Protection of human endothelial cells from oxidant injury by adenovirus-mediated transfer of the human catalase cDNA

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ABSTRACT
In a variety of disorders, endothelial cells are exposed to high levels of oxidants, generated within the cells and/or consequent to local inflammation. In the context of the sensitivity of endothelial cells to oxidant stress, particularly related to \( \text{H}_2\text{O}_2 \), we have designed a replication deficient recombinant adenovirus containing the human catalase cDNA (AdCL) to transfer the catalase cDNA to the endothelial cells, in order to augment intracellular anti-\( \text{H}_2\text{O}_2 \) protection. Human umbilical vein endothelial cells that were not infected or infected with control adenovirus maintained low levels of catalase mRNA. Endothelial cells infected with AdCL expressed AdCL-driven exogenous catalase mRNA, as early as 24 hr and at least for 7 days. Catalase protein levels were increased significantly over controls in cells infected with AdCL, as were catalase activity levels, with catalase activity correlated closely with levels of catalase protein. Importantly, when the endothelial cells were exposed to 500 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), all the AdCL infected endothelial cells survived, compared to only 37\% of the control cells. Thus, a recombinant adenovirus containing the human catalase cDNA is able to infect human endothelial cells \textit{in vitro} and express high levels of functional intracellular catalase, protecting the cells against \( \text{H}_2\text{O}_2 \)-mediated oxidant stress. These observations support the feasibility of the transfer of catalase cDNA to human endothelium to protect against oxidant injury.

INTRODUCTION
Oxidant-mediated injury to endothelial cells contributes to the pathogenesis of a variety of disease processes, including atherosclerosis, reperfusion injury after myocardial ischemia and stroke, and increased microvascular permeability states such as septic shock, burn injury and adult respiratory distress syndrome (ARDS) (1–10). The importance of oxidants in these disorders relates not only to the anatomic location of the endothelial cells, but also to a large extent to the sensitivity of the endothelium to oxidants generated by inflammatory cells such as neutrophils, as well as within the endothelial cell itself such as via the xanthine oxidase pathway (11–13).

As with other oxidant-mediated processes, hydrogen peroxide is a major oxidant involved in endothelial cell damage (14,15). By virtue of the anti-\( \text{H}_2\text{O}_2 \) catalase and the glutathione systems (16–19), the endothelial cell can deal with physiologic cytosolic levels of \( \text{H}_2\text{O}_2 < 20 \mu \text{M} \) (20), but these antioxidant defenses can be overwhelmed by inappropriate generation of \( \text{H}_2\text{O}_2 \) within the endothelial cell or by the generation of high levels of \( \text{H}_2\text{O}_2 \) by inflammatory cells in the local milieu. For example, local \( \text{H}_2\text{O}_2 \) levels of 100 \( \mu \text{M} \) are easily achieved by activated neutrophils (20).

One strategy to protect endothelial cells from \( \text{H}_2\text{O}_2 \)-mediated injury is to augment endothelial anti-\( \text{H}_2\text{O}_2 \) activity levels by transferring to the endothelial cell the cDNA for catalase. While this could be achieved with a variety of gene transfer methods in cell lines that are replicating, the endothelial cell represents a difficult target for gene transfer \textit{in vivo} because of its slow rate of proliferation, estimated to be 1\% of the endothelium/day (21). One strategy to circumvent this hurdle is to use a gene transfer vector which does not require host cell proliferation for gene expression. The replication deficient recombinant adenovirus fulfills this criterion (22–24). The adenovirus genome can be rendered replication deficient and can be manipulated to accommodate genes up to 7.5 kb. Live adenovirus has been used safely as human vaccine in >5 million individuals, and there is no known association of human malignancy with adenovirus infection. Importantly, the human endothelial cells are susceptible to gene transfer with this type of adenovirus vector (25).

