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Porcine adenovirus serotype 3 internalization is independent of CAR and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin

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Abstract

Nonhuman adenoviruses including porcine adenovirus serotype 3 (PAd3) are emerging vectors for gene delivery. PAd3 efficiently transduces human and murine cells in culture, and circumvents preexisting humoral immunity in humans. The coxsackievirus–adenovirus receptor (CAR) serves as a primary receptor and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin as a secondary receptor for several human adenovirus (HAd) subtypes including HAd5. In this study, we deduced the role of CAR, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin in PAd3 internalization. Transduction experiments were conducted in human mammary epithelial (MCF-10A) cells using replication-defective PAd-GFP (PAd3 vector expressing green fluorescent protein [GFP]) and HAd-GFP (HAd5 vector expressing GFP). MCF-10A cells were treated with or without anti-human CAR, or anti-$\alpha_v\beta_3$ or anti-$\alpha_v\beta_5$ integrin antibodies prior to infection with HAd-GFP or PAd-GFP. Significant ($P < 0.05$) inhibition in transduction by HAd-GFP was observed in antibody-treated cells as compared to untreated cells, whereas transduction by PAd-GFP remained to similar levels irrespective of the treatment. To study the adenoviral fiber knob-mediated virus interference, MCF-10A cells were treated with or without the recombinant HAd5 or PAd3 knob followed by infection with HAd-GFP or PAd-GFP. Significant ($P < 0.05$) inhibition was observed only in transduction of the homologous vector. These results suggested that PAd3 internalization was CAR- as well as $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin-independent and the primary receptor for HAd5 and PAd3 were distinct. CAR- and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin-independent entry of PAd3 vectors may have implications in targeting cell types that are not efficiently transduced by other adenoviral vectors.

Keywords: Porcine adenovirus; CAR; $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin

Introduction

High-affinity binding of the adenoviral fiber knob to the coxsackievirus-adenovirus receptor (CAR) is the primary event in virus internalization into the host cells (Bergelson et al., 1997) for human adenovirus (HAd) serotype 5 (HAd5), the widely studied HAd vectors for gene therapy. In fact, CAR serves as a primary receptor for the majority of HAd in subgroups A, C, D, E, and F but not B (Bergelson et al., 1997; Roelvink et al., 1998; Tomko et al., 1997). CAR is a 46-kDa type I integral transmembrane protein belonging to the Ig superfamily (Bergelson et al., 1997). While its exact cellular function is unknown, it appears that CAR might serve as a cell adhesion molecule. Expression of CAR is variable in human cell types (Tomko et al., 1997), and it appears to be developmentally regulated (Honda et al., 2000). In addition to CAR, several other cell surface molecules, which have been implicated as primary receptors for HAd include MHC class I α2 domain (Hong et al., 1997), sialic acid saccharides (Arnberg et al., 2000), CD46 (Gaggar et al., 2003), CD80/CD86 (Short et al., 2004), and heparan sulfate glycosaminoglycans (Dechecchi et al., 2000; Smith et al., 2003).

Following CAR binding, the HAd penton base protein interacts with vitronectin-binding integrins, specifically $\alpha_v\beta_3$ and $\alpha_v\beta_5$, leading to receptor-mediated uptake of virus particles (Hong et al., 1997; Wickham et al., 1993).

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This process is facilitated by the tripeptide Arg-Gly-Asp (RGD) motif of the adenovirus penton base. The integrin-binding RGD motif is also found in a number of adhesion molecules that are known to interact with integrins (Bai et al., 1993). It has been shown that the interaction of the HAd penton base with integrin promotes actin cytoskeletal reorganization via activation of several signaling molecules (Li et al., 2001).

Due to ubiquitous nature of HAd, a majority of the human population is exposed to one or more HAd serotypes leading to the development of a HAd-specific immune responses (Harvey et al., 1999). This preexisting vector immunity significantly reduces the uptake of HAd vectors following the first inoculation. The early region 1 (E1)-deleted replication-defective HAd vectors are capable of expressing the early and late viral genes at a magnitude sufficient to stimulate cellular and humoral immune responses (Kafri et al., 1998; Yang et al., 1995). The cellular immune response eliminates the target cells expressing viral and transgene products, whereas neutralizing antibodies significantly inhibit virus uptake following readministration of the same vector (Moffatt et al., 2000; Sailaja et al., 2002; Walter et al., 1996). This leads to a rapid loss of transgene expression in experimental animals (Crystal, 1995). In order to elude preexisting vector immunity and to effectively transduce cell types that are poorly infected by HAd5 vectors, a number of human and nonhuman adenoviruses, such as HAd35 (Reddy et al., 2003), bovine adenovirus serotype 3 (BAd3) (Mittal et al., 1995; Reddy et al., 1999), canine adenovirus serotype 2 (Hemminki et al., 2003; Klonjkowski et al., 1997; Kremer et al., 2000), chimpanzee adenovirus (Farina et al., 2001; Xiang et al., 2002), ovine adenovirus (Hofmann et al., 1999; Loser et al., 2003), and porcine adenovirus serotype 3 (PAd3) (Bangari and Mittal, 2004; Reddy et al., 1999; Zakhartchouk et al., 2003) have been developed.

