind to sialic acid residues (25), which therefore represent an alternative cellular target for reovirus loading. Treatment of DC and T cells with sialidase to remove sialic acid prior to virus loading, significantly reduced reovirus retention by all 3 cells types (Fig. 1E), suggesting sialic acid is important for effective loading of these carrier cells.

Human DC and T cells deliver reovirus for tumor cell killing
Human neutralizing serum protected melanoma cells from reovirus-induced cell death (Fig. 2A), suggesting that reovirus may be neutralized following systemic delivery in patients. Therefore, the potential delivery of reovirus via human cell carriage was examined. In the absence of human serum, both virus-loaded DC and T cells were able to deliver reovirus for melanoma cell killing as shown by
the percentage of propidium iodide (PI)-positive cells (Fig. 2B) and by microscopy (Fig. 2C). In the mouse, delivery of reovirus via cell carriage was more efficient than direct viral delivery in vitro (21). Similar results were seen here in the human system, where the percentage of virus retained by the DC and T cells after loading was low (approximately 4%; Fig. 1C), but the resulting tumor target cell death was as high as that induced by direct virus at the full loading dose (Fig. 2B). However, the presence of 2% human serum abrogated reovirus-induced tumor cell lysis when the virus was delivered either directly or via T cells. By contrast, both iDC and mDC were able to deliver reovirus for melanoma killing in the presence of serum, although cell death was partially inhibited (Fig. 2B). Efficient reovirus delivery via DC (but not T cells) was also shown in the presence of the more physiologic level of 30% human serum (Fig. 2D). Interestingly, target melanoma cells express JAM-1 (Fig. 2E), and blocking this JAM-1 significantly reduced productive viral handoff (Fig. 2F), indicating that JAM-1 expression by tumor cells is important for reovirus-dependent tumor cell killing.

**DC internalize reovirus thus protecting it from neutralization**

The efficacy of DC compared with T cells as carriers of reovirus in the presence of neutralizing serum, indicated that DC were somehow able to protect the virus from elimination, suggesting that the 2 cell types might employ different mechanisms for delivery. Owing to their phagocytic nature, particularly for virus-sized particles (26), it seemed possible that internalization of the virus by DC might be a mechanism whereby DC could “hide” the virus during delivery to tumor cells. In addition, several viruses are internalized by DC following binding to DC-SIGN (27–29), which facilitates their dissemination (30, 31). On the contrary, T cells might transport the virus on the surface leaving it accessible to neutralization. Therefore, the location of reovirus on loaded DC and T cells was examined. Following reovirus loading, reovirus was detected both at the surface and internally in iDC and mDC (Fig. 3A, C, and D).

**Reovirus does not inhibit DC maturation or function**

Although it has previously been shown that reovirus can directly activate DC (20), tumor cells can impair DC maturation and function (32). Figure 4A shows that reovirus loaded onto iDC was able to induce their maturation even in the presence of tumor cells, inducing upregulation of MHC-II, CD80, and CD86. Moreover, the presence of tumor cells did not alter the activated phenotype of previously matured DC (Fig. 4A). Hence, carrier DC entering the tumor site, or endogenous DC recruited to the tumor following therapy, should be activated for potential...
antitumor immune priming. In addition, reovirus loading did not impair the phagocytic function of DC, because both iDC and mDC loaded with reovirus phagocyted melanoma cells at the same level as nonloaded DC (Fig. 4B). Thus, reovirus-loaded DC remain competent for antigen uptake, an essential component of antitumor immune cross-priming. The absence of double-positive cells in the PFA-fixed controls (Fig. 4B) indicated that tumor cell uptake was an active process and not merely passive adhesion of the melanoma cells to DC. The ability of reovirus-loaded DC to prime an antitumor immune response in the context of viral handoff and target killing was investigated. Isolated autologous T cells, rather than complete PBMC, were used as responders for CTL generation, to exclude the possibility of any noncarrier, endogenous DC within the PBMC fraction contributing to priming. Reovirus-loaded DC were cultured with Mel-888 cells for 24 hours; nonadherent cells (including carrier cells and dying melanoma cells) were then harvested and cultured with responder T cells. Both reovirus-loaded iDC and mDC were effective at priming specific antitumor CTL (Fig. 4C). Thus reovirus is directly cytotoxic to tumor cells and can also act as an adjuvant to induce functional DC maturation leading to induction of specific antitumor immunity. Hence, reovirus carriage by human DC has the potential to support immune-mediated and direct cytotoxic therapy.
Reovirus-loaded DC prime antitumor immunity in the presence of human serum

The potential of reovirus-loaded T cells or direct reovirus to support specific antitumor immune priming was investigated, initially in the absence of serum, using whole PBMC rather than isolated T cells as responders for CTL generation, thus providing antigen presenting cells for antigen uptake and priming. Under these serum-free conditions, both direct reovirus and reovirus-loaded T cells induced antitumor immune priming to a level comparable with that of reovirus-loaded DC (Fig. 5A). However, consistent with the ability of DC, but not T cells, to protect reovirus from serum neutralization for direct cytotoxicity (Fig. 2B), only DC loaded with reovirus were effective in priming-specific antitumor immunity in the presence of neutralizing serum (Fig. 5B and C). This suggests that in this in vitro system, reovirus handoff and tumor cell lysis are required for efficient priming. Intracellular IFN-γ levels from priming cultures were assayed by ELISA (Fig. 5D). Increased IFN-γ secretion was seen in cultures containing DC loaded with reovirus, consistent with the development of a Th1 adaptive immune response. However, although skin lesions are readily accessible for direct viral injection, oncolytic viral therapy is seriously curtailed by an immune response if given systemically. Hence, novel delivery mechanisms which protect virus from neutralization by the immune system are required. Among the cell types that are competent for viral delivery to tumors, it has recently been shown that DC can deliver reovirus for the clearance of melanoma metastases from the lymph nodes of reovirus-immune mice, and in this model DC were more efficient than T cells over a range of viral loads (21). Therefore, in this study, human DC were investigated as potential carriers for the therapeutic delivery of reovirus.