With the knowledge that endothelial cells are sensitive to increased levels of \( \text{H}_2\text{O}_2 \) and that catalase is a potent anti-\( \text{H}_2\text{O}_2 \) enzyme, the present study is directed toward constructing a replication deficient recombinant adenovirus containing the human catalase cDNA, and evaluating whether transfer and expression of catalase cDNA by this recombinant adenovirus could augment endothelial cell catalase mRNA, protein and activity levels. The data demonstrate that this is indeed possible, and gene transfer by such an adenovirus protects human endothelial cells from levels of \( \text{H}_2\text{O}_2 \) which otherwise would destroy the cell.

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METHODS

Adenovirus vectors

The replication deficient recombinant vector AdCL is based on adenovirus (Ad) type 5. The overall construction is similar, with minor modifications, to Ad-α1AT, a replication deficient recombinant adenovirus containing the human α1-antitrypsin cDNA (23). AdCL was constructed (left to right) by ligation of the ClaI cut plasmid pPB369 [including the left inverted terminal repeat and origin of replication from Ad5, the encapsidation signal and Ela enhancer from Ad5, the major late promoter and tripartite leader sequences from Ad5 followed by the entire 2.3 kb human catalase cDNA (26)] with the large ClaI fragment of the Ad5 E3 deletion mutant AddD327 (23,27) (Figure 1). The pPB369-AddD327 ligation product was transfected into the transformed human embryonic kidney cell line 293 (28) (ATCC RL1573) grown in Improved Minimal Essential Medium (Biofluids) containing 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Individual plaques were isolated, amplified on 293 cells, and screened for the correct Ela- ligation product by polymerase chain reaction for AdCL-specific and Ela DNA sequences, and by assay for catalase activity (29). AdCL was propagated in 293 cells and recovered 36 hr after infection by freeze-thawing. All preparations were purified by CsCl density centrifugation and dialyzed and stored in virus dialysis buffer (10 mM Tris–HCl, pH 7.4, 1 mM MgCl₂) with 10% glycerol at −70°C prior to use. Titers of viral stocks were determined by plaque assay using 293 cells (30). Ad-CFTR [an Ela-, partial Elb-, E3-, replication deficient Ad5-based virus containing the human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA] (24) was used as a control. Ad-CFTR was purified, stored and assessed in a fashion similar to that for AdCL.

Cell culture and in vitro infection

Human umbilical vein endothelial cells were obtained and cultured as previously described (25). Identification of the cultures as endothelial cells was confirmed by positive staining with the anti-factor VIII-related antigen (von Willebrand factor) antibody.

Figure 1. Construction of the replication deficient recombinant adenovirus vector AdCL containing the human catalase cDNA. The plasmid pPB369 contains the left inverted terminal repeat, origin of replication, encapsidation signal, Ela enhancer, the major late promoter, and tripartite leader sequences (all from Ad5), followed by the entire 2.3 kb human catalase cDNA. After the ClaI site, there is a segment of Ad5 and a segment of the plasmid pBluescript II SK+ (Stratagene) not used in the construction of AdCL. ClaI cut pPB369 was ligated to the large ClaI fragment of AddD327, an Ad5-based mutant virus with a deletion of 66% of the E3 region [78.4—84.3 map units (mu) of Ad5; 360 bp/mu]. The pPB369-AddD327 ligation product, AdCL, is therefore similar to adenovirus type 5, but it has the majority of the E1 region deleted (0—2.67 mu), and the majority of the E3 region deleted. At the left end (left to right), AdCL contains the left end inverted terminal repeat (ITR) and origin of replication, encapsidation signal and Ela enhancer, the major late promoter and the tripartite leader sequence, followed by the entire human catalase cDNA comprised of sequences coded by exons 1—13 of the catalase gene, including 71 bp sequences 5' to the ATG, the TGA stop at bp 1655 (of the catalase cDNA) and the endogenous consensus polyadenylation signal (AATAAA) at bp 2258 (see reference 26 for numbering of the catalase cDNA). To the right of exon 13 is a linker sequence, the position of the ClaI cut used to construct AdCL, and the entire remaining sequence of AddD327.
(Dako) and immunoperoxidase technique as previously described (25). After the cells were grown to 70 to 80% confluence, they were infected with AdCL, or Ad-CFTR at 25 plaque-forming units (pfu)/cell or sham infected with no virus. The cells were incubated for 90 min with medium containing the virus and 2% bovine calf serum; the incubation was then continued for the specified time with the addition of the regular culture medium with 20% bovine calf serum (25).

