

Expression of Id1 Results in Apoptosis of Cardiac Myocytes through a Redox-dependent Mechanism*

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Koichi Tanaka‡, John B. Pracyk‡, Kazuyo Takeda§, Zu-Xi Yu§, Victor J. Ferrans§, Shailesh S. Deshpande¶, Michitaka Ozaki¶, Paul M. Hwang¶, Charles J. Lowenstein¶, Kaikobad Irani¶, and Toren Finkel‡¶

From the ‡Cardiology Branch and the §Pathology Section, NHLBI, National Institutes of Health, Bethesda, Maryland 20892, and the ¶Cardiology Division, Johns Hopkins School of Medicine, Baltimore, Maryland 21205

We have constructed a recombinant adenovirus (Ad.Id1) that allows for efficient expression of the helix-loop-helix protein Id1. After infection with Ad.Id1, neonatal cardiac myocytes display a significant reduction in viability, which was proportional to the level of Id1 expression. A similar effect was observed in adult myocytes. Morphological and biochemical assays demonstrated that Id1 expression resulted in myocyte apoptosis. In contrast, expression of Id1 in endothelial cells, vascular smooth muscle cells, or fibroblasts did not affect the viability of these cells. Along with the induction of apoptosis, the expression of Id1 in neonatal cardiac myocytes resulted in an increase in the level of intracellular reactive oxygen species. The source of these reactive oxygen species appears to be the mitochondria. Reducing the ambient oxygen concentration or treatment with a cell-permeant H₂O₂ scavenger prevented Id1-stimulated apoptosis in cardiac myocytes. These results suggest that the expression of Id1 leads to the induction of apoptosis in cardiac myocytes through a redox-dependent mechanism.

The Id family of proteins belongs to a class of nuclear proteins known as helix-loop-helix (HLH)¹ proteins, which regulate differentiation and tissue-specific gene expression. To date, four different Id family members have been identified (1–6). Although these four proteins share a high degree of homology, especially within the HLH domain, evidence suggests that they exert nonoverlapping functions (7–9).

The HLH domain acts as a protein dimerization motif. Members of the HLH protein family which act to regulate transcription positively also contain an additional basic domain that mediates binding to DNA. In contrast, members of the Id family lack this domain and therefore are unable to bind DNA. These structural differences have led to a model in which Id proteins act in a dominant negative fashion to inhibit differentiation (1, 2). Under these circumstances, expression of high levels of Id1 would result in the binding and subsequent inactivation of basic domain HLH proteins.

Consistent with this model of Id function is the observation

that Id expression decreases when skeletal muscle cells differentiate (10). In addition, forced expression of Id has been shown to inhibit tissue-specific gene expression or differentiation of a variety of cell types (1, 10–12).

Previous reports have demonstrated that Id levels are high in embryonic hearts and decrease after birth (13–15). Such a shift in Id expression is consistent with its role as an inhibitor of differentiation. Although Id expression is low or absent in adult cardiac myocytes in culture, its expression can be reactivated by certain stimuli, such as α -adrenergic agonists (14, 15). A similar induction of Id expression has been demonstrated in skeletal muscle after treatment with the cardiotoxic chemotherapeutic agent doxorubicin (16, 17).

Although previous studies have demonstrated that forced expression of Id proteins can block differentiation, relatively little is known about the effects of Id expression in the context of a terminally differentiated cell phenotype. This is perhaps because of the difficulty of efficient gene transfer into most terminally differentiated cell types. The use of recombinant adenoviruses, which allows for high efficiency gene transfer, provides one strategy to address these issues in cardiac myocytes (18–20). These studies were motivated initially by the possibility that reexpression of Id in cardiac myocytes potentially could lead to a dedifferentiated phenotype and possible reentry into the cell cycle. Such results could have important potential therapeutic implications. In this report, however, we demonstrate that overexpression of Id1 in cardiac myocytes results in the induction of apoptosis.

MATERIALS AND METHODS

Cells—Ventricular neonatal myocytes were harvested from 2–3-day-old Sprague-Dawley rats by trypsin and collagenase digestion, purified by differential preplating, and cultured as described previously (21). Staining with MF-20 antibody to myosin heavy chain revealed the purity of the myocyte preparation to be in the range of 90–95% (data not shown). Cells that adhered during preplating were used as a source for primary cultures of neonatal ventricular fibroblasts. Adult rat myocytes were prepared as described previously (22, 23). Cultures of human umbilical vein endothelial cells and human coronary artery smooth muscle cells were obtained from Clonetics.

