Ionizing Radiation-Induced Adenovirus Infection Is Mediated by Dynamin 2

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Abstract

Specific viral targeting into intrahepatic tumors remains critical for adenovirus gene therapy in liver cancer. We previously showed that ionizing radiation increases adenovirus uptake and transgene expression in cells and colon cancer xenografts. Here, we tested whether radiation induces viral uptake through virus-cell membrane interaction. We found that radiation (8 Gy) induced adenoviral gene transfer in rat hepatocytes (WB) and human colon carcinoma cells (LoVo). This induction (24.4- and 6.5-fold, respectively) and viral uptake were significantly diminished by preincubation with antibody for Dynamin 2 but not for Coxackie adenovirus receptor or for integrin αv. Radiation-induced Dynamin 2 expression was detected by immunohistochemical staining and by increased mRNA levels for Dynamin 2 in WB (1.5-fold) and LoVo (2.2-fold) cells. Specific small interference RNA (siRNA) transfection significantly inhibited Dynamin 2 expression of other integrins, such as α2β3 in murine melanoma cells (15) and p1 in human lung tumor cells (16), leading to enhanced cell adhesion to extracellular matrix or recruitment of leukocytes during radiation-mediated inflammation. However, there is no direct evidence for the radiation regulation of Dynamin 2. In this study, we first explored the roles of CAR, αvβ3 integrins, and dynamins in viral endocytosis, it seemed possible that the radiation-induced increase in uptake that we observed previously may be mediated via effects on one or all of these receptors. Although a firm link between radiation and these receptors does not exist, they can respond to environmental stresses. For instance, CAR expression can be regulated upon progression through the cell cycle (13) and by inflammatory mediators (14). Radiation does, in fact, induce the expression of other integrins, such as α2β3 in murine melanoma cells (15) and p1 in human lung tumor cells (16), leading to enhanced cell adhesion to extracellular matrix or recruitment of leukocytes during radiation-mediated inflammation. Therefore, there is no direct evidence for the radiation regulation of Dynamin 2.

In this study, we first explored the roles of CAR, integrin, and Dynamin 2 in radiation-induced adenoviral uptake and gene transfer in cells. When our cell culture experiments suggested a leading role for Dynamin 2, its expression and function were further investigated in nude mice bearing tumor xenografts.

Materials and Methods

Cell culture and viral gene expression. Human cancer cell lines, LoVo (colon), D54 (brain), and MCF-7 (breast), and rat hepatocyte WB were preincubated with antibodies for CAR at 1:100 (RmCB, American Type Culture Collection, Manassas, VA), αv integrin at 1:100 (H-75, Santa Cruz Biotechnology, Santa Cruz, CA), or Dynamin 2 at 1:100 (BD Bioscience PharMingen, San Diego, CA) at 37°C for 1 hour followed by irradiation with 8 Gy. Cells were then infected by a type 5 adenoviral vector (CMV-GFP) at multiplicity of infection (MOI) of 0.2 and 4.0 at 37°C for 4 hours followed by washing. Cells were either lysed for cellular DNA extraction for real-time PCR analysis of viral DNA, or were cultured for an additional 48 hours before quantification of green fluorescent protein (GFP)-positive cells. The efficacy of gene transfer is expressed as the ratio of GFP-positive irradiated cells compared with nonirradiated cells.

Human tumor xenografts and adenoviral infusion. LoVo cells (2 × 106) were implanted s.c. in the flanks of nude mice. After 3 weeks, tumors were irradiated with 10 Gy and animals were infused through the tail vein with 1 × 106 plaque-forming units of adenovirus (CMV-GFP) 3 or 24 hours later. Four hours after infusion, tumors were excised and subjected to real-time PCR analysis of viral DNA content or Western blotting. The use of animals was in compliance with the regulations of the University of Michigan and with the NIH guidelines.

Note: J. Qian and J. Yang contributed equally to this work.

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Radiation treatment. Radiations were carried out using an Ortho-voltage Unit Pantak DXT 300 producing with 300-kV X-rays (East Haven, CT).

Inhibition of Dynamin 2 by small interference RNA transfection. LoVo, D54, and MCF-7 were transfected with Dynamin 2–specific small interference RNA (siRNA; Dharmacon, Lafayette, CO) using oligofectamine reagent (Invitrogen, Carlsbad, CA). At 24 hours after transfection, cells were irradiated or left untreated before incubation with GFP adenovirus at the MOI described above. Radiation induction of viral gene transfer in siRNA-transfected cells is expressed as a ratio compared with parental cells.