Catalase gene expression in endothelial cells

Expression of endogenous catalase mRNA and AdCL driven catalase mRNA was evaluated in endothelial cells by Northern analysis. Total RNA was extracted from uninfected cells or cells infected with Ad-CFTR or AdCL, purified on CsCl gradients, electrophoresed in formaldehyde/agarose gels (25), blotted onto nylon membranes (Duralon, Stratagene). Total RNA was then probed with either a 32P-labeled human catalase cDNA probe (26) or as a control, a γ-actin probe (25). To distinguish the endogenous catalase mRNA from the AdCL-directed catalase mRNA in the mRNA transcripts which hybridized with the human catalase cDNA probe, the nylon membrane was then stripped and rehybridized with an AdCL-specific cDNA oligonucleotide-probe coding for the majority of the 5′ linker sequence of AdCL (GAATTCTGCAGCCCGGGGGATCC-AGTTTCTAGA).

Catalase protein amounts and function in endothelial cells

Catalase protein levels were determined in endothelial cells at various time-points after infection with AdCL, control virus (Ad-CFTR), or in the absence of virus. The endothelial cells were lysed in lysis buffer [10 mM ethylenediaminetetraacetic acid, 2% Triton X, and 0.05% deoxycholic acid, in phosphate buffered saline, pH 7.4]. After lysis, the samples were centrifuged at 700g for 5 minutes to remove nuclei and cell debris. Amounts of catalase in endothelial cell lysates were quantified with a specific double antibody sandwich enzyme linked immunoassay (ELISA) using 2 antibodies against human erythrocyte catalase (first antibody from Athens Research and Technology, Athens, Ga, and second antibody from Calbiochem, LaJolla, Ca). The purity of the first antibody was verified by demonstration of a single arc by immunoelectrophoresis against purified catalase and erythrocyte homogenates (done by Athens Research). Purified human erythrocyte catalase (Calbiochem, LaJolla, Ca) was used as a standard. All assays were done in duplicate. The amount of catalase in the samples was expressed relative to cell lysate protein (BCA protein assay reagent, Pierce, Rockford, IL).

Catalase activity in the endothelial cells was quantified by the method of Aebi (29) in which H2O2 was reacted with the catalase enzyme linked immunoassay (ELISA) using 2 antibodies against human erythrocyte catalase (first antibody from Athens Research and Technology, Athens, Ga, and second antibody from Calbiochem, LaJolla, Ca). The purity of the first antibody was verified by demonstration of a single arc by immunoelectrophoresis against purified catalase and erythrocyte homogenates (done by Athens Research). Purified human erythrocyte catalase (Calbiochem, LaJolla, Ca) was used as a standard. All assays were done in duplicate. The amount of catalase in the samples was expressed relative to cell lysate protein (BCA protein assay reagent, Pierce, Rockford, IL).

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Protection against oxidant stress

Endothelial cells were split into culture plates at equal density and infected when 70 to 80% confluent with either AdCL, Ad-CFTR or no virus. The incubation was continued for 48 hr at 37°C and 5% CO2. After 48 hr, H2O2 was added to the media at concentration of 0, 500 or 1000 μM and the culture continued for 24 hr. The cells were then evaluated qualitatively by microscopy and quantitatively by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) cleavage assay for cell viability (31). The amount of MTT reduced to its blue formazan derivative by viable cells during an additional 4 hr of culture was quantified spectrophotometrically at 540 nm using an ELISA reader; there is a linear relationship between the formazan generated and the number of viable cells present (31). The fraction of cells surviving H2O2 exposure was expressed as the ratio of the optical density at 540 nm (OD540) of cells with H2O2 over the OD540 of control cells (no H2O2).

Statistical analysis

Comparisons within groups were performed with Analysis of Variance and where significant differences were found, the means compared by the Student-Newman—Keuls test. Correlation between catalase antigenic levels and activity were performed by linear regression analysis.

