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Novel approaches to augment adeno-associated virus type-2 endocytosis and transduction

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Abstract

Recombinant adeno-associated virus (rAAV) receptor binding, endocytosis, nuclear trafficking and second strand gene conversion have been described as potential rate-limiting steps in rAAV type-2 (rAAV-2) transduction. Several strategies have been developed to enhance rAAV-2 intracellular trafficking and gene conversion in an attempt to increase the efficiency of this virus as a gene therapy vector. To this end, the current study has investigated novel methods for augmenting rAAV transduction by enhancing endocytosis of rAAV-2. A selective trypsinization assay demonstrated that the abundance of internalized rAAV ssDNA was increased only in cells treated with both pyrrolidinedithiocarbonate (PDTC) and a genotoxic agent. Treating cells with each of these agents alone had no effect on rAAV endocytosis in comparison to controls. To investigate the mechanisms of this synergistic effect on rAAV transduction, the involvement of Rac1 protein was evaluated. Inhibition of the Rac1 pathway by expression of a dominant negative mutant of Rac1 (N17Rac1) decreased rAAV transduction. In contrast, expression of a dominant active form of Rac1 (V12Rac1) alone mimicked the up-regulated response seen in the presence of PDTC and genotoxic agents. These studies provide potential insights into the importance of the Rac1 pathway to enhance uptake of rAAV-2.

Keywords: Adeno-associated virus; Endocytosis; Rac 1; Gene therapy; PDTC

1. Introduction

Adeno-associated virus (AAV) is a non-pathogenic human parvovirus with a single stranded DNA genome of approximately 4.7 kb size. Its diverse organ tropism, ability to transduce non-dividing cells, and capacity for integration are among the main reasons why AAV is favored as a vector for gene therapy (Alexander et al., 1994; Kaplitt et al., 1994; Lai et al., 2002; Xiao et al., 1996). Recombinant AAV (rAAV) mediated gene delivery to certain tissues, including muscle, brain, and eye has been fairly successful in recent years (Bennett et al., 1997; Cucchiarini et al., 2003; Fisher et al., 1997; Hauck and Xiao, 2003; Kaplitt et al., 1994; Xiao et al., 1996). In these tissues, rAAV mediates high level and long lasting gene expression. However, the transduction of other organs with rAAV, such as the liver or lung, is relatively inefficient for reasons that are not yet fully understood. It has been suggested that the low abundance of cell surface AAV receptor(s) and differences in intracellular proteins (i.e., a D-sequence binding protein named ssD-BP) are two of several possible reasons that may account for inefficient rAAV transduction (Qing et al., 1997; Smith et al., 2003; Summerford and Samulski, 1998). For instance, the phosphorylation status of ssD-BP has been shown to be important in the conversion of AAV single stranded DNA genomes to duplex forms required for gene expression (Qing et al., 1997).

Several types of environmental damage, including UV, hydroxyurea (HU), and ionizing irradiation, as well as some viral gene products (such as adenovirus E4orf6), have been shown to augment rAAV transduction in cell types exhibiting inefficient transduction (Alexander et al., 1994; Ferrari et al., 1996; Fisher et al., 1996; Musonda and Chipman, 1998; Qing et al., 1998; Russell et al., 1995; Zhang et al.,

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1997). Originally, it was thought that all of these agents acted by a similar mechanism involving the conversion of AAV single stranded DNA into linear duplex replicative forms (R_f) of AAV (Ferrari et al., 1996; Fisher et al., 1996; Yakinoglu et al., 1988; Yakobson et al., 1987). However, despite the ability of both E4orf6 and UV to enhance rAAV gene expression, the mechanisms involved appear to be quite diverse. UV irradiation augments the abundance of AAV circular genomes, whereas E4orf6 increases the abundance of linear AAV replicative forms (Sanlioglu et al., 1999; Sanlioglu et al., 2001a). Despite these differences, there are also several interesting similarities in the action of genotoxic agents to induce rAAV gene expression. For example, several genotoxic agents, including UV, HU, and H_2O_2 have been shown to have redox components required for the activation of rAAV transduction. Previously, we have shown that the hydroxyl radical scavenger *N*-acetyl-L cysteine (NAC) blocks the augmentation of rAAV transduction by UV, HU, and H_2O_2 (Sanlioglu and Engelhardt, 1999). Surprisingly, during an evaluation of additional antioxidant molecules, the anti-HIV drug, pyrrolidinedithiocarbamate (PDTC) (Lee et al., 1997; Schreck et al., 1992) was found to synergistically increase the augmentation of rAAV transgene expression produced by UV or H_2O_2 treatments. In combination with PDTC treatment, low doses of these environmental stimuli were sufficient to significantly augment rAAV transduction in HeLa cells. To gain insight into the mechanisms of this synergistic interaction, we investigated the involvement of the cellular redox modulating protein Rac1, which has been demonstrated to be important for the endocytosis of rAAV (Sanlioglu et al., 2000). Findings from these studies suggest that activation of Rac1 can lead to an enhancement of AAV-2 endocytosis, which leads to substantial increases in transduction.

2. Materials and methods

2.1. Generation of recombinant AAV stocks

The construction of the *cis*-acting proviral plasmid (pCisAV.GFP3ori) used for rAAV production has been previously described (Duan et al., 1998). Recombinant AAV stocks were generated by co-transfection of pCisAV.GFP3ori and pRep/Cap together with infection of recombinant Ad.CMVlacZ in 293 cells (Fisher et al., 1996). The rAV.GFP3ori virus was subsequently heated at 60 °C for 1 h and then purified by HSPG column chromatography as previously described (Clark et al., 1999). Typical yields from this viral preparation were 10^{12} DNA molecules/ml. Particle titers were determined by viral DNA slot blot hybridization with an EGFP P^{32}-labeled probe, using copy number plasmid standards. The absence of helper adenovirus or wtAAV in rAAV stocks (sensitivity of less than one infectious unit per 10^{10} recombinant DNA particles) was confirmed as previously described (Duan et al., 1997).