Real-time PCR and reverse transcription-PCR assays. Adenoviral DNA content in infected cells or tumors was detected by real-time PCR analysis as described previously (1) using primers for the β-gal gene sequence (5′-CACGGCAGATACACTTGCTG and 3′-ATGCGCATTTGACEACTAC). Viral DNA copies were calculated from a standard curve of purified adenovirus vector (CMV-β-gal) and were further adjusted by the protein concentration of each lysate. Dynamin 2 mRNA were quantified from 50 ng of total RNAs by real-time reverse transcription-PCR (RT-PCR) assay (Opticon, MJ Research, Inc., San Francisco, CA) using Quantitect SYBR Green RT-PCR kit (Qiagen, Inc., Valencia, CA) and specific primers for Dynamin 2 (5′-AGGAGTACTGGTTTGTGCTGACTG and 3′-TGTGGTGCATGAAGACATGCC). The levels of mRNA for Dynamin 2 were normalized by each α-actin mRNA detected by specific primers (5′-CGAGATCCCTCACAAATCCA and 3′-GTGATCTAGCTCTTCCA) and were expressed as a ratio compared with nonirradiated controls.

Immunohistochemistry staining and Western blotting for Dynamin 2. WB and LoVo cells were fixed in 4% paraformaldehyde at 2 or 4 hours after irradiation and were incubated with a rabbit anti-Dynamin 2 antibody at (1:50 dilution; BD Bioscience PharMingen) followed by incubation with a FITC-conjugated mouse anti-rabbit IgG antibody (1:100 dilution; Sigma, St. Louis, MO). Dynamin 2 signals were assessed by fluorescence microscopy. For immunoblotting, cell lysates were fractionated on a 7.5% polyacrylamide gel and electrotransferred onto a polyvinylidene difluoride membrane. Dynamin 2 was detected by an anti-Dynamin 2 antibody (1:500 dilution) followed by incubation with a horseradish peroxidase–conjugated goat anti-rabbit antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) and visualized by enhanced chemiluminescence (Pierce, Rockford, IL). Expression of Dynamin 2 was normalized to β-actin expression as detected by an antibody against β-actin (Sigma) and was quantified by densitometry.

Data analysis and statistics. Each experiment was carried out four times. Values are expressed as mean ± SE and were compared by ANOVA analysis. Data were considered significantly different when P < 0.05.

Results

Anti-Dynamin 2 antibody blocked radiation-induced adenoviral gene transfer and viral uptake in cells. Consistent with our previous findings, radiation significantly increased gene transfer in WB (hepatocyte) and LoVo (colon cancer) cells by as much as 36.9 ± 2.6- and 6.5 ± 1.0-fold, respectively, compared with unirradiated control cells (Fig. 1A). Although preincubation of cells with anti-CAR antibody decreased adenoviral gene transfer in nonirradiated WB and LoVo cells by ∼37% and 54% (data not shown), preincubution with anti-CAR or anti-αv integrin antibody in WB cells only slightly decreased radiation induction to 28.3 ± 3.0- or 27.0 ± 4.5-fold and had no inhibitory effect in LoVo cells (Fig. 1A). In contrast, preincubation with anti-Dynamin 2 antibody significantly decreased this radiation induction in both WB and LoVo cells to 10.0 ± 1.8- and 3.5 ± 1.0-fold, respectively (Fig. 1A). These data implied that radiation-induced adenoviral gene transfer could be substantially inhibited by blocking Dynamin 2 function.

Similarly, radiation significantly induced viral uptake, measured by quantitative PCR analysis of intracellular viral DNA content, in WB and LoVo cells at 4 hours after infection by as much as 7.2 ± 1.1- and 3.1 ± 0.2-fold, respectively (Fig. 1B). Pretreatment of cells with anti-CAR and anti-αv integrin antibodies had little or no inhibitory effect on viral uptake in irradiated WB and LoVo cells. However, preincubation of cells with anti-Dynamin 2 antibody significantly reduced the induction of viral uptake by radiation in both WB (3.7 ± 0.7-fold) and LoVo (1.8 ± 0.1-fold) cells (Fig. 1B) compared with controls. This inhibition was consistent with the reduction of gene transfer in cells, suggesting that radiation induced adenoviral gene transfer in partially through Dynamin 2–mediated viral uptake.