PAd3 is a *Mastadenovirus* and was originally isolated from a healthy piglet (Clarke et al., 1967) and is not known to cause any serious disease in its natural host (Derbyshire et al., 1975). Its genome organization and the transcription map are similar to those of subtype C HAd (Reddy et al., 1998). PAd3 has been shown to effectively circumvent the HAd-specific immune response (Moffatt et al., 2000). HAd-specific preexisting neutralizing antibodies in humans did not cross-neutralize PAd3 (Bangari and Mittal, 2004), and PAd3 vectors efficiently transduce both human and murine cells in culture (Bangari and Mittal, 2004). We observed that the transduction efficiency of a PAd3 vector in mouse NIH 3T3 cell line was significantly higher than that of a HAd5 vector (Bangari and Mittal, 2004), suggesting that these two vectors may use different primary receptors for receptor-mediated internalization. Since HAd5 vectors enter susceptible cells via CAR-mediated endocytosis and NIH 3T3 cells are known to express low level of CAR, these results indicated that PAd3 entry into these cells might be CAR-independent. The nature of primary receptor(s) for PAd3 is unknown. Since the PAd3 penton base lacks the integrin binding RGD motif (Reddy et al., 1998), the nature of secondary interactions in PAd3 internalization is not clear. The present study shows that PAd3 internalization in MCF-10A cell line (originated from human normal breast epithelial cells) is independent of CAR and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin. The results of fiber knob-mediated virus interference studies using the recombinant HAd5 or PAd3 fiber knob domain suggest that the receptor(s) for HAd5 and PAd3 internalization into susceptible cells are distinct.

Results

Comparative sequence analysis of PAd3 and HAd5 fiber knobs

While entry of HAd5, HAd2, and several other HAd serotypes is mediated by their initial binding to CAR (Bergelson et al., 1997), receptors involved in PAd3 attachment and internalization are currently unknown. Our recent observation demonstrates that PAd-GFP efficiently transduce NIH 3T3 cells, a low CAR expressing cell line (Bangari and Mittal, 2004), suggesting that PAd3 vector entry into this cell line may be CAR-independent. Since the adenoviral fiber knob domain is believed to initiate virus binding on the cell surface, we began with a comparison of deduced amino acid sequences of PAd3 and HAd5 fiber knob domains. Based on the structural and mutagenesis studies of knob domains of various CAR-binding HAd subtypes, amino acid residues that are critical for CAR binding have been identified (Kirby et al., 2000; Roelvink et al., 1998). Comparative sequence analysis of the fiber knob domains of PAd3 and HAd5 (Fig. 1) revealed that the residues critical in CAR-binding (Ser_{408}, Pro_{409}, Tyr_{477}, and Leu_{485}) or those that peripherally influence CAR-binding (Ala_{406}, Arg_{412}, and Arg_{481}) were not conserved in the PAd3 fiber knob (Fig. 1). The Thr-Leu-Trp-Thr (TLWT) motif that marks the beginning of the knob domain in most *Mastadenovirus* species (Chroboczek et al., 1995) was conserved in the PAd3 fiber, but there was only 23.1% amino acid sequence similarity with the PAd3 fiber knob compared to its HAd5 counterpart. Furthermore, the PAd3 fiber is expected to be shorter (448 residues with 15 repeats in the shaft region) (Reddy et al., 1995, 1998), compared to the HAd5 fiber (581 residues with 22 repeats in the shaft region) (Chroboczek et al., 1995). Moreover, the integrin-binding motif such as the classical Arg-Gly-Asp (RGD) or Leu-Asp-Val (LDV) motif is not present in the PAd3 penton base (Reddy et al., 1998). In the light of these striking differences at the amino acid sequence level, we hypothesized that PAd3 internalization into susceptible cells may be CAR- as well as integrin-independent.

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CLUSTAL W (1.82) multiple sequence alignment
HAd5 Fiber Knob TLWTTPAPSPNCRLN--AEKDAKLTLVLTKCGSQILATVSVLAVKGSLAPISGTVQSAHL 457
                **** .:*:.*  *.  :  :.:: * **: *. :*.: : :*:*.*:   *.:  : ::
PAd3 Fiber Knob TLWTGASPTANVILTNTTTPNGTFFLCLTRVGGLVLG---SFALKSSIDLTS---MTKKV 314
HAd5 Fiber Knob IIRFDENGVLLNNSFLDPEYWNFRNGD--LTEGTAYTNAVGFMPNLSAYPKSHGKTAKSN 515
                 : **  * * .:*  . .: .**..*  :   :*  ... :**.   **:: .:*: *.
PAd3 Fiber Knob NFIFDGAGRLQSDSTYKGRFG-FRSNDSVIEPTAAGLSPAWLMPSTFIYPR---NTSGSS 370
HAd5 Fiber Knob IVSQVYLNGD--KTKPVTLTITLNGTQETGDTTPSAYSMSFSWDWSGHNYINEIFATSSY 573
                :.* **:*    :..  . *:: ** .   :    ::* .** .:. . *: .  :  .
PAd3 Fiber Knob LTSFVYINQTYVHVDIKVNTLSTNGYSLEFNFQNMSFSAPFSTSYGTFCYVPRRTTHRPR 430
HAd5 Fiber Knob TFSYIAQE---------- 581
                  .:  :*
PAd3 Fiber Knob HGPFSLRERRHLFQLLQQ 448
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Fig. 1. Homology comparison of the HAd5 fiber knob with the corresponding region of PAd3 fiber using CLUSALW alignment software. Identical, similar, and related residues are indicated by asterisks, colons, and dots, respectively. The HAd5 fiber knob residues that are known to be critical for CAR binding or that peripherally affect CAR binding are in bold or are underlined, respectively.