Adenovirus—An adenovirus encoding the mouse Id1 was constructed by homologous recombination in 293 cells (24). The Id1 cDNA (a gift of R. Benzeva) was placed under the control of the cytomegalovirus immediate-early promoter. Positive plaques were identified by polymerase chain reaction, subsequently amplified in 293 cells, and then purified by double cesium gradients (25). A control virus, Ad.d1312, which is E1-negative but lacks a recombinant transgene, was used as a control (26). Unless stated otherwise, all infections were at a multiplicity of infection (m.o.i.) of 100. Expression of Id1 was confirmed by Western blot analysis using an Id-specific antibody (Santa Cruz, CA, 1 μ g/ml) and enhanced chemiluminescence (Amersham Pharmacia Biotech). Infection with an adenovirus containing a marker gene product (β -galactosidase) demonstrated a neonatal cardiac myocyte gene transfer efficiency of greater than 90% (data not shown).

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¶ To whom correspondence should be addressed: Cardiology Branch, National Institutes of Health, 10 Center Dr., Bldg. 10, Rm. 7B15, Bethesda, MD 20892-1650. Tel.: 301-402-4081; Fax 301-402-0888.

¹ The abbreviations used are: HLH, helix-loop-helix; m.o.i., multiplicity of infection; ELISA, enzyme-linked immunosorbent assay; ROS, reactive oxygen species; DCF, dichlorofluorescein; DHR, dihydrorhodamine.

Viability and Apoptosis Assays—Measurements of cell viability were obtained using XTT (Cell Proliferation Kit, Boehringer Mannheim) according to the manufacturer's instructions.

To demonstrate apoptosis, a fluorescence-based TUNEL assay was

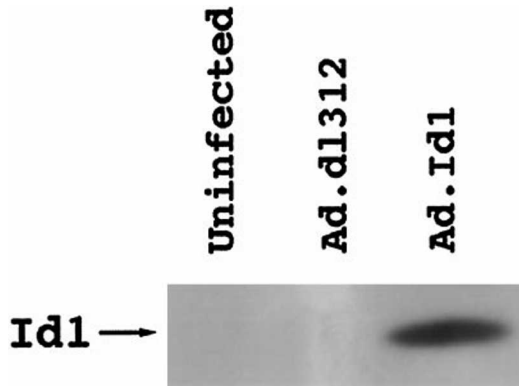


FIG. 1. Expression of Id1 in cardiac myocytes after adenovirus-mediated gene transfer. Western blot analysis of protein lysates (20 μ g) from uninfected myocytes or myocytes infected 48 h earlier with 100 m.o.i. of a control adenovirus (Ad.d1312) or with Ad.Id1 is shown.

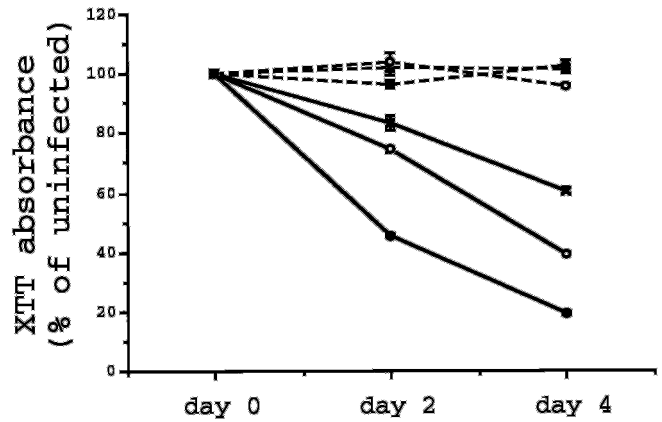


FIG. 2. Expression of Id1 results in a reduction in cardiac myocyte viability. Cells infected with the indicated m.o.i. of either Ad.d1312 (broken lines) or Ad.Id1 (solid lines) were assayed over 4 days for viability using XTT absorbance. Results are the mean \pm S.D. of triplicate cultures from one of four similar experiments and are expressed as the percentage of XTT absorbance compared with that of uninfected cultures. \times , 25 m.o.i.; open circles, 50 m.o.i.; closed circles, 100 m.o.i.