2.2. Generation of recombinant adenoviral stocks

Four recombinant adenoviral vectors expressing β-galactosidase (Ad.CMVLacZ) (Engelhardt et al., 1993b) or E4orf6 (Ad.dl802) (Fisher et al., 1996) a dominant negative mutant of Rac1 (Ad.N17Rac1) (Kim et al., 1998; Sulciner et al., 1996) and a dominant active form of Rac1 (Ad.V12Rac1) (Pracyk et al., 1998) were used for functional studies. Ad.CMVLacZ vector is a first generation E1 deleted adenovirus vector (Ad. serotype 5) carrying alkaline phosphatase under the control of CMV promoter. Ad.dl802 is a replication defective E2 deleted adenovirus (Ad serotype 5) expressing high levels of E4orf6. Ad.N17Rac1 construct carrying an epitope-tagged dominant negative rac1 c-DNA was constructed by homologous recombination in 293 cells using a plasmid with an Ad5 backbone (JM17). This vector is also an E1 deleted first generation adenovirus vector. Ad.V12Rac1 is an E1 deleted first generation adenovirus vector carrying the dominant active form of rac1 c-DNA. The construction scheme of this vector was similar to that of Ad.N17Rac1. Recombinant adenoviral stocks were generated as previously described (Engelhardt et al., 1993b). Viral stocks were stored in 10 mM Tris with 20% glycerol at −80 °C. The particle titers of adenoviral stocks were determined by A_{260} readings and were typically 10^{13} DNA particles/ml. The functional titers of adenoviral stocks were determined by plaque titering on 293 cells. Typically, the particle/pfu ratio was equal to 25.

2.3. PDTC, UV, and H_2O_2 treatments

HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and a 1% penicillin/streptomycin mixture. The cells were plated the day before infection at a density of 1×10^6 cells per 35 mm plate. On the following day, the cells were treated with either 0.1 or 0.2 mM concentrations of PDTC (Sigma) for 1 h. The cells were then washed several times in PBS and were either treated with H_2O_2 (0.1 or 0.2 mM) for another hour or were UV irradiated at indicated doses (5 or 15 joules/m^2) just prior to infection with AV.GFPori3 at an MOI of 50 DNA particles per cell. For studies evaluating combined effects of PDTC and E4orf6, cells were infected with either Ad.CMVLacZ or Ad.dl802 virus at MOIs as indicated in the figures for 24 h prior to treatment with PDTC and infection with AV.GFP3ori virus.

2.4. Virus infections and FACS analysis

For functional assays evaluating transgene expression, HeLa cells were infected with AV.GFP3ori virus at an MOI of 50 DNA particles per cell for 24 h. Adenovirus infections (Ad.CMVLacZ, Ad.dl802, or Ad.V12Rac1 viruses) were performed using various MOIs (as indicated in figures) for 24 h prior to infection with rAAV. Adenoviral infections were carried out in DMEM with 2% fetal calf serum for

2 h, followed by increasing the level of FCS to 10% (v/v) final concentration and continued incubation for an additional 24 h. At the termination of the experiment, cells were washed with PBS, trypsinized, and the percentage of GFP positive cells was analyzed by fluorescent activated cell sorting (FACS).

Hirt DNA preparations and Southern blotting for viral DNA. HeLa cells were treated with PDTC, UV, and/or H_2O_2 or infected with Ad.LacZ or Ad.dl802 for 24 h prior to treatment with PDTC, as described in the methods above. Cells were then washed and incubated with AV.GFP3ori virus at an MOI of 100 DNA particles per cell for 1 h at 4 °C. An MOI of 100 DNA particles per cell was used in this experiment to allow visualization of AAV ssDNA by Hirt Southern blotting. Following binding, cells were either harvested directly to quantify viral binding or washed with PBS three times and shifted to 37 °C for 2 h to promote internalization of virus prior to harvest. Two harvesting protocols were used, which involved either scraping the plates directly for collection or trypsinizing cells followed by washing with PBS to remove extracellularly bound virus. Extraction of low molecular weight Hirt DNA (viral DNA) and Southern blotting were performed according to the protocols described previously (Hirt, 1967; Sanlioglu et al., 1999).

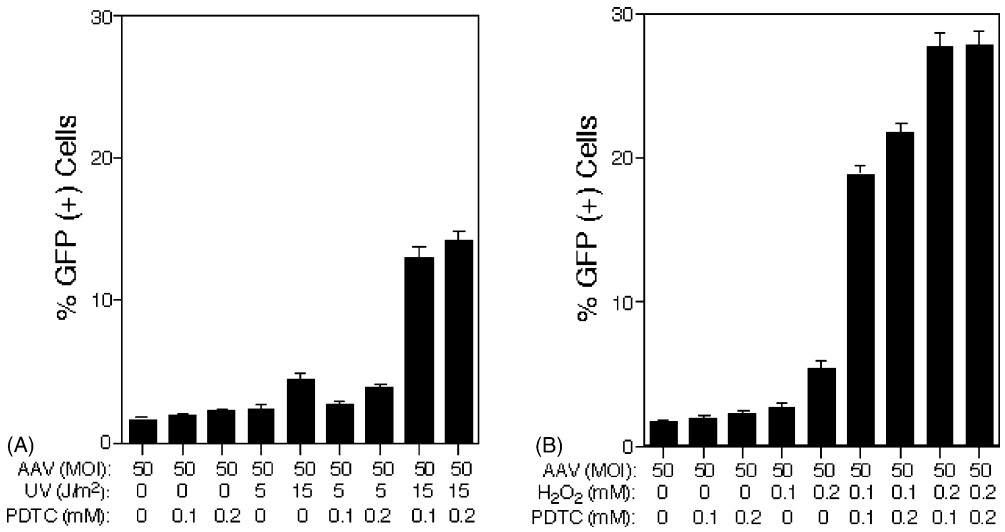

Fig. 1. PDTC synergistically activates rAAV transduction induced by UV and H_2O_2. HeLa cells were treated with the indicated doses of PDTC for 1 h prior to treatment of cells with either UV irradiation (panel (A)) or H_2O_2 for 1 h interval (panel (B)). The medium was then replaced and cells were infected with AV.GFP3ori virus at an MOI of 50 DNA particles per cell. The percentage of cells expressing the GFP transgene was determined 24 h post-infection by FACS analysis. Data represent the mean (±S.E.M.) of six independent data points for each experimental value ($N = 6$).