RESULTS

Evaluation of AdCL-directed human catalase mRNA transcripts

Uninfected endothelial cells and endothelial cells infected for 24 hr with the control virus, Ad-CFTR, expressed catalase mRNA, with one transcript at 2.4 kb, the expected size for the endogenous human catalase mRNA (26) (Figure 2, lanes 1,2). Endothelial cells infected with AdCL also expressed a catalase mRNA of 2.4 kb, but also produced a transcript of 3.2 kb, the expected size of AdCL-directed catalase mRNA (lane 3). By 72 hr, the

![Figure 2. Evaluation of human catalase mRNA transcripts in human endothelial cells after in vitro infection with AdCL. Shown is a Northern analysis of total cellular RNA (5 μg/lane) with a human catalase cDNA probe (lanes 1—4), an AdCL-specific probe (lanes 5—8), and a γ-actin probe (lanes 9—12). The AdCL-specific probe was designed to detect catalase mRNA transcripts directed by AdCL, and not to detect the endogenous catalase mRNA (see Methods). Lane 1—uninfected human endothelial cells; lane 2—endothelial cells infected with a control replication deficient adenovirus containing the human CFTR cDNA (Ad-CFTR); lane 3—AdCL infected endothelial cells, 24 hr after infection; lane 4—AdCL infected endothelial cells, 72 hr after infection. Lanes 5—8—identical to lanes 1—4, but with AdCL-specific probe. Lanes 9—12—identical to lanes 1—4 but with γ-actin cDNA probe.](https://academic.oup.com/nar/article-abstract/21/7/1607/2385927/Protection-of-human-endothelial-cells-from-oxidant)
expression of the 3.2 kb transcript was greater than the 2.4 kb transcript, with also an increase in expression of the 2.4 kb transcript compared to controls (lane 4). Hybridization with the AdCL-specific probe showed no hybridization in uninfected cells and cells infected with Ad-CFTR (lanes 5,6). In contrast, in cells infected with AdCL, both 2.4 kb and 3.2 kb hybridized with the AdCL-specific probe, demonstrating that AdCL-directed catalase mRNA were expressed at 2 different sizes, likely by alternative polyadenylation (lanes 7,8). Control γ-actin transcripts were at similar levels in each sample (lanes 9–12). The AdCL driven mRNA continued to be expressed 1 wk after infection (not shown).

**Catalase protein production and activity**

The amount of catalase protein in uninfected and control Ad-CFTR infected endothelial cells was similar over time (p > 0.1) and ranged between 25 to 56 ng/mg total protein. However, in cells infected with AdCL, expression of catalase protein increased significantly over controls at 0 d (p = 0.03), 2 d (p = 0.02), 3 d (p < 0.001), and 7 d (p = 0.01) (Figure 2A).

Catalase activity in control uninfected or Ad-CFTR infected groups was similar (p > 0.3) and ranged between 1 to 3.5 mU anti-H$_2$O$_2$ activity/mg total protein, similar to that previously reported for endothelial cells (32). In contrast, the catalase activity in AdCL infected cells was significantly higher than controls at 2 d (p = 0.01), 3 d (p = 0.001) and 7 d (p < 0.001) (Figure 2B).

Catalase activity in the endothelial cell lysates paralleled the amounts of catalase present (correlation coefficient 0.85, p < 0.001). The slope of the linear regression indicated that there was a 0.09 mU increase in anti-H$_2$O$_2$ activity for each 1 ng increase in protein level (Figure 2C).

**AdCL expressed catalase protection of endothelial cells against oxidant injury**

Uninfected endothelial cells, Ad-CFTR infected or AdCL infected cells were exposed to lethal doses of H$_2$O$_2$, then evaluated after 24 hr. In the control groups (i.e., no H$_2$O$_2$) all three groups of cells (no infection, Ad-CFTR infected, or AdCL infected) were confluent when examined after 24 hr (Figure 3, panels 1,3,5). In marked contrast, in the presence of 500 μM H$_2$O$_2$, most of uninfected endothelial cells and Ad-CFTR infected cells were dead or significantly damaged (panels 2,4). However, the AdCL infected endothelial cells were remarkably well preserved (panel 6).