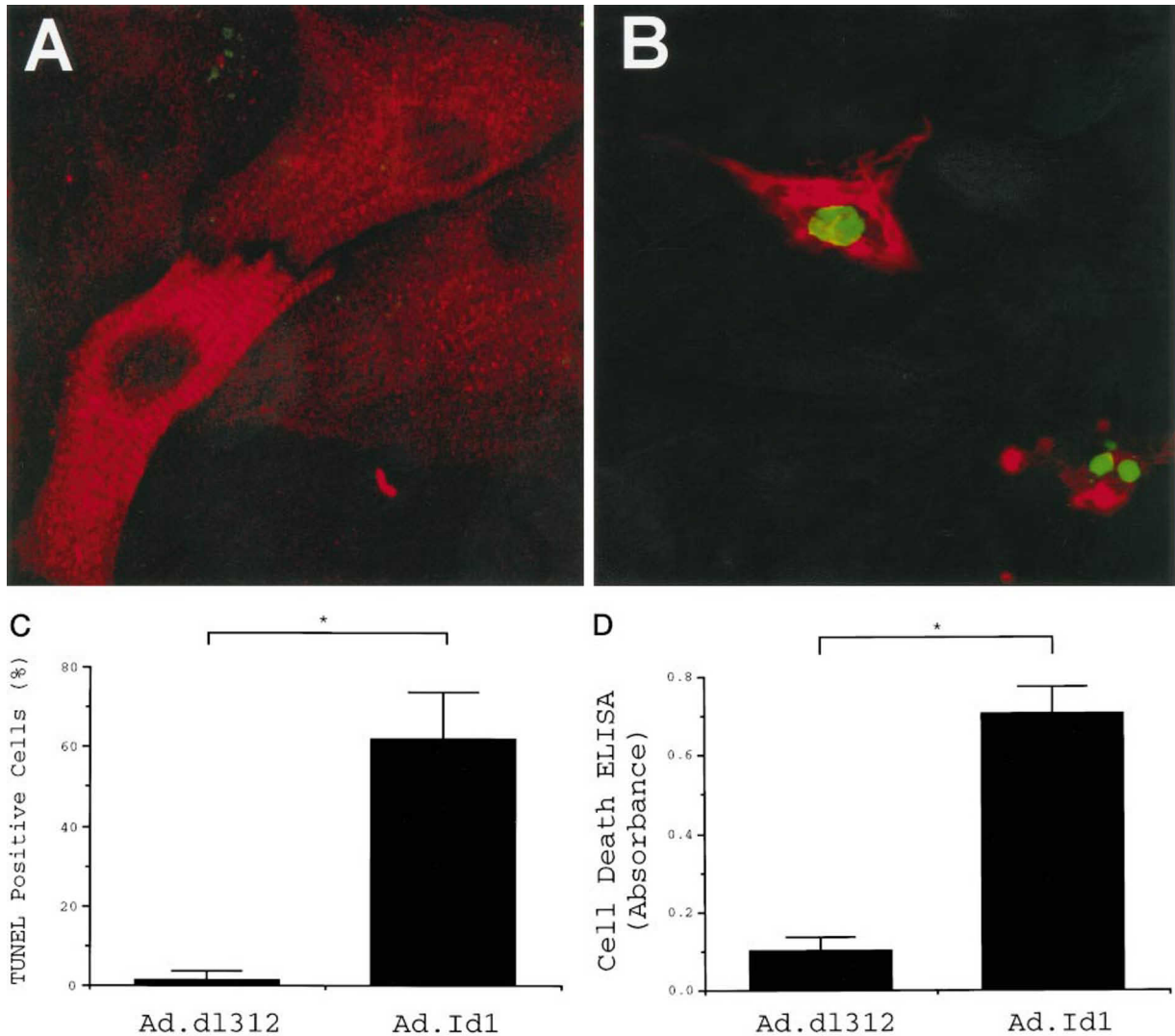


FIG. 3. Id1 induces neonatal myocyte apoptosis. Primary cultures of myocytes infected 72 h earlier with Ad.d1312 (panel A) or Ad.Id1 (panel B) were assayed with a fluorescence-based TUNEL assay. Apoptotic nuclei are green. Cultures were stained concurrently for the expression of the cytoplasmic myosin heavy chain (red). Panel C, quantification TUNEL-positive nuclei. Shown are the percentages of TUNEL-positive nuclei (mean \pm S.D.) obtained by counting approximately 200 myosin heavy chain-positive cells from three separate experiments. The asterisk (*) indicates $p < 0.05$. Panel D, absorbance measurement (arbitrary units) quantifying the amount of apoptosis in myocytes infected with 100 m.o.i. Ad.d1312 or Ad.Id1 using the cell death ELISA.