CAR expression in various cell lines

In order to conduct CAR-blocking experiments, we needed a cell line, which was efficiently transduced by both PAd3 and HAd5 vectors, and expressed moderate levels of CAR. Although our focus was to determine whether CAR or integrins play a role in PAd3 internalization, we included HAd-GFP as a control to validate our experimental methodology. Initially, we compared expression levels of CAR in a number of cell lines of human, murine, porcine, or bovine origin by immunofluorescence and Western blot using an anti-CAR polyclonal antibody. We observed significant differences in expression levels of CAR in various human and nonhuman cell lines. The human embryonic kidney (293), fetal bovine retinal (FBRT HE1), and murine breast tumor (MT1A2) cell lines appeared to be high-CAR expressing; human malignant breast cancer (MDA-MB231), human mammary epithelial (MCF-10A), and fetal porcine retinal (FPRT HE1-5) cells as intermediate-CAR expressing; and the mouse fibroblast (NIH 3T3) cell lines as low-CAR expressing cells as detected by immunofluorescence assay (Fig. 2A) and Western blot (Fig. 2B). On the basis of our previous finding that transduction efficiencies of HAd-GFP and PAd-GFP in MCF-10A cell line were approximately 83% and 88%, respectively (Bangari and Mittal, 2004), and the results of levels of CAR expression shown here, this cell line was chosen for the present study.

To further confirm the expression of CAR, and to determine the expression of $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin in MCF-10A cells, we performed flow cytometric analysis using an anti-human CAR (hCAR), or anti-$\alpha_v\beta_3$ integrin or anti-$\alpha_v\beta_5$ integrin antibody. The results demonstrated that MCF-10A cells expressed CAR (Fig. 3A), $\alpha_v\beta_3$ integrin, and $\alpha_v\beta_5$ integrin (Fig. 3B). Of these three cell surface molecules, $\alpha_v\beta_5$ integrin appeared to be in most abundance.

CAR- and integrin-independent transduction by PAd-GFP

Having confirmed the presence of the CAR, $\alpha_v\beta_3$ integrin, and $\alpha_v\beta_5$ integrin on MCF-10A cells, we sought to determine the effect of antibody-mediated blocking of these cell surface molecules on the transduction ability of PAd-GFP. The mouse monoclonal antibody, RmcB, is known to bind CAR receptors on the cell surface and thereby block infection of subtype C and other CAR-

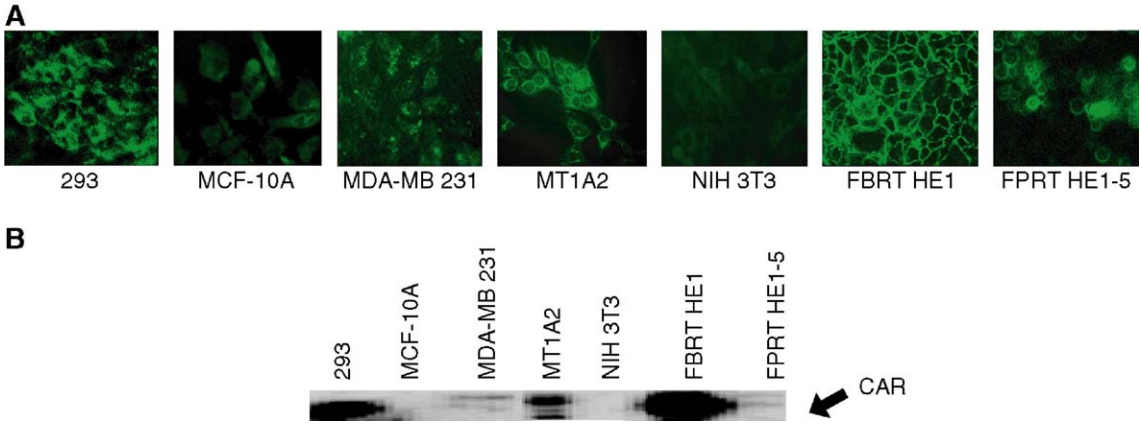

Fig. 2. Level of CAR expression in various cell lines. (A) Various cell lines of human [293, MDA-MB-231 and MCF-10A], murine [MT1A2 and NIH 3T3], bovine [FBRT-HE1-1], and porcine [FPRT-HE1-5] origin were grown to approximately 75% confluence on the Lab-tek chambered slide, fixed with formaldehyde, and analyzed by immunofluorescence assay using a rabbit anti-human CAR antibody. The slides were visualized at ×200 under a fluorescence microscope. (B) Various cell lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and analyzed by Western blot using a polyclonal rabbit anti-human CAR antibody and a secondary HRP-conjugated anti-rabbit antibody. The signal was detected by chemiluminescence using Kodak Image Station.

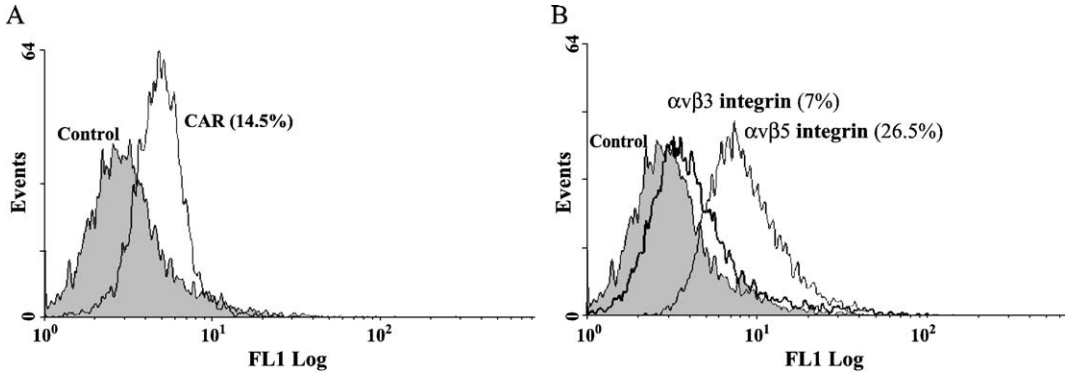

Fig. 3. Flow cytometric analysis for CAR, $\alpha_v\beta_3$ integrin or $\alpha_v\beta_5$ integrin expression on MCF-10A cells. MCF-10A cells were harvested and analyzed for the presence of CAR, $\alpha_v\beta_3$ integrin or $\alpha_v\beta_5$ integrin molecules using a CAR-, $\alpha_v\beta_3$ integrin-, or $\alpha_v\beta_5$ integrin-specific antibody and an FITC-labeled secondary antibody followed by flow cytometry. Unstained cells or the cells stained only with the secondary antibody served as negative controls.