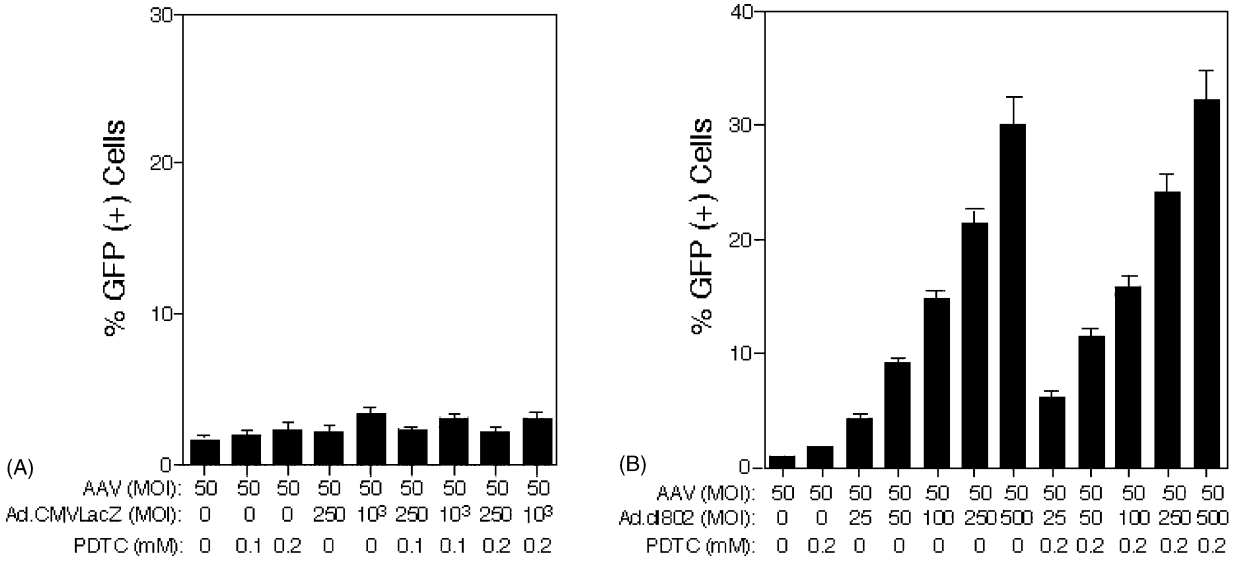

Fig. 2. PDTC has no effect on rAAV transduction induced by adenoviral E4orf6 gene expression. HeLa cells were infected with either Ad.CMVLacZ virus (panel (A)) or Ad.dl802 virus (panel (B)) at the indicated doses for 24 h followed by PDTC treatment for 1 h prior to infection with AV.GFP3ori virus as explained in Section 2. The percentage of GFP expressing cells was quantified by FACS analysis 24 h after infection with AV.GFP3ori virus. Values indicate the mean (±S.E.M.) for four independent data points for each condition ($N = 4$).

3. Results

3.1. PDTC synergistically activates rAAV transduction induced by genotoxic agents

Reactive oxygen intermediates (ROIs), induced by UV irradiation or H_2O_2 treatment, have been demonstrated to enhance rAAV transduction through as yet an unknown mechanism (Sanlioglu and Engelhardt, 1999). To begin to elucidate what type(s) of ROI might be responsible for augmentating rAAV transduction under these conditions, HeLa cells were treated with a known antioxidant, PDTC, prior to treatment with genotoxic agents and infection with rAAV. As seen in Fig. 1, contrary to our original hypothesis that PDTC would inhibit UV and H_2O_2 mediated induction, pre-treatment with PDTC synergistically activated rAAV transduction mediated by UV (Fig. 1, panel (A)) and/or H_2O_2 (Fig. 1, panel (B)). Since PDTC did not activate rAAV transduction alone at either dose tested (0.1 and 0.2 mM), we hypothesized that both pathways induced by PDTC and genotoxic agents (UV and H_2O_2) must merge at some point to synergistically activate rAAV transduction.

3.2. PDTC has no effect on rAAV transduction induced by adenovirus

In addition to genotoxic agents, the adenoviral gene product E4orf6 has also been demonstrated to augment rAAV transduction. However, E4orf6 and UV exposure increase rAAV transduction by two distinct pathways (Sanlioglu et al., 1999). In order to test whether PDTC could also synergistically activate rAAV transduction induced by adenovirus, HeLa cells were first infected with Ad.CMVLacZ or Ad.dl802 virus (a replication defective E2 deletion mutant that expresses a high level of E4orf6) at increasing doses 24 h before PDTC treatment and infection with rAAV (Fig. 2). As previously demonstrated, Ad.dl802 induced high levels of rAAV mediated GFP expression when compared to the control E1-deleted recombinant Ad.CMVlacZ vector (Fisher et al., 1996). Interestingly, in neither case did PDTC pre-treatment affect rAAV transduction. These results suggest that the mechanism of induction following combined treatments with PDTC and UV or H_2O_2 is mechanistically distinct from that of E4orf6 induction. Several potential explanations could account for these results. First, enhancement of rAAV transduction by E4orf6 is thought to be a strictly regulated nuclear event involving lytic phase replication of the second DNA strand. In contrast, increased circular intermediate formation following UV and H_2O_2 treatments has been suggested to be associated with latent phase of AAV infection (Sanlioglu et al., 1999; Sanlioglu