performed (Oncor, Gaithersburg, MD). Briefly, cells were fixed overnight at 4 °C with 4% *p*-formaldehyde in 0.1 M phosphate buffer, pH 7.2, and subsequently stained overnight at 4 °C with a myosin heavy chain (MF-20) antibody conjugated indirectly with Texas red. The samples were then processed for the TUNEL assay. Apoptotic myocytes were imaged with a laser scanning confocal microscope (Leica, TCS-4D). Negative controls for the TUNEL assay consisted of cells incubated in the absence of the terminal deoxynucleotide transferase. These preparations gave negative results. Alternatively, apoptosis was measured according to the manufacturer's recommendations using 2×10^5 neonatal myocytes or 2×10^4 adult myocytes and an enzyme immunoassay that allowed for the quantification of cytoplasmic histone-associated DNA fragments composed of mono- or oligonucleosomes generated by apoptosis-induced endonucleases (Cell Death Detection ELISA, Boehringer Mannheim).

For electron microscopic studies to confirm the occurrence of apoptotic changes in the cardiac myocytes, cells were postfixed with 1% osmium tetroxide and ultrathin sections stained with uranyl acetate and lead citrate. The cells were then examined by transmission electron microscope.

Measurements of Reactive Oxygen Species (ROS)—For measurement of intracellular ROS, cells were loaded with 5 $\mu\text{g/ml}$ 2',7'-dichlorofluorescein diacetate (Molecular Probes) for 5 min. Cells were subsequently imaged and the fluorescence of dichlorofluorescein (DCF) quantified using a Leica laser scanning confocal microscope with false color imaging as described previously (27). In certain cases, cells were treated with the mitochondrial electron transport inhibitor rotenone (1 μM) for 1 h before assessment with DCF. Direct visualization of mitochondrial ROS was made using dihydrorhodamine 123 (DHR123). Previous studies have demonstrated that this compound accumulates selectively in the mitochondria where it is oxidized by mitochondrial ROS to the fluorescent rhodamine (28, 29). Cells were incubated with DHR123 (1 μM) for 1 h before visualization by confocal microscopy. For manipulation of ambient O_2 levels, the cells were placed in a sealed hypoxia chamber (Billups-Rothenberg) immediately after infection. This chamber was flushed for 20 min with a gas mixture containing 5% CO_2 , the indicated O_2 concentration, and the balance composed of nitrogen gas. Levels of lipid peroxides were measured using a lipid peroxidation assay kit (Calbiochem) according to the manufacturer's recommendation.

RESULTS

In an effort to induce efficient expressions of HLH proteins in cardiac myocytes, we constructed a recombinant adenovirus encoding Id1. Infection of neonatal ventricular myocytes with Ad.Id1 adenovirus resulted in an increase in the level of Id1 protein detected in myocyte lysates (Fig. 1). Whereas levels of Id were undetectable in uninfected myocytes or in myocytes infected with a control adenovirus (Ad.d1312), infection with Ad.Id1 adenovirus resulted in significant expression of Id1.

We next assessed the biological effects of Id1 expression. Within 48–72 h after Ad.Id1 infection, there was a marked reduction in myocyte viability (Fig. 2). The reduction in viability was proportional to the m.o.i. and hence the level of Id1 expression (see also Fig. 4A). In contrast, infection with the control adenovirus (Ad.d1312) did not affect cell viability.

Because expression of Id1 resulted in a loss of cell viability, we next determined whether myocytes were undergoing apoptosis. Cultures of Ad.d1312-infected cells and cells infected with Ad.Id1 were assessed using a fluorescence-based TUNEL assay. To confirm that only cardiac myocytes were analyzed, cells were counterstained with a Texas red-conjugated antibody recognizing myosin heavy chain. As demonstrated in Fig. 3A, there were very few TUNEL-positive nuclei in control-infected myocytes. In contrast, myocytes infected with Ad.Id1 (Fig. 3B) demonstrated a high percentage of TUNEL-positive cells. Quantification of TUNEL-positive nuclei obtained from three separate experiments demonstrated a marked increase in the Ad.Id1-infected cell cultures (Fig. 3C). Direct measurement of cell death using an ELISA capable of detecting cytoplasmic histone-associated DNA fragments also demonstrated significant Id1-induced apoptosis (Fig. 3D).