binding HAd (Bergelson et al., 1997; Tomko et al., 1997). We observed a drastic inhibition in HAd-GFP transduction of MCF-10A cells preincubated with various concentrations of RmcB (Fig. 4). More than 50% reduction in the transduction efficiency of HAd-GFP was observed at the lowest concentration of RmcB antibody (1 μg/ml) used in our experiment. Further reduction in transduction efficiency was observed at higher concentrations of RmcB. This finding supported the pre-established paradigm of CAR-mediated entry of HAd5 (Bergelson et al., 1997; Tomko et al., 1997). Interestingly, RmcB blocking of CAR molecules on MCF-10A cells did not significantly ($P < 0.05$) affect the transduction efficiency of PAd-GFP (Fig. 4), thus implying a CAR-independent entry pathway for PAd3. We also conducted adenoviral vector transduction experiments with an m.o.i. of 1 or 5 p.f.u. per cell of HAd-GFP or PAd-GFP following antibody-mediated blocking of CAR and found that the inhibitory effect on HAd-GFP transduction remained to similar levels (data not shown). These experiments were also conducted in MDBK (Madin Darby bovine kidney) cell line with similar results (data not shown).

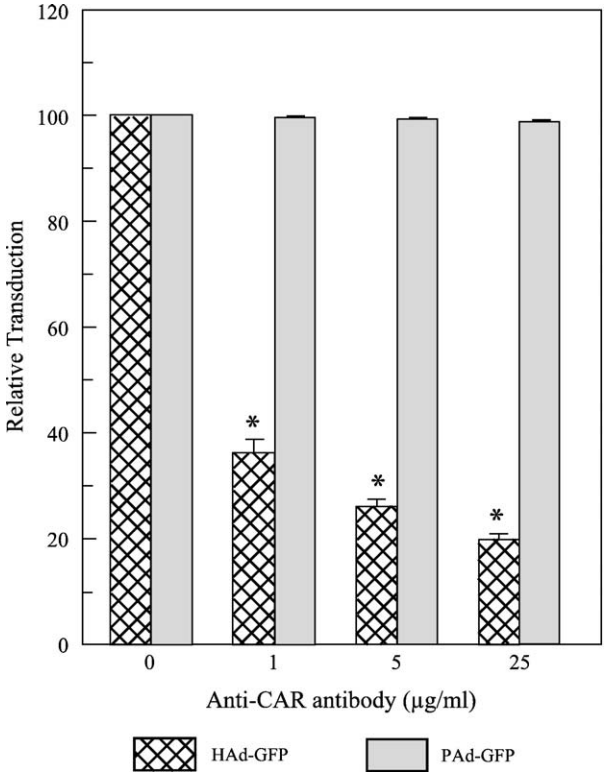

Fig. 4. Ablation of CAR-mediated internalization using a CAR-specific antibody. MCF-10A cells in monolayer cultures were treated with 1, 5, or 25 μg/ml of CAR-specific antibody at 4 °C for 1 h followed by infection either with HAd-GFP or PAd-GFP at an m.o.i. of 20 p.f.u. per cell. At 36 h post-infection, cells were harvested by trypsinization and the cells expressing GFP were sorted by flow cytometry. Cell monolayers treated without the CAR-specific antibody but were infected similarly with HAd-GFP or PAd-GFP served as positive controls. The transduction efficiencies of positive controls were taken as 100% for calculating relative transduction of various treatment groups. The results are shown as the mean ± standard deviation of the percent relative transduction of at least three independent observations. *, $P < 0.05$.

Next, we tested if the function-blocking antibody against $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin could affect infectivity of PAd-GFP. Since integrins have been suggested as secondary receptors mediating HAd5 internalization (Wickham et al., 1993), it was expected that preincubation of cells with an anti-$\alpha_v\beta_3$ or anti-$\alpha_v\beta_5$ integrin antibody would diminish HAd5 infectivity. To rule out the possibility that CAR-binding itself was sufficient to mediate internalization, we performed all anti-integrin antibody-mediated blocking assays in the presence of the anti-CAR antibody. MCF-10A cells were incubated with RmcB antibody (20 μg/ml) prior to the addition of 10 or 25 μg/ml of an anti-$\alpha_v\beta_3$ or anti-$\alpha_v\beta_5$ integrin antibody. Significant ($P < 0.05$) dose-dependent inhibition in HAd-GFP transduction was observed and the effect was more pronounced with the anti-$\alpha_v\beta_3$ integrin antibody (Fig. 5A) than with the anti-$\alpha_v\beta_5$ integrin antibody (Fig. 5B). This difference could partly be attributed to the low abundance of $\alpha_v\beta_3$ integrin molecules on MCF-10A cells (Fig. 3B). Importantly, no significant ($P > 0.05$) inhibition in PAd-GFP infectivity was noticed with either antibody. These data indicated that PAd3 did not use CAR, $\alpha_v\beta_3$ integrin, or $\alpha_v\beta_5$ integrin for internalization.