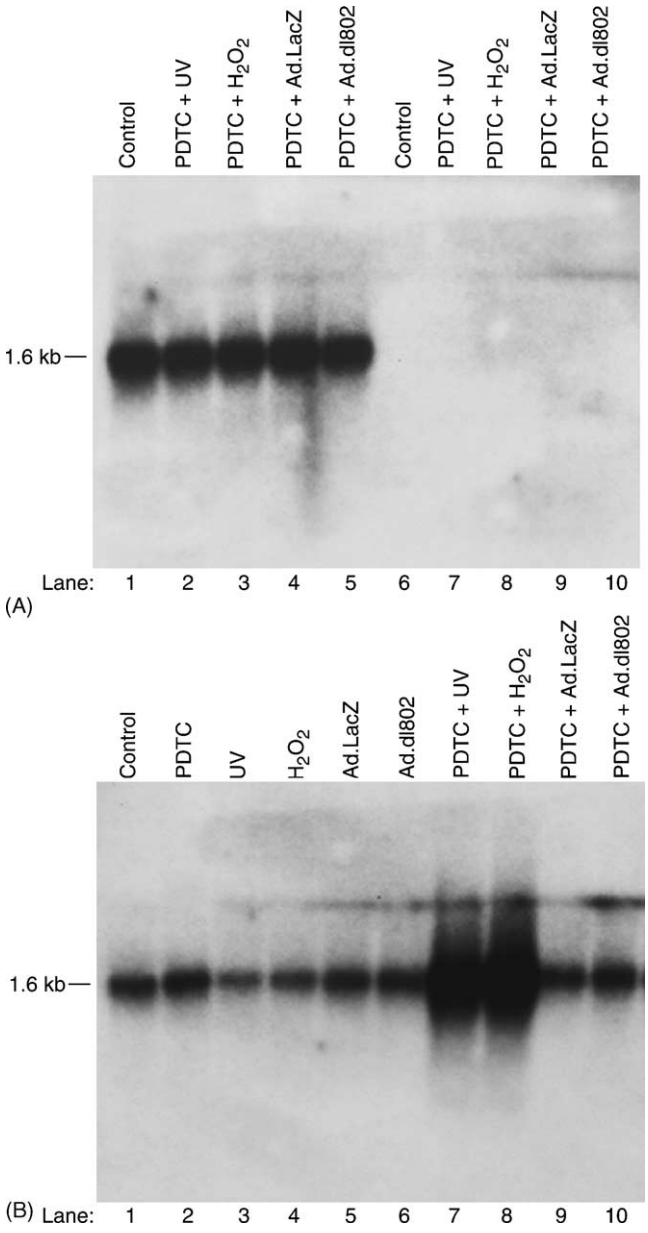

Fig. 3. rAAV uptake is enhanced by combined use of PDTC with UV or H_2O_2 treatment. Selective trypsinization assays followed by Southern blots of purified Hirt DNA were performed to determine the amount of binding and internalization of AAV virus to HeLa cells. HeLa cells were infected with AV.GFP3ori virus (MOI of 100 DNA particles per cell) for 1 h at 4 °C, washed, and either harvested directly to determine viral binding (panel (A)) or shifted to 37 °C for 2 h for assessment of viral internalization (panel (B)). Cells were harvested by either direct scraping (−trypsin, panel (A) lanes 1–5) or by trypsinization to remove extracellularly bound virus (+trypsin, panel (A) lanes 6–10, panel (B) lanes 1–10). Treatment conditions for cells are given above each lane and the control lane represents untreated cells. HeLa cells were treated with 0.1 mM PDTC and/or 0.1 mM H_2O_2 for 1 h prior to infection with rAAV. For combined UV treatment studies, cells were washed several times in PBS following treatment with PDTC, and were then UV irradiated at 15 J/m^2 just prior to infection with rAAV. Cells were infected with either Ad.LacZ (MOI of 1000 DNA particles per cell) or Ad.dl802 virus (MOI of 500 DNA particles per cell) for 24 h prior to treatment with PDTC and infection with AV.GFP3ori virus (MOI of 100 DNA particles per cell). Hirt DNA was prepared from cells subjected to these conditions, and Southern blots were hybridized with a P^{32}-labeled EGFP cDNA probe. The position of single stranded rAAV genomes (1.6 kb hybridizing band) is marked to the left of the blots.

and Engelhardt, 1999). We hypothesized that synergistic induction of rAAV mediated gene expression by PDTC and UV or H_2O_2 treatments occurs at a time point prior to nuclear circularization events. This might include effects on viral binding, endocytosis, and/or nuclear trafficking. To begin to investigate this hypothesis, we examined the effects of combined treatments with PDTC and genotoxic agents on viral binding and endocytosis.

3.3. Combined treatment with PDTC and genotoxic agents enhances the endocytosis of rAAV

Selective trypsinization assays followed by Southern blotting of viral DNA were performed to assess viral binding and endocytosis of rAAV. Trypsin has previously been shown to effectively remove externally bound AAV-2 from HeLa cells (Duan et al., 1999). Therefore, this assay can easily distinguish trypsin-resistant, internalized AAV genomes from trypsin-sensitive, externally bound AAV. As seen in Fig. 3A, none of the treatment conditions in any combination altered the binding of trypsin-sensitive rAAV particles to HeLa cells. In contrast, combined treatments of PDTC with UV irradiation or H_2O_2 led to increased internalization of trypsin-resistant rAAV ssDNA in HeLa cells after incubation for 2 h at 37 °C (Fig. 3B, lanes 7 and 8). At the 2 h time points analyzed, treatment of cells with each of these agents individually had no measurable effect on rAAV endocytosis (Fig. 3B, lanes 2–4). In addition, when cells were pre-infected with Ad.CMVLacZ or Ad.dl802 adenoviral constructs 24 h prior to the infection with rAAV, no increase in rAAV uptake was detected (Fig. 3B, lanes 9 and 10). These results substantiate the GFP expression data, documenting synergistic augmentation and indicating that enhanced endocytosis of rAAV accounts for a substantial portion of the increase in rAAV transduction associated with this phenomenon.