In accord with the results of the TUNEL and cell death

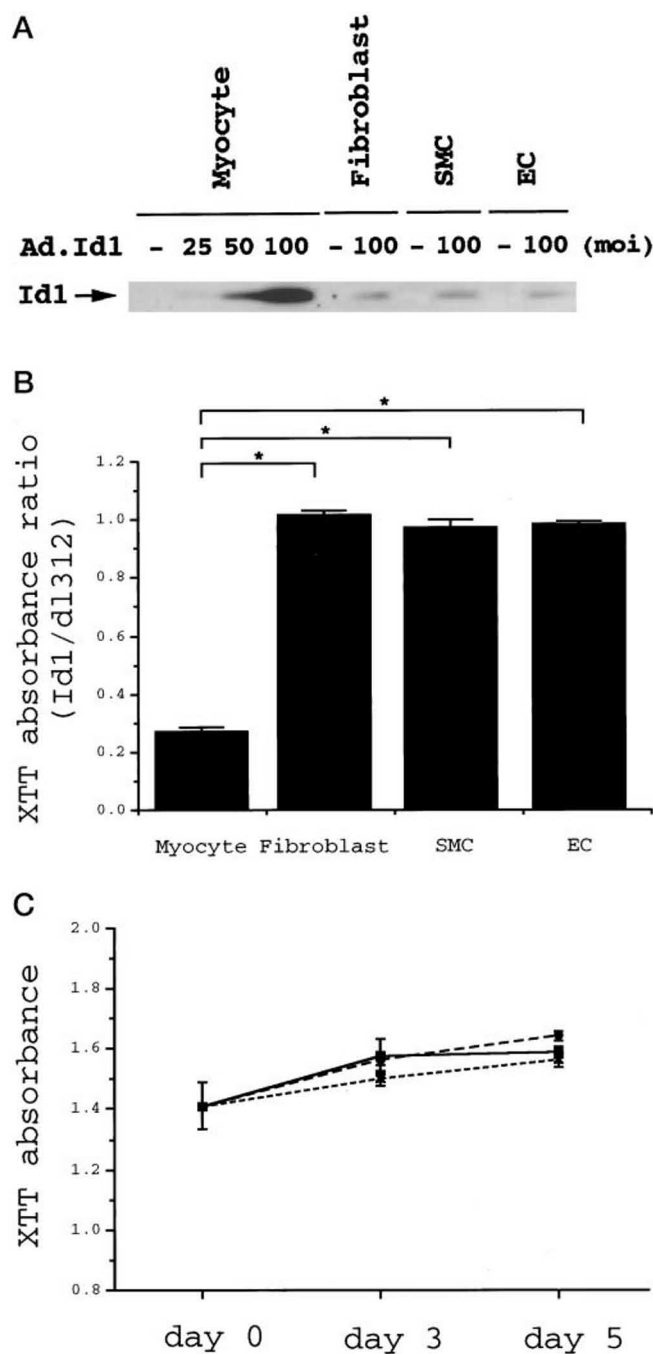


FIG. 4. Id1 expression does not affect the viability of other cell types. Panel A, Western blot analysis after infection at the indicated m.o.i. with either Ad.d1312 or Ad.Id1 in neonatal myocytes, fibroblast, vascular smooth muscle cells (SMC), or endothelial cells (EC). Panel B, ratio of XTT absorbance (arbitrary units) of Ad.Id1- to Ad.d1312-infected cells 5 days after infection. Results are from one of three similar experiments, each performed in triplicate. The asterisk (*) indicates $p < 0.05$. Panel C, primary cultures of endothelial cells were grown to confluence and subsequently infected with Ad.d1312 or Ad.Id1. Viability of cells was assessed by XTT absorbance after infection. Results (mean \pm S.D.) are from one of two similar experiments, each performed in triplicate. \times , uninfected; closed circles, Ad.d1312; closed squares, Ad.Id1.

ELISA, myocytes infected with Ad.Id1 showed morphological changes consistent with apoptosis. By light microscopy, the apoptotic cells appeared to round up, with evidence of membrane blebbing and nuclear condensation; electron micrographs of myocytes infected with Ad.Id1 demonstrated rounded