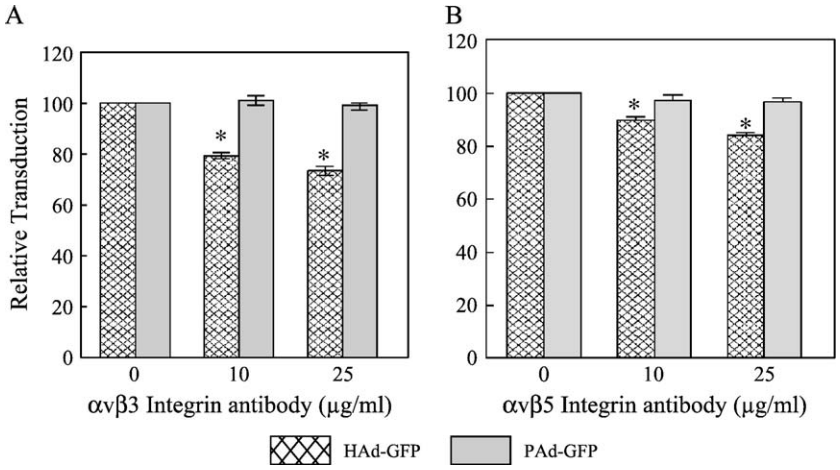

Fig. 5. Ablation of $\alpha_v\beta_3$ integrin- or $\alpha_v\beta_5$ integrin-mediated internalization using specific antibodies. MCF-10A cells in monolayer cultures were treated with a fixed amount of RmcB antibody (20 μg/ml) and (A) $\alpha_v\beta_3$ integrin-specific (10 or 25 μg/ml) or (B) $\alpha_v\beta_5$ integrin-specific (10 or 25 μg/ml) antibody at 4 °C for 1 h followed by infection either with HAd-GFP or PAd-GFP at an m.o.i. of 20 p.f.u. per cell. At 36 h post-infection, cells were harvested by trypsinization and the cells expressing GFP were sorted by flow cytometry. Cell monolayers treated without the specific antibody but were infected similarly with HAd-GFP or PAd-GFP served as positive controls. The transduction efficiencies of positive controls were taken as 100% for calculating relative transduction of various treatment groups. The results are shown as the mean ± standard deviation of the percent relative transduction of at least three independent observations. *, $P < 0.05$.

Inhibition in transduction efficiency of HAd-GFP or PAd-GFP by homologous fiber knob

Competitive inhibition of adenoviral binding and internalization by the soluble viral fiber or knob domain has been utilized as the most convincing evidence for the distinct receptor usage by various HAd serotypes (Arnberg et al., 2000; Bergelson et al., 1997; Philipson et al., 1968; Roelvink et al., 1998). The primary receptor seeking moiety of the adenovirus resides in the fiber knob (Henry et al., 1994). Therefore, preincubation of susceptible cells with soluble trimeric knobs leads to saturation of the virus receptors resulting in a dose-dependent inhibition in the parent (homologous) virus attachment and infectivity (Bergelson et al., 1997; Henry et al., 1994). We needed both HAd5 and PAd3 trimeric knob domains to pursue virus interference studies. Both the monomeric and the trimeric forms of purified PAd3 knob (P3 knob) and HAd5 knob (H5 knob) were observed on SDS-PAGE gels stained with Coomassie blue (Fig. 6A). The expected molecular weights of P3 and H5 knob monomers were 20.9 and 19.8 kDa, respectively. The knob trimers seen on stained gels were confirmed to be specific by Western blot using virus-specific rabbit hyperimmune sera (Figs. 6B,C), indicating that recombinant P3 and H5 knobs were capable of trimerization into functionally active forms.

Inability of anti-CAR antibody to inhibit transduction of PAd-GFP suggested that the primary receptor for HAd5 and

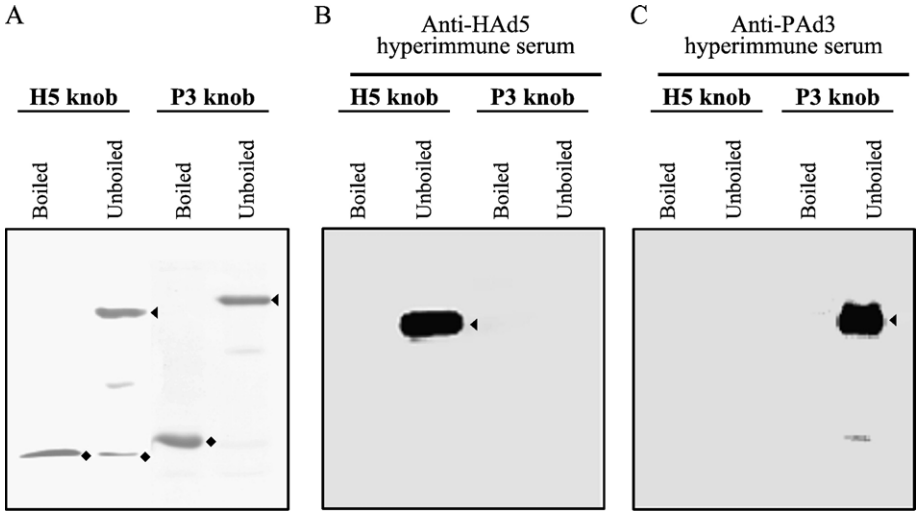

Fig. 6. Expression and specificity of recombinant H5 and P3 knobs. (A) Purified recombinant H5 and P3 knob proteins with (boiled) or without (unboiled) dithiothreitol treatment were analyzed by SDS-PAGE and stained with Coomassie blue. Western blot analyses of purified recombinant H5 and P3 knob proteins were conducted using (B) anti-HAd5 or (C) anti-PAd3 hyperimmune serum. Trimers (◄), monomers (◆).