3.4. Synergistic activation of rAAV transduction by PDTC and genotoxic agents involves Rac1 pathway

It has previously been reported that the small intracellular GTP-binding signaling molecule Rac1 is activated by rAAV infection and is required for AAV endocytosis (Sanlioglu et al., 2000). In this study, inactivation of Rac1 by expression of a dominant negative form of Rac1, Ad.N17Rac1 (Sulciner et al., 1996), did not affect binding, but led to the inhibition of AAV endocytosis. Rac1 is also well known for its ability to regulate ROI formation in cells through activation of NADPH-oxidase (Joneson and Bar-Sagi, 1998; Toporik et al., 1998). Based on the fact that PDTC is an ROI modulating agent and synergistically activates endocytosis in the presence of H_2O_2, we hypothesized that the site of action by PDTC and genotoxic stimuli (UV or H_2O_2) might reside at the point of Rac1 regulation. To this end, HeLa cells were infected with either Ad.N17Rac1 virus (Sulciner et al., 1996) or Ad.CMVLacZ virus (Engelhardt et al., 1993a) 24 h prior to treatment with UV, H_2O_2 and/or PDTC and infection with rAAV. The results indicate that N17Rac1 expression significantly reduces the augmentation of rAAV transduction induced by PDTC/UV (Fig. 4A) or PDTC/H_2O_2 (Fig. 4B) treatments. In contrast, no such inhibition was observed with Ad.CMVLacZ infected cells, suggesting that the effect was specific to N17Rac1 expression and that Rac1 is either directly involved in the action of PDTC and genotoxic agents or alternatively lies proximal to the site of action. Furthermore, since N17Rac1 did not completely block the synergistic activation, additional players or pathways might also be involved in this induction.

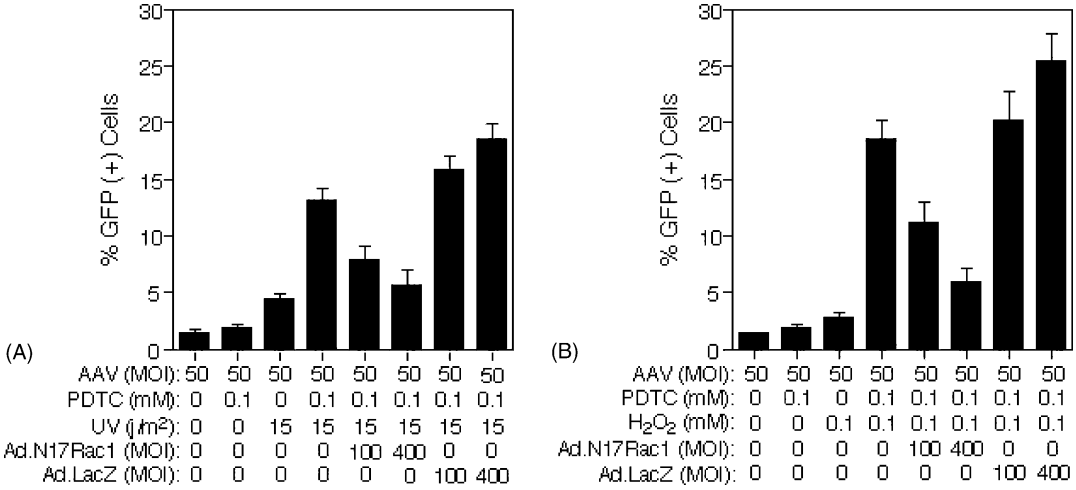

Fig. 4. Expression of N17Rac1 inhibits the synergistic activation of rAAV transduction by PDTC and genotoxic agents. HeLa cells were infected with either Ad.N17Rac1 or Ad.CMVLacZ virus (MOIs of 100 and 400 DNA particles per cell) for 24 h. Following adenoviral infection, cells were treated with PDTC and genotoxic agents followed by infection with AV.GFP3ori virus (MOI of 50 DNA particles per cell). Treatments of HeLa cells with PDTC, UV, and H_2O_2 were carried as described in Section 2. The percentage of cells expressing GFP was determined by FACS analysis 24 h following AV.GFP3ori virus infection. Data represent the mean (±S.E.M.) of four independent data points ($N = 4$).