Radiation enhanced Dynamin 2 expression in cells. We next wished to determine if radiation had a direct effect on Dynamin 2

Figure 1. Antibody blocking of radiation-induced adenoviral gene transfer and viral uptake. WB and LoVo cells were preincubated with antibodies against CAR (Anti-CAR), αv integrin (Anti-αv Integrin), Dynamin 2 (Anti-Dyn 2), or no antibody (control) for 1 hour before irradiation with 8 Gy or no radiation control (No RT) followed by immediate adenovirus (CMV-GFP) infection for 4 hours. A, antibody blocking of radiation-induced adenoviral gene transfer. GFP-positive cells were measured as fold increase in comparison with nonirradiated cells. B, antibody blocking of radiation-induced adenoviral uptake. Viral DNA content in total cellular DNA extract was quantified by real-time PCR and expressed as fold increase in comparison with nonirradiated cells ∗, P < 0.05, significantly different from control (n = 4).
expression at the mRNA and protein level. We found that Dynamin 2 mRNA levels were increased at 2 hours after irradiation in both WB (1.5 ± 0.2-fold) and LoVo (1.3 ± 0.1-fold) and were maximized at 4 hours after irradiation at 2.2 ± 0.3- and 1.5 ± 0.1-fold, respectively, compared with nonirradiated cells (Fig. 2B). Furthermore, immunohistochemistry revealed that membrane Dynamin 2 signals in both WB and LoVo cells were intensified at 2 and 4 hours after radiation (Fig. 2A). The fact that radiation increased Dynamin 2 gene expression is consistent with a role for Dynamin 2 in radiation-induced adenoviral uptake in cells.

Small interference RNA inhibition of Dynamin 2 abolished radiation induction of adenoviral gene transfer. To determine if the increase in Dynamin 2 expression was the cause of radiation-induced viral uptake, we used siRNA to block induction. In fact, Dynamin 2 expression was significantly inhibited in tumor cell lines LoVo, D54, and MCF-7 transfected with Dynamin 2–specific siRNA compared with parental cells by 24 hours after transfection (Fig. 3A) and remained inhibited for up to 72 hours after transfection regardless of radiation treatment. Radiation induced viral gene transfer in parental cells by as much as 6.8 ± 0.5-, 5.5 ± 0.7-, and 9.0 ± 0.8-fold, respectively, whereas induction was significantly impaired in siRNA-treated cells (2.6 ± 0.3-, 3.7 ± 0.5-, and 5.0 ± 0.6-fold, respectively; Fig. 3B). These reductions were well correlated with the decreased viral uptake (data not shown), suggesting that Dynamin 2 may play an essential role during radiation induction of adenoviral infection.

Radiation increased Dynamin 2 expression and adenoviral uptake in colon cancer xenografts. To determine if Dynamin 2 expression was critical to radiation-induced viral uptake in vivo, we carried out experiments using s.c. LoVo cell xenografts. Radiation significantly increased viral presence, quantified by real-time PCR analysis, 4 hours after systemic viral infusion. The induction was significant when virus was given 3 hours after radiation (5,579 ± 543 particles/μg protein) compared with nonirradiated tumors (1,439 ± 245 particles/μg protein) and was enhanced maximally when virus was infused at 24 hours after irradiation (15,675 ± 765; Fig. 4A). This is consistent with our previous finding that adenoviral gene expression in intrahepatic colon xenografts was maximally induced when viral infusion took place at 24 hours post irradiation (1). As we anticipated, Dynamin 2 protein expression was induced by ~ 2-fold in tumors irradiated for 24 hours compared with nonirradiated tumor or tumors being irradiated for shorter period of time (3 hours) by densitometry scanning of Dynamin 2 bands (corrected by β-actin level in each sample) from Western blotting (Fig. 4B). Consistently, Dynamin 2 level was increased in tumors at 24 hours after liver irradiation by immunohistochemistry staining (Fig. 4C). It was noted also that this increase was detected mostly in interstitial tissues, although a moderate increase was also observed in tumor cells, implying that endothelium might be the target of radiation. These findings suggested that radiation stimulated Dynamin 2 gene expression could facilitate an enhanced adenoviral presence or uptake in tumors.

Discussion

Our previous studies indicated that radiation induces adenovirus infection through adenovirus-specific mechanism(s) (1). Here we
found that antibody blockages of CAR can reduce adenoviral infection in both WB and LoVo cells by ~37% and 54% evidenced by decreased viral gene transfer (data not shown). However, antibody blockages for CAR and integrin had little or no effects on radiation induction of viral gene transfer and uptake in cells. These inductions are mediated chiefly through an induction of Dynamin 2 function, based on the findings that this induction can be blocked by specific antibody and siRNA. Radiation also significantly increased tumor viral content after i.v. administration at the time of induction for Dynamin 2.