PAd3 were distinct. The H5 or P3 knob domain was used for homologous and heterologous competition experiments in order to confirm this finding. MCF-10A cells were incubated with various concentrations of recombinant H5 or P3 knob domain to saturate their respective primary receptors prior to infection with HAd-GFP or PAd-GFP. As expected, the H5 knob domain significantly ($P < 0.05$) inhibited HAd-GFP transduction in MCF-10A cells in a dose-dependent manner, whereas there was no significant ($P > 0.05$) inhibition in PAd-GFP transduction even at the highest concentration of the H5 knob domain. HAd-GFP transduction was reduced to approximately 40% in cells preincubated with 25 μg/ml of the H5 knob (Fig. 7A). Similarly we observed a significant ($P < 0.05$) dose-dependent inhibition in PAd-GFP transduction in MCF-10A preincubated with the P3 knob (Fig. 7B), while HAd-GFP transduction was not significantly ($P > 0.05$) affected. Interestingly, higher concentrations of P3 knob were needed to obtain similar levels of inhibition in transduction with homologous vector as compared to those of H5 knob. This may be attributed to high abundance of PAd3 receptor(s) compared to CAR on MCF-10A cells.

Discussion

Although PAd3 has been used as a vaccine vector in swine (Hammond et al., 2001) and is being developed as a potential gene delivery vector (Bangari and Mittal, 2004), its tissue tropism and the determinants of cell entry have not been investigated. Among various HAd subtypes, there are significant differences in the fiber structure, both in the overall fiber shaft length and the amino acid composition (Roelvink et al., 1999) accounting for the differences in their tissue tropism. This is supported by the fact that different serotypes preferentially infect different tissue types in human and animals, including the ocular, respiratory, gastrointestinal, and genitourinary tissues (Mei et al., 1998). PAd3 was originally isolated from the rectal swab (Clarke et al., 1967), whereas HAd5 is known to infect the respiratory system (Horwitz, 2001). A comparison of the receptor-binding knob domain of PAd3 fiber with that of HAd5 revealed that the residues involved in CAR binding were not conserved in the PAd3 fiber knob, while most of the CAR-binding HAd subtypes show conservation at these CAR-binding residues (Roelvink et al., 1999). Therefore, we anticipated that PAd3 internalization into susceptible cells might be independent of CAR expression on the cell surface. Our results clearly demonstrated that antibody-mediated blocking of CAR molecules on the cell surface did not affect PAd-GFP transduction, while HAd-GFP transduction was substantially reduced.

In addition to CAR, various integrins are believed to be involved as secondary receptors for HAd5 entry. The integrin-binding RGD or LDV motif is absent in the penton base of PAd3, suggesting that interaction with integrin molecules may not be important for PAd3 internalization. However, it has been suggested that adenovirus could bind integrins even in the absence of the RGD motif (Soudais et al., 2000). Our study involving the antibody-mediated

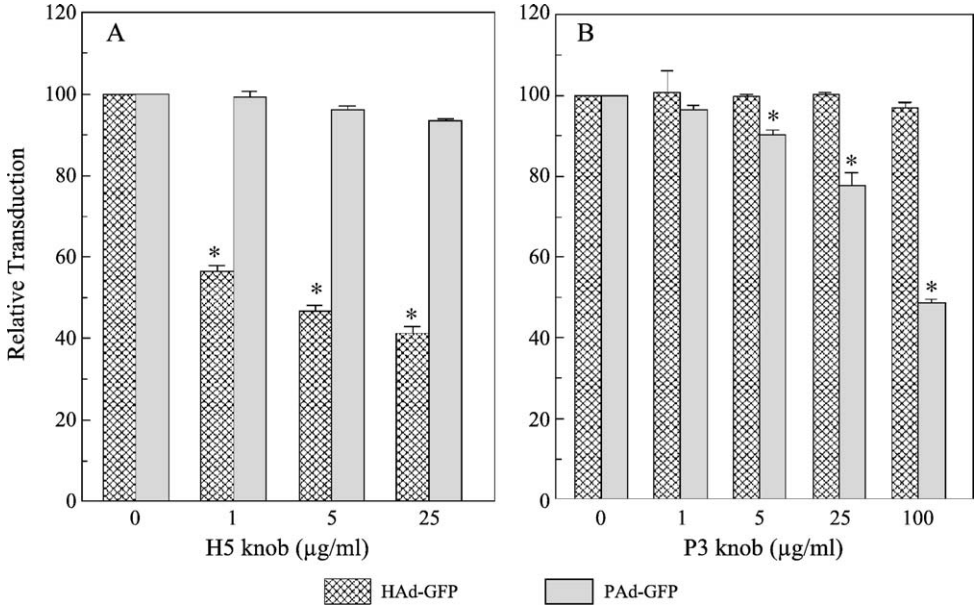

Fig. 7. Ablation of CAR-mediated internalization using the recombinant HAd5 or PAd3 fiber knob. MCF-10A cells in monolayer cultures were treated with (A) 1, 5, or 25 μg/ml of the recombinant HAd5 fiber knob or (B) 1, 5, 25, or 75 μg/ml of the recombinant PAd3 fiber knob at 4 °C for 1 h followed by infection either with HAd-GFP or PAd-GFP at an m.o.i. of 20 p.f.u. per cell. At 36 h post-infection, cells were harvested by trypsinization and the cells expressing GFP were sorted by flow cytometry. Cell monolayers treated without the recombinant HAd5 fiber knob but were infected similarly with HAd-GFP or PAd-GFP served as positive controls. The transduction efficiencies of positive controls were taken as 100% for calculating relative transduction of various treatment groups. The results are shown as the mean ± standard deviation of the percent relative transduction of at least three independent observations. *, $P < 0.05$.

blocking of $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin did not significantly reduce PAd-GFP transduction as compared to HAd-GFP transduction. It has been shown that human HeLa and A549 cell lines that efficiently expressed CAR, $\alpha_v\beta_3$ integrin, and $\alpha_v\beta_5$ integrin (Davison et al., 2001; Soudais et al., 2000) were poorly transduced with PAd3 (Zakhartchouk et al., 2003), whereas, murine NIH 3T3 cells that poorly expressed CAR, $\alpha_v\beta_3$ integrin, and $\alpha_v\beta_5$ integrin were efficiently transduced with PAd3 (Bangari and Mittal, 2004; unpublished data). These observations and the fact that the RGD or LDV motif is absent in the PAd3 penton base clearly suggest that $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin might not play a significant role in PAd3 internalization. In addition, our fiber-knob-mediated virus interference experiments using the recombinant H5 or P3 knob showed that there was no inhibition in vector transduction with the heterologous virus, indicating that the primary receptor(s) for PAd3 and HAd5 were distinct.