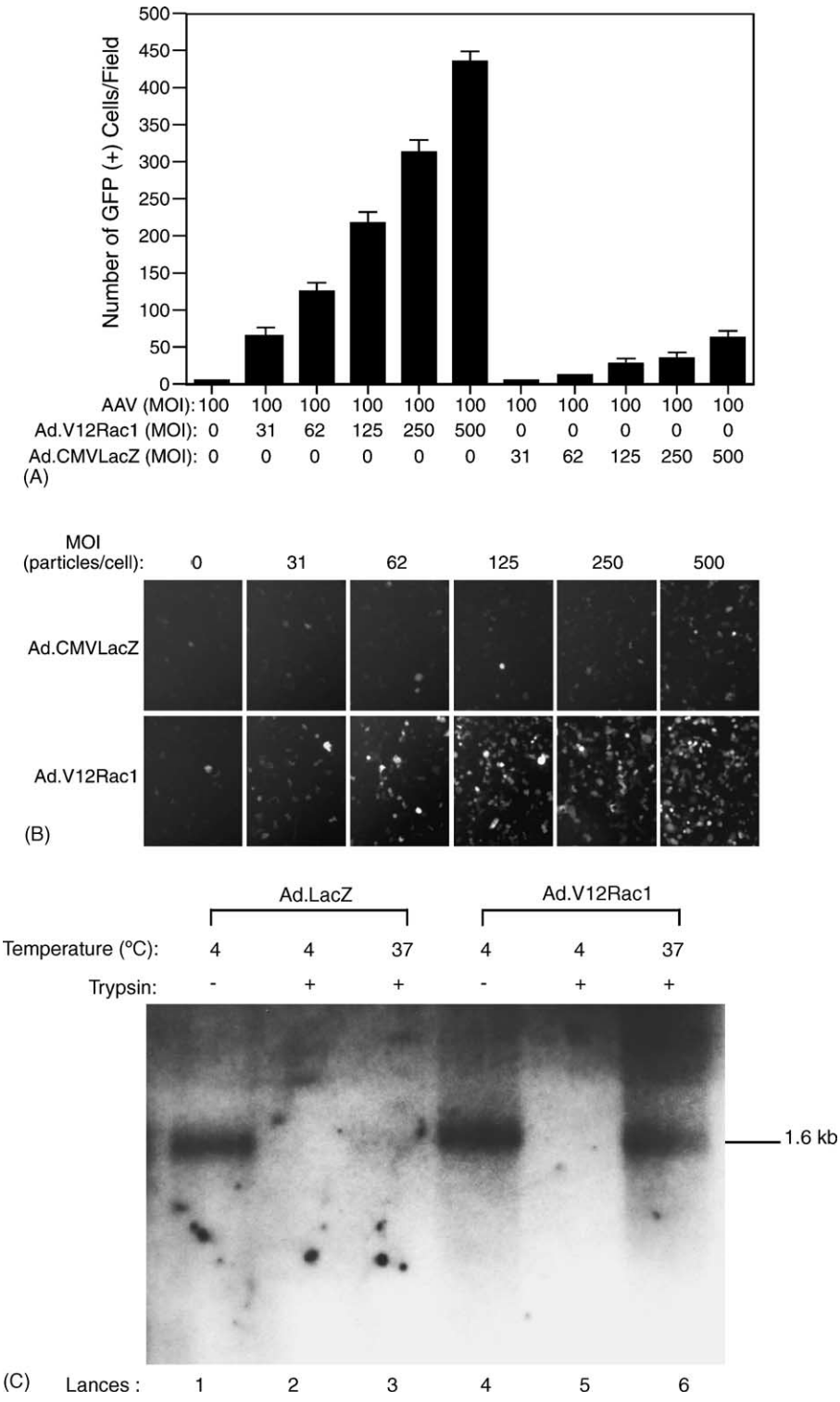

Fig. 5. V12Rac1 augments rAAV transduction. HeLa cells were infected with Ad.V12Rac1 or Ad.CMVLacZ for 48 h at the indicated MOIs. Cells were then infected with AV.GFP3ori virus (MOI of 100 DNA particles per cell) for 24 h. The number of GFP positive cells was determined by fluorescent microscopy. The *y*-axis in panel (A) represents the transduction efficiency based on the number of EGFP positive cells/20× field. Various experimental conditions are given on the *x*-axis. Values represent the mean (±S.E.M., $N = 6$). Panel (B) illustrates fluorescent photomicrographs of the data summarized in panel (A). MOIs are indicated above each set of panels illustrating infection with Ad.CMVLacZ (top) or Ad.N17Rac1 (bottom). Selective trypsinization assays followed by Southern blotting of Hirt DNA were performed to compare the extent of rAAV endocytosis in V12Rac1 and LacZ expressing HeLa cells (panel (C)). 48 h following infection with Ad.V12Rac1 or Ad.CMVLacZ, HeLa cells were infected with AV.GFP3ori virus (MOI of 100 DNA particles per cell) for 1 h at 4 °C, washed, and either harvested directly to determine virus binding or shifted to 37 °C for 2 h for assessment of virus internalization. Cells were harvested by either direct scraping (−trypsin, lanes 1 and 4) or by trypsinization to remove extracellularly bound virus (+trypsin, lanes 2, 3, 5, and 6). Hirt DNA was then prepared and Southern blots were hybridized with a P^{32}-labeled EGFP c-DNA probe. The location of single stranded rAAV genomes is marked to the right of the figure (1.6 kb).

3.5. Constitutively active Rac1 augments rAAV transduction by enhancing the endocytosis of rAAV virus

One hypothesis for the function of Rac1 in the synergistic interactions of PDTC and genotoxic agents includes the possibility that these agents directly activate Rac1. To investigate this hypothesis, we tested whether direct constitutive activation of Rac1, by expressing a dominant active form of Rac1 (V12Rac1) (Pracyk et al., 1998) would enhance rAAV transduction in the absence of genotoxic agents. As seen in Fig. 5, Ad.V12Rac1 infection significantly increased rAAV transduction in a dose dependent fashion. In contrast, rAAV transduction of Ad.CMVLacZ infected cells was 10-fold lower than that following Ad.V12Rac1 infection. Experiments analyzing the extent of rAAV endocytosis under these conditions demonstrated that V12Rac1 expression increased the amount of endocytosed trypsin-resistant rAAV DNA within cells by 30 min post-infection (Fig. 5C). In contrast, no such increase was evident when cells were infected with Ad.CMVLacZ virus. Furthermore, neither the expression of V12Rac1 nor LacZ affected the binding of rAAV to HeLa cells. Taken together, these results demonstrate that the constitutively active form of Rac1 has the ability to augment rAAV transduction by enhancing the endocytosis of rAAV in the absence of genotoxic agents.

We next tested if these agents would biochemically activate the Rac1 pathway. Using a Rac1 activation assay (Sanlioglu et al., 2001b) we were able to demonstrate that UV, H_2O_2, and PDTC activated Rac1 individually (data not shown). However, we did not observe that PDTC in combination with genotoxic agents, synergistically activated Rac1. This might suggest that Rac1 might only be a component of the synergistic activation cascade.

4. Discussion

Research elucidating the transduction biology of AAV-2 has uncovered several cellular barriers to infection preventing wide range use of this vector for gene therapy. These include the abundance of receptors (Summerford and Samulski, 1998) and co-receptors (Qing et al., 1999; Summerford et al., 1999) at the cell surface, ubiquitination of endocytosed virus (Duan et al., 2000), nuclear trafficking (Hansen et al., 2000), and gene conversion to duplex expressible forms (Alexander et al., 1994; Ferrari et al., 1996; Fisher et al., 1996; Musonda and Chipman, 1998; Zhang et al., 1997). However, little is known about potential barriers involving endocytosis of AAV-2. Endocytosis of AAV-2 has previously been demonstrated to be dependent on Rac1 (Sanlioglu et al., 2000), a small GTPase recruited to the cell surface by a GTP-exchange protein called PAK-1. Given the known contribution of Rac1 involvement in reactive oxygen species generation through NADPH-oxidase (Joneson and Bar-Sagi, 1998), we were intrigued by the possibility that H_2O_2 and UV redox mediated augmentation of rAAV transduction—a previous findings by our group—might involve pathways associated with Rac1 (Sanlioglu and Engelhardt, 1999).