Quantitation of the relative proportion of viable cells confirmed that at 500 μM H$_2$O$_2$, AdCL infected endothelial cells clearly had better survival compared to uninfected and Ad-CFTR infected endothelial cells (Figure 5, p = 0.004). At 1000 μM H$_2$O$_2$, very few cells survived in all groups (AdCL survival 1.8 ± 1%, uninfected 0.1 ± 0.1%, Ad-CFTR 0.8 ± 1%, p = 0.1).

**DISCUSSION**

Vascular endothelial cells are a major target of oxidant injury, particularly that mediated by H$_2$O$_2$ (11–13). H$_2$O$_2$ is the major cytoytic oxidant product of inflammatory cells and of the xanthine oxidase pathway within cells (14,15). H$_2$O$_2$ can injure cells directly or indirectly (through formation of HOCl and OH·), and is particularly dangerous because it can move freely through membranes, so all components of the cell are vulnerable (11,33). Among antioxidants, catalase has been shown to be the major pathway for removal of H$_2$O$_2$ at high (but still physiologic) levels, as opposed to the glutathione system which is active in removal of H$_2$O$_2$ at lower levels (<20 μM) (20). The data in the present study demonstrate that AdCL, a replication deficient recombinant adenovirus containing the human catalase cDNA, is able to direct the expression of sufficient levels of functional catalase within human endothelial cells to protect the cells against high levels of exogenous H$_2$O$_2$.

The concept of augmenting catalase levels within endothelial cells to protect against H$_2$O$_2$ mediated injury is not new (34); the problem has been how to achieve this goal. To achieve pharmacologic value in protecting the endothelium, catalase must be available in sufficient quantity at sites of oxidant generation. In vitro, addition of catalase itself to endothelial cells will afford protection, but this is very difficult to achieve in vivo. For example, continuous infusions of bovine catalase to human endothelial cells were effective in vitro in protecting the endothelium from neutrophil mediated injury and high levels of H$_2$O$_2$ (14,18,19). However, when injected in vivo intravenously in rats, catalase was unable to protect the animals against damages...
due to hyperoxia, likely because the half-life of catalase in vivo is very short (10 to 20 min) due to clearance by the kidneys (35).

Further, catalase is large (240 kDa) and impermeable to cell membranes, and it is especially labile due to sensitivity to proteases (36–38). Conjugation of catalase to liposomes or mono-methoxy-polyethylene glycol is able to prolong the in vivo half-life to 30 to 40 hr, and deliver a small percentage of catalase into the cell (35,38,39). However this strategy increases maximum intracellular catalase levels for a short period, and at most, doubles catalase activity. In contrast, temporary transfection of an epidermal cell line with a plasmid containing a human catalase cDNA increased catalase activity 2 to 4-fold and protected the transfected cells against oxidant stress (40).

With this in mind, it is reasonable to suggest that transfer of the catalase gene to raise intracellular endothelial catalase levels for periods up to 1 wk may be useful for many common acute and subacute diseases afflicting humans. In this regard, catalase gene therapy in the vasculature may be appropriate for protecting against reperfusion ischemia, such as myocardial ischemia and cerebrovascular events, as well as to shock states related to microvasculature increased permeability as in sepsis, burn injury and ARDS.

The endothelium represents an ideal target for transfer of genes due to its easy accessibility. The major technical hurdle is based on the inherent biology of the endothelium in its resting state in vivo. Endothelial cells replicate slowly, limiting the use of gene transfer vectors that depend on cell proliferation for expression of transferred genes. The adenovirus does not require host cell proliferation to express the exogenous gene (22–24). In the context of our previous data that Ad vectors can transfer exogenous marker genes with high efficiency to the endothelium in intact human umbilical veins (25), it should be possible to use AdCL to transfer the human catalase cDNA to human endothelium in intact blood vessels. Since many diseases potentially treatable with catalase are acute in nature, therapy with AdCL would only require one ‘dose’ for a protective effect that would last for several days to weeks.

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