A variety of cell surface molecules other than CAR have been implicated in adenoviral binding and internalization. Cell surface major histocompatibility (MHC) class I molecules bind HAd5 knob (Hong et al., 1997); heparan sulfate glycosaminoglycans (HSG) can interact with the fiber shaft of HAd subtypes 2 and 5 (Dechecchi et al., 2000; Smith et al., 2003); sialic acid has been shown to mediate attachment and infection of adenovirus in subgroup D (Arnberg et al., 2000); CD46 has been implicated as a receptor for adenoviruses of subgroup B (Gaggar et al., 2003); and recently CD80 and CD 86 have been shown to be the attachment receptors for HAd3 (Short et al., 2004). The role of any of these molecules as the primary PAd3 receptor/s is yet to be explored, but CD46 may not be important in PAd3 internalization since PAd3 efficiently transduced murine fibroblast cells, which are known to be deficient in CD46.

Taken together, the results of this study suggest that PAd3 entry in human MCF-10A cells is CAR- as well as $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin-independent and the primary receptors for HAd5 and PAd3 are different. Cellular tropism is one of the important factors that determine the usefulness of viral vectors for gene delivery. Promiscuous transduction of nontarget tissues, and poor transduction of several clinically relevant tissue types including many cancers with HAd5 vectors is largely attributed to the CAR-dependent tropism of HAd (Hemminki and Alvarez, 2002). Some of the strategies to ablate CAR-dependent tropism of HAd vectors include development of chimeric vectors carrying fibers from non-CAR-binding adenoviruses such as HAd35 (Koizumi et al., 2003; Shayakhmetov et al., 2004), HAd16 (Goossens et al., 2001), or HAd3 (Kanerva et al., 2002) as well as from nonhuman adenoviruses such as canine adenovirus serotype 2 (Glasgow et al., 2004), bovine adenovirus serotype 4, duck adenovirus type 1, and fowl adenovirus type 1 (Renaut et al., 2004). As reported in this paper, CAR-independent entry of PAd3 should expand the possibility of developing novel vectors with an extended or modified tropism by engineering the chimeric PAd/HAd knob or fiber.

To harness the potential of PAd3 vectors for application in human gene delivery, it will be important to determine the repertoire of cell types transduced by PAd3 vectors and whether these vectors can efficiently transduce cell types, which are refractory to HAd5 transduction. In vivo studies in experimental animals will be necessary to explore whether biodistribution of PAd3 vectors is different than that of HAd5. It is anticipated that CAR-independent entry of PAd3 may significantly lower the innate immune response and hepatotoxicity, which are commonly observed with a number of HAd vectors.

Materials and methods

Adenoviral genome sequence alignment

The complete genomic sequences of HAd5 (accession no. NC_001405) and PAd3 (accession no. AF083132) were obtained from GenBank to compare amino acid sequences of their receptor-binding fiber knob domains using CLUSALW alignment software (Thompson et al., 1994).

Cells and viruses

Various cell lines used in this study were as follows: human embryonic kidney (293), human mammary epithelium (MCF-10A), human malignant breast cancer (MDA-MB231), murine fibroblast (NIH 3T3), murine breast cancer (MT1A2) (Addison et al., 1995), fetal bovine retina (FBRT HE1) (van Olphen et al., 2002), and fetal porcine retina (FPRT HE1–5) (Bangari and Mittal, 2004). These cell lines were propagated as described previously (Bangari and Mittal, 2004). The construction of replication-defective HAd5 (HAd-GFP) and PAd3 (PAd-GFP) vectors carrying the green fluorescent protein (GFP) gene driven by the cytomegalovirus promoter is described elsewhere (Bangari and Mittal, 2004). These vectors were propagated and purified as described previously (Bangari and Mittal, 2004).

Antibodies

A rabbit polyclonal antibody against human CAR (hCAR) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) was used for Western blot and immunofluorescence assays. For blocking studies, the mouse hybridoma RmcB that expresses anti-hCAR antibody (Hsu et al., 1988) was purchased from ATCC and propagated as per supplier's recommendations. This monoclonal antibody was purified from the hybridoma supernatant by protein G-sepharose (Zymed Laboratories, Inc., San Francisco, CA) affinity column chromatography following the manufacturer's instructions. The antibody concentration was determined by Coomassie Blue Protein Assay Reagent (Pierce Bio-

technology, Inc., Rockford, IL). Sodium azide (1%) and bovine serum albumin (BSA; 0.1%) were added to the purified antibody for prolonged storage. For determining integrin expression and function blocking assays, the mouse anti-human $\alpha_v\beta_3$ integrin (clone LM609) and the mouse anti-human $\alpha_v\beta_5$ integrin (clone P1F6) monoclonal antibodies (Chemicon International, Inc., Temecula, CA) were used.