With the goal of finding more specific antioxidants that might inhibit redox mediated induction of AAV infection, we tested whether PDTC treatment could block UV or H_2O_2 mediated effects on rAAV. To our surprise, PDTC treatment in conjunction with UV or H_2O_2 treatment synergistically activated rAAV transduction, PDTC alone or in conjunction with adenoviral E4orf6 expression had no effect on rAAV transduction. This is in agreement with a previous report concluding that genotoxic agents and adenovirus regulate rAAV transduction through distinct pathways (Sanlioglu et al., 1999). To further elucidate the mechanisms of PDTC synergistic action with genotoxic agents, selective trypsinization assays followed by Hirt Southern blotting of viral DNA were performed to assess the extent of viral binding and endocytosis. These studies clearly indicated that rAAV endocytosis was enhanced when cells were treated with combination of PDTC and UV or PDTC and H_2O_2. No increase in rAAV endocytosis was observed when these agents were individually used to treat cells. As viral binding was unaffected by treatment with these agents, receptor abundance at the cell surface did not seen to be a critical factor. The enhancement of rAAV endocytosis was also correlated with the synergistic activation of rAAV transduction as assessed by gene expression.

Based on the hypothesis that Rac1 was a central effector involved in the activation of rAAV endocytosis by PDTC and UV or H_2O_2, we next performed several experiments to support the functional involvement of Rac1. First, expression of N17Rac1 effectively blocked synergistic activation of rAAV transduction induced by PDTC and genotoxic agents. As V12Rac1 expression activated rAAV endocytosis and transduction in the absence of the other agents, Rac1 was found to be involved in the endocytosis of rAAV-2 and as combined PDTC and UV or H_2O_2 treatments augment the endocytosis of rAAV-2, we sought to directly evaluate whether Rac1 was biochemically activated by treatment with PDTC and genotoxic agents. Findings from Rac1 activation assays confirmed the hypothesis that PDTC, UV, and H_2O_2 all increased the GTP-bound form of Rac1 (data not shown).

In summary, the reported experiments substantiated functional studies evaluating the synergistic activation of AAV-2 endocytosis and transduction following treatment with these agents. Further insights into these molecular mechanisms may lead to new approaches for enhancing endocytosis and transduction efficiency of rAAV-2 as a gene therapy vector.

Acknowledgements

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References

Alexander, I.E., Russell, D.W., Miller, A.D., 1994. DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. J. Virol. 68, 8282–8287.

Bennett, J., Duan, D., Engelhardt, J.F., Maguire, A.M., 1997. Real-time, noninvasive in vivo assessment of adeno-associated virus-mediated retinal transduction. Invest. Ophthalmol. Vis. Sci. 38, 2857–2863.

Clark, K.R., Liu, X., McGrath, J.P., Johnson, P.R., 1999. Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. Hum. Gene Ther. 10, 1031–1039.

Cucchiarini, M., Ren, X.L., Perides, G., Terwilliger, E.F., 2003. Selective gene expression in brain microglia mediated via adeno-associated virus type 2 and type 5 vectors. Gene Ther. 10, 657–667.

Duan, D., Fisher, K.J., Burda, J.F., Engelhardt, J.F., 1997. Structural and functional heterogeneity of integrated recombinant AAV genomes. Virus Res. 48, 41–56.

Duan, D., Li, Q., Kao, A.W., Yue, Y., Pessin, J.E., Engelhardt, J.F., 1999. Dynamin is required for recombinant adeno-associated virus type 2 infection. J. Virol. 73, 10371–10376.

Duan, D., Sharma, P., Yang, J., Yue, Y., Dudus, L., Zhang, Y., Fisher, K.J., Engelhardt, J.F., 1998. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long term episomal persistence in muscle. J. Virol. 72, 8568–8577.

Duan, D., Yue, Y., Yan, Z., Yang, J., Engelhardt, J.F., 2000. Endosomal processing limits gene transfer to polarized airway epithelia by adeno-associated virus. J. Clin. Invest. 105, 1573–1587.

Engelhardt, J.F., Simon, R.H., Yang, Y., Zepeda, M., Weber-Pendleton, S., Doranz, B., Grossman, M., Wilson, J.M., 1993a. Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: biological efficacy study. Hum. Gene Ther. 4, 759–769.

Engelhardt, J.F., Yang, Y., Stratford-Perricaudet, L.D., Allen, E.D., Kozarsky, K., Perricaudet, M., Yankaskas, J.R., Wilson, J.M., 1993b. Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses. Nat. Genet. 4, 27–34.

Ferrari, F.K., Samulski, T., Shenk, T., Samulski, R.J., 1996. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. J. Virol. 70, 3227–3234.

Fisher, K.J., Gao, G.P., Weitzman, M.D., DeMatteo, R., Burda, J.F., Wilson, J.M., 1996. Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. J. Virol. 70, 520–532.

Fisher, K.J., Jooss, K., Alston, J., Yang, Y., Haecker, S.E., High, K., Pathak, R., Raper, S.E., Wilson, J.M., 1997. Recombinant adeno-associated virus for muscle directed gene therapy. Nat. Med. 3, 306–312.

Hansen, J., Qing, K., Kwon, H.J., Mah, C., Srivastava, A., 2000. Impaired intracellular trafficking of adeno-associated virus type 2 vectors limits efficient transduction of murine fibroblasts. J. Virol. 74, 992–996.

Hauck, B., Xiao, W., 2003. Characterization of tissue tropism determinants of adeno-associated virus type 1. J. Virol. 77, 2768–2774.

Hirt, B., 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26, 365–369.

Joneson, T., Bar-Sagi, D., 1998. A Rac1 effector site controlling mitogenesis through superoxide production. J. Biol. Chem. 273, 17991–17994.

Kaplitt, M.G., Leone, P., Samulski, R.J., Xiao, X., Pfaff, D.W., O'Malley, K.L., During, M.J., 1994. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. Nat. Genet. 8, 148–154.