Human DC and T cells were able to deliver reovirus for melanoma killing in the absence of human blocking serum. By contrast, only iDC or mDC acted as efficient cell carriers in the presence of neutralizing serum, and delivered the virus for melanoma killing, albeit with lower efficacy than in the absence of serum. We have previously shown in mice that mDC, but not iDC, act as carriers for reovirus delivery in reovirus-immune mice, and this model DC were more efficient than T cells over a range of viral loads (21). Therefore, in this study, human DC were investigated as potential carriers for the therapeutic delivery of reovirus.

Discussion

There is currently little effective therapy for metastatic melanoma and new approaches are urgently required. Oncolytic viruses are currently under investigation as a novel therapy for the disease. Among recent clinical trials, intratumoral injection of herpes simplex virus has shown promising results (33) and reovirus is also potentially useful in melanoma (34). However, although skin lesions are readily accessible for direct viral injection, oncolytic viral therapy is seriously curtailed by an immune response if given systemically. Hence, novel delivery mechanisms which protect virus from neutralization by the immune system are required. Among the cell types that are competent for viral delivery to tumors, it has recently been shown that DC can deliver reovirus for the clearance of melanoma metastases from the lymph nodes of reovirus-immune mice, and in this model DC were more efficient than T cells over a range of viral loads (21). Therefore, in this study, human DC were investigated as potential carriers for the therapeutic delivery of reovirus.

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Figure 3. iDC (A), mDC (C and D), and T cells (B) were loaded with or without reovirus. The cells were then washed, resuspended in medium, and placed at 37°C for 6 hours to allow internalization of the virus to take place, after which the cells were fixed, immunogold labeled, and analyzed by electron microscopy. Scale bars, 500 nm. N, nucleus; M, mitochondria; G, Golgi.
localized on the carrier cells. It seemed possible that DC might internalize reovirus prior to delivery to tumor cells. Electron microscopy indicated that this was indeed the case, with reovirus being located at the surface of T cells where it would be susceptible to neutralizing antibodies, whereas significant levels of virus were detected internally.
in the DC and thus presumably protected from elimination. This is an important finding with regard to the systemic delivery of reovirus, but may also have implications for therapy with other oncolytic viruses, because even if patients have had no prior exposure to the therapeutic virus, antibody titers increase rapidly following the first dose (14) complicating multiple dosing strategies. Internalization of virus by DC is likely to be an important factor in determining their value as carriers for the systemic administration of oncolytic viruses.

In addition to a role as passive carrier cells for the delivery of reovirus for oncolysis, DC may also improve therapy by priming both innate and adaptive antitumor immunity. In the mouse, long-term purging of metastases correlated with induction of adaptive tumor-specific immunity (21). In addition, human in vitro innate tumor cell killing by both natural killer cells and T cells can be induced by reovirus-activated DC (20, 35). This study shows that reovirus-loaded DC, cultured with melanoma cells, could effectively prime specific antitumor immunity.

Tumor-resident DC are often functionally impaired (32); therefore, the ability of reovirus-loaded carrier DC to prime specific CTL, indicates an important potential contribution to effective cancer immunotherapy in addition to direct oncolysis.

One criticism of oncolytic viral therapy is that any immune response generated will be predominantly against the virus rather than the tumor. Although the induction of antiviral immunity was not examined in this study, it is significant that (i) specific antitumor immunity could be generated in the presence of reovirus and (ii) reovirus-loaded DC were competent for adaptive antitumor immune priming. The current data do not clearly indicate whether iDC or mDC was superior for viral handoff/tumor cytotoxicity or for support of adaptive priming in the presence of neutralizing serum. For clinical studies, iDC, without the use of an additional maturation signal, therefore seems to be a reasonable, straightforward choice.

Figure 5. Reovirus (reo) at 0 or 10 pfu/cell, or DC, or T cells loaded at 0 or 10 pfu/cell were cultured with Mel-888 cells for 24 hours and used to prime autologous PBMC. A, CTL were then used in a CD107 assay. Graph shows mean ± SE of data from 5 independent experiments. B, autologous PBMC were primed as in (A) but with blocking human serum added to the Mel-888 cells during coculture with reovirus or carrier cells. Graph shows mean ± SE of data from 5 independent experiments. *, P < 0.05 between iDC or mDC and T cells or reovirus. C, representative plots showing CD107 degranulation by CD8 cells from priming cultures, against Mel-888 (relevant) or SKOV (irrelevant) targets. D, supernatants from priming cultures were analyzed for IFN-γ by ELISA. Graph shows mean ± SE of data from 5 independent experiments.
Dendritic Cells Protect Reovirus from Neutralization

The delivery of reovirus via cell carriage on DC could be a promising new development in systemic reoviral therapy for metastatic disease, both by efficient delivery of virus for tumor cell lysis in the presence of neutralizing antibodies and by generation of adaptive antitumor immunity. Furthermore, DC delivery may also be applicable to other oncolytic viruses and is worthy of further research and consideration in the design of future clinical trials.

Disclosure of Potential Conflicts of Interest

M. Coffey is an employee of Oncolytics Biotech. The other authors disclosed no potential conflicts of interest.

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