Expression, purification, and characterization of recombinant PAd3 and HAd5 fiber knobs

PAd3 fiber sequences encoding the knob domain and the last shaft motif between nucleotides 29656 and 30285 (corresponding to the PAd3 genome sequence in GenBank accession no. AF083132) was amplified by PCR using the forward 5′ -TGGATCCGACTTTCCTGTGGACAAT-3′ and the reverse 5′ AAGCTTTCATTGGAGTACTTGA-3′ primers, and the genomic PAd3 DNA as a template. The PCR product was ligated into the *Bam*HI–*Hin*dIII site of pQE30 vector (Qiagen, Inc., Valencia, CA) to obtain pQE30.P3 knob, and expressed in *E. coli* M5 (pREP4) (Qiagen) following the supplier's recommendations. Briefly, the bacterial culture was grown to an OD_{600} of 0.5–0.6 followed by induction of protein expression by 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and the incubation was continued for 3–4 h at 37 °C. Due to the presence of the 6× His-tag in-frame with the knob, the recombinant protein was purified from the soluble fraction by nickel-nitrilotriacetic acid (Ni-NTA) metal chelation chromatography using His.Bind protein purification kit (Novagen, Madison, WI) as per supplier's instructions.

The plasmid pQE30.H5 knob that contains the HAd5 fiber knob domain sequence along with the last shaft motif (Krasnykh et al., 1996) was generously provided by David T. Curiel, University of Alabama, Birmingham, AL. Recombinant HAd5 knob (H5 knob) was expressed and purified from the soluble fraction as described above for the P3 knob.

Purified knobs were dialyzed against PBS (137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl, pH 7.4) and protein concentrations were determined by Coomassie Protein Assay Reagent (Pierce) using BSA as a standard. The ability of the recombinant P3 or H5 knob to form trimers was verified using both boiled and unboiled samples followed by Coomassie staining of SDS-PAGE gels and Western blot using a rabbit anti-PAd3 or anti-HAd5 hyperimmune serum (Moffatt et al., 2000).

Western blot for CAR

Crude cell lysates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with the blocking buffer made with 4% skimmed milk powder in TBS (150 mM sodium chloride, 10 mM Tris–Cl, pH 8.0 and 0.05% Tween-20) followed by incubation with a 1:100 dilution of a rabbit polyclonal anti-hCAR antibody in the blocking buffer for 1 h at room temperature. After washing the membrane in TBS for 30 min, the membrane was incubated for 1 h with a 1:2000 dilution of an HRP-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA). After the final wash, the signal was detected by chemiluminescence using Enhanced Chemiluminescence Assay Reagent (Pierce) and Kodak Image Station (Eastman Kodak Co., Rochester, NY).

Antibody-mediated blocking experiments

To examine the ability of an anti CAR antibody to block transduction by HAd-GFP or PAd-GFP, MCF-10A cells grown to approximately 90% confluence in 12-well tissue culture plates, were incubated with various concentrations of the anti-CAR monoclonal antibody (RmcB) in 100 μl PBS–BSA (PBS containing 1% BSA) for 45 min at 4 °C. PAd-GFP or HAd-GFP was added at a multiplicity of infection (m.o.i.) of 20 plaque forming units (p.f.u.) per cell in 100 μl PBS++ (PBS containing 0.1% $MgCl_2$ and 0.1% $CaCl_2$) and incubated at 37 °C for 45 min. The cells were then washed once with PBS and supplemented with minimum essential medium (MEM) containing 2% fetal calf serum (FCS; Hyclone, Logan, UT) and incubated further at 37 °C. At 36 h post-infection, the cells were trypsinized, resuspended in PBS, and the cells expressing GFP were sorted by flow cytometry. For blocking studies using anti-integrin antibodies, cells were preincubated with RmcB (20 μg/ml) for 30 min at 4 °C prior to the addition of indicated concentrations of an anti-$\alpha_v\beta_3$ or anti-$\alpha_v\beta_5$ antibody. After 45-min incubation at 4 °C, virus infection followed by sorting of GFP expressing cells was carried out as above.

Fiber-knob-mediated virus interference assays

To analyze the ability of recombinant P3 or H5 knob to competitively inhibit transduction of homologous and heterologous adenoviral vectors, MCF-10A cells in 12-well plate were incubated with increasing concentrations of the recombinant H5 or P3 knob in 100 μl PBS–BSA for 45 min at 4 °C. PAd-GFP or HAd-GFP was added at an m.o.i. of 20 p.f.u./cell in 100 μl PBS++ and further incubated at 37 °C for 45 min. The cells were then washed once with PBS and supplemented with MEM containing 2% FCS and incubated further at 37 °C. Cells were trypsinized at 36 h post-infection and the cells expressing GFP were sorted by flow cytometry.

Immunofluorescence labeling and flow cytometry

Approximately 10^6 MCF-10A cells, in 100 μl staining buffer (PBS with 2% inactivated FCS and 0.1% sodium azide), were incubated with the primary (anti-CAR, anti-$\alpha_v\beta_3$, or anti-$\alpha_v\beta_5$) antibody (10 μg/ml) for 30 min at 4 °C.

After two washes with the staining buffer, cells were incubated in 100 μl of a FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) (10 μg/ml) for 30 min at 4 °C. Cells were then washed twice with the staining buffer and analyzed by flow cytometry. Unstained cells or the cells stained only with the secondary antibody served as negative controls.

Analysis of GFP expression by flow cytometry

Flow cytometry analyses were performed essentially as described (Bangari and Mittal, 2004). Briefly, cells were trypsinized, washed once with PBS, and resuspended at a density of approximately 10^6 cell per milliliter in PBS containing 2% formaldehyde and immediately used for flow cytometry at Purdue University Flow Cytometry Laboratories using Cytomics FC-500 Flow Cytometer (Beckman Coulter, Inc., Fullerton, CA) with Ar 488-nm excitation filter and FL-1 525 nm BP emission filter. For each sample, at least 5000 cells were counted.

Statistical analysis

Data were compared by Student's *t* test for statistical significance. $P < 0.05$ was considered significant.

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