Kim, K.S., Takeda, K., Sethi, R., Pracyk, J.B., Tanaka, K., Zhou, Y.F., Yu, Z.X., Ferrans, V.J., Bruder, J.T., Kovesdi, I., Irani, K., Goldschmidt-Clermont, P., Finkel, T., 1998. Protection from reoxygenation injury by inhibition of rac1. J. Clin. Invest. 101, 1821–1826.

Lai, C.M., Lai, Y.K., Rakoczy, P.E., 2002. Adenovirus and adeno-associated virus vectors. DNA Cell Biol. 21, 895–913.

Lee, R., Beauparlant, P., Elford, H., Ponka, P., Hiscott, J., 1997. Selective inhibition of l kappaB alpha phosphorylation and HIV-1 LTR-directed gene expression by novel antioxidant compounds. Virology 234, 277–290.

Musonda, C.A., Chipman, J.K., 1998. Quercetin inhibits hydrogen peroxide (H2O2)-induced NF-kappaB DNA binding activity and DNA damage in HepG2 cells. Carcinogenesis 19, 1583–1589.

Pracyk, J.B., Tanaka, K., Hegland, D.D., Kim, K.S., Sethi, R., Rovira, I.I., Blazina, D.R., Lee, L., Bruder, J.T., Kovesdi, I., Goldshmidt-Clermont, P.J., Irani, K., Finkel, T., 1998. A requirement for the rac1 GTPase in the signal transduction pathway leading to cardiac myocyte hypertrophy. J. Clin. Invest. 102, 929–937.

Qing, K., Khuntirat, B., Mah, C., Kube, D.M., Wang, X.S., Ponnazhagan, S., Zhou, S., Dwarki, V.J., Yoder, M.C., Srivastava, A., 1998. Adeno-associated virus type 2-mediated gene transfer: correlation of tyrosine phosphorylation of the cellular single-stranded D sequence-binding protein with transgene expression in human cells in vitro and murine tissues in vivo. J. Virol. 72, 1593–1599.

Qing, K., Mah, C., Hansen, J., Zhou, S., Dwarki, V., Srivastava, A., 1999. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. Nat. Med. 5, 71–77.

Qing, K., Wang, X.S., Kube, D.M., Ponnazhagan, S., Bajpai, A., Srivastava, A., 1997. Role of tyrosine phosphorylation of a cellular protein in adeno-associated virus 2-mediated transgene expression. Proc. Natl. Acad. Sci. U.S.A. 94, 10879–10884.

Russell, D.W., Alexander, I.E., Miller, A.D., 1995. DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. Proc. Natl. Acad. Sci. U.S.A. 92, 5719–5723.

Sanlioglu, S., Benson, P.K., Yang, J., Atkinson, E.M., Reynolds, T., Engelhardt, J.F., 2000. Endocytosis and nuclear trafficking of adeno-associated virus type 2 are controlled by rac1 and phosphatidylinositol-3 kinase activation. J. Virol. 74, 9184–9196.

Sanlioglu, S., Duan, D., Engelhardt, J.F., 1999. Two independent molecular pathways for recombinant adeno-associated virus genome conversion occur after UV-C and E4orf6 augmentation of transduction. Hum. Gene Ther. 10, 591–602.

Sanlioglu, S., Engelhardt, J., 1999. Cellular redox state alters recombinant adeno-associated virus transduction through tyrosine phosphatase pathways. Gene Ther. 6, 1427–1437.

Sanlioglu, S., Monick, M.M., Luleci, G., Hunninghake, G.W., Engelhardt, J.F., 2001a. Rate limiting steps of AAV transduction and implications for human gene therapy. Curr. Gene Ther. 1, 137–147.

Sanlioglu, S., Williams, C.M., Samavati, L., Butler, N.S., Wang, G., McCray Jr., P.B., Ritchie, T.C., Hunninghake, G.W., Zandi, E., Engelhardt, J.F., 2001b. Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor-alpha secretion through IKK regulation of NF-kappa B. J. Biol. Chem. 276, 30188–30198.

Schreck, R., Grassmann, R., Fleckenstein, B., Baeuerle, P.A., 1992. Antioxidants selectively suppress activation of NF-kappa B by human T-cell leukemia virus type I tax protein. J. Virol. 66, 6288–6293.

Smith, A.D., Collaco, R.F., Trempe, J.P., 2003. Enhancement of recombinant adeno-associated virus type 2-mediated transgene expression in a lung epithelial cell line by inhibition of the epidermal growth factor receptor. J. Virol. 77, 6394–6404.

Sulciner, D.J., Irani, K., Yu, Z.X., Ferrans, V.J., Goldschmidt-Clermont, P., Finkel, T., 1996. rac1 regulates a cytokine-stimulated, redox-dependent pathway necessary for NF-kappaB activation. Mol. Cell Biol. 16, 7115–7121.

Summerford, C., Bartlett, J.S., Samulski, R.J., 1999. AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. Nat. Med. 5, 78–82.

Summerford, C., Samulski, R.J., 1998. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J. Virol. 72, 1438–1445.

Toporik, A., Gorzalczany, Y., Hirshberg, M., Pick, E., Lotan, O., 1998. Mutational analysis of novel effector domains in Rac1 involved in the activation of nicotinamide adenine dinucleotide phosphate (reduced) oxidase. Biochemistry 37, 7147–7156.

Xiao, X., Li, J., Samulski, R.J., 1996. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. J. Virol. 70, 8098–8108.

Yakinoglu, A.O., Heilbronn, R., Burkle, A., Schlehofer, J.R., zur Hausen, H., 1988. DNA amplification of adeno-associated virus as a response to cellular genotoxic stress. Cancer Res. 48, 3123–3129.

Yakobson, B., Koch, T., Winocour, E., 1987. Replication of adeno-associated virus in synchronized cells without the addition of a helper virus. J. Virol. 61, 972–981.

Zhang, X., Rosenstein, B.S., Wang, Y., Lebwohl, M., Wei, H., 1997. Identification of possible reactive oxygen species involved in ultraviolet radiation-induced oxidative DNA damage. Free Radic. Biol. Med. 23, 980–985.