Although Dynamin 2 induction accounts for the majority of the radiation-induced increase in viral uptake, radiation could trigger multiple mechanisms to induce adenoviral infection. In addition to CAR and clathrin-dependent viral endocytosis, adenovirus uses macropinocytosis, the non–clathrin-dependent pathway that involves αv, integrin, filamentous actin, and the amiloride-sensitive Na+/H+ exchanger, for both viral uptake and endosomal escape independent of Dynamin 2 function (17). Radiation could provide energy to regulate these molecules in endosomal events.

Dynamin 2 is a large GTPase that is distinguished from other small GTPases such as Ras-like GTPases by its low GTP-binding affinities and ability to interact with the lipid layer of the membrane. It is activated by protein oligomerization (self-assembly; ref. 18), leading to GTP hydrolysis as required for endocytosis. Dynamin 2 is also implicated in variety of pathophysiologic processes, including actin-associated cytoskeleton rearrangement (19) and p53-dependent apoptotic signaling (20). Therefore, activation of Dynamin 2 could be the result of cell apoptosis in response to environmental stressors such as radiation or viral infection itself.

Radiation may regulate Dynamin 2 function through distinctive mechanisms. One possibility may involve a direct self-activation of by radiation (quick response) through oligomerization of this molecule. Another possibility may involve the activation of Dynamin 2 gene expression (late response) that requires protein synthesis for increased function, leading to a later viral infection. In cell culture, induced viral gene transfer and uptake were achieved maximally during the first 4 hours after radiation. This may result from a rapid induction of Dynamin 2 self-assembly as reflected by immunohistochemistry staining. Inhibition of Dynamin 2 by siRNA significantly reduced cellular Dynamin 2 content and therefore may have impaired Dynamin 2 oligomerization and subsequent viral uptake. However, increased viral uptake in tumor xenografts was observed maximally at 24 hours after irradiation, concurrent with increased Dynamin 2 levels in irradiated tumors. We also noted that mRNA levels were enhanced at 4 hours after radiation and remained moderately high at 24 hours, suggesting that new synthesis of Dynamin 2 could be triggered by radiation treatment in tumor xenografts, leading to a late induction of viral uptake. Moreover, we found that radiation may induce Dynamin 2 expression in interstitial tissue within tumors, suggesting that endothelium could be a potential target for radiation in facilitating adenoviral uptake in tumor cells. The cell type with increased Dynamin 2 expression in response to radiation needs to be addressed.

Our current finding that ionizing radiation induced adenoviral uptake through an elevated Dynamin 2 function may improve selectivity of adenovirus targeting in tumors, thus, a potential benefit in the combination of radiation therapy with enzyme/prodrug adenovirus gene therapy. One potential disadvantage of this approach is the liver toxicity due to radiation-induced viral uptake in normal liver, potentially leading to subacute or fulminate hepatitis. As conformal radiation technology continues to advance, a highly targeted delivery of radiation should benefit for liver

Figure 4. Radiation induced Dynamin 2 (Dyn 2) expression and adenoviral uptake in tumor xenografts. S.c. LoVo tumors were established in nude mice. Adenovirus was infused through tail vein at various time (0, 3, and 24 hours) after 10 Gy of irradiation. Nonirradiated tumors (No RT) were used as controls. Tumors were harvested at 4 hours after viral infusion. A, viral uptake measured by real-time PCR for viral DNA content (particles) in tumors receiving no or 10 Gy of radiation followed by viral infusion at various time (0, 3, and 24 hours) after irradiation. B, Western blotting of Dynamin 2 in tumors receiving no or 10 Gy radiation with β-actin expression in tumors as internal controls. Quantification of Dynamin 2 was done by density scanning of specific bands that were adjusted by β-actin level in each sample. C, immunohistochemistry staining of Dynamin 2 in intrahepatic LoVo tumors at 24 hours after radiation (10 Gy) or no radiation control (No RT) by monoclonal antibody against Dynamin 2 and FITC-conjugated anti-mouse IgG. Tumor nodules (representatively outlined) were connected by interstitial tissue. *, P < 0.05, significantly different from nonirradiated tumors (n = 4).
Radiation and Adenovirus Infection through Dynamin 2


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References


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