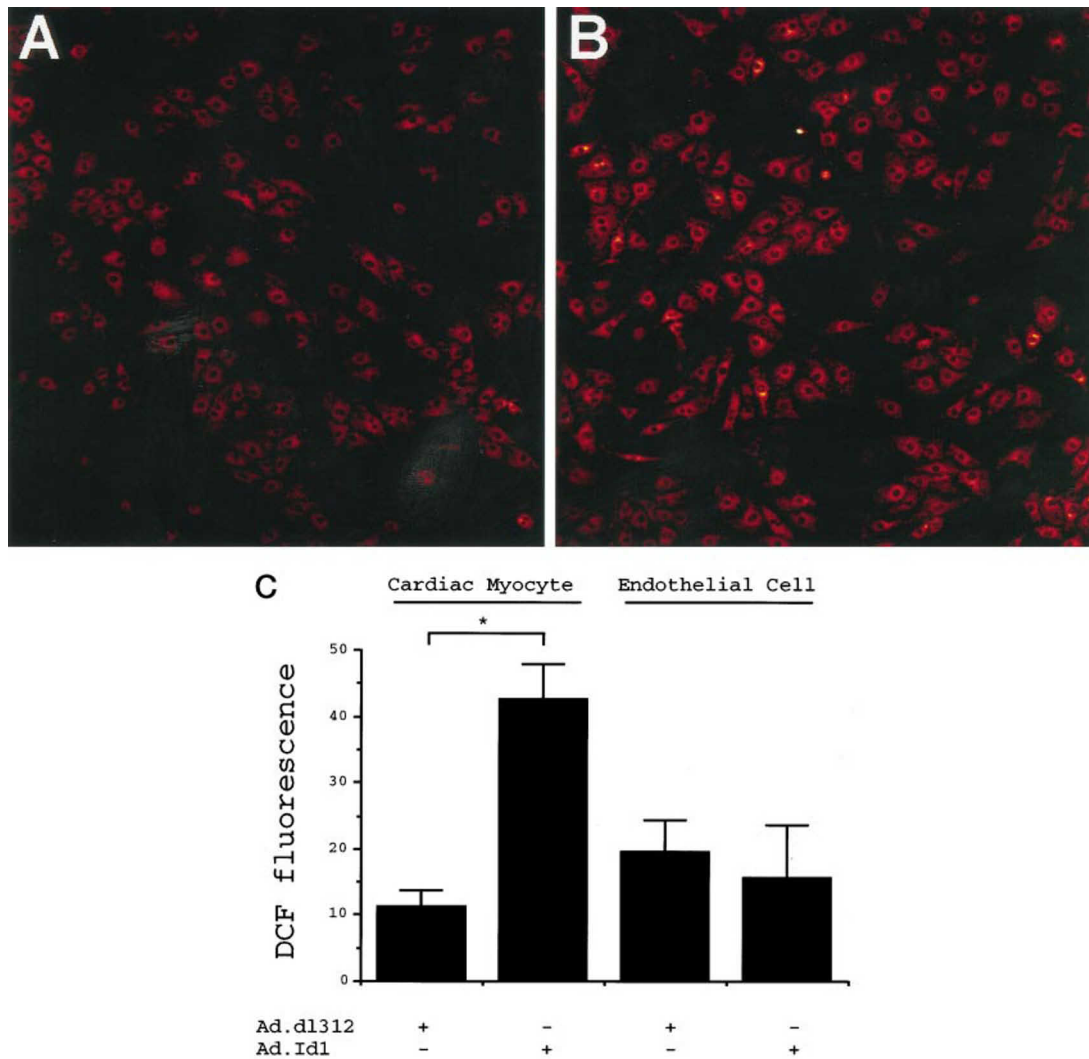


FIG. 5. Id1 affects the level of ROS in myocytes. Representative field demonstrating DCF fluorescence in cardiac myocyte-infected Ad.d1312 (panel A) or Ad.Id1 (panel B). Panel C, quantification of the DCF fluorescence (arbitrary units) of 60 cells in three separate fields of Ad.Id1- or Ad.d1312-infected cardiac myocytes or endothelial cells. The asterisk (*) indicates $p < 0.05$.

cell shapes and highly condensed chromatins (see Fig. 3B and data not shown).

We next sought to assess whether the apoptotic effects of Id1 expression could be demonstrated in other cell types. Cultures of fibroblasts, vascular smooth muscle cells, and endothelial cells were infected with a control virus or with Ad.Id1. All cell types were transduced efficiently by recombinant adenoviruses; however, the level of Id1 expression was less than what was observed in neonatal myocytes (Fig. 4A). As demonstrated in Fig. 4B, in these three different cell types there was no appreciable effect on viability after Ad.Id1 infection. Although these differences in viability may merely reflect differences in the absolute level of expression, it should be noted that the level of Id1 expression appeared to be higher in these other cell types infected at 100 m.o.i. than in neonatal cardiac myocytes infected at 25 m.o.i. Nonetheless, myocytes infected at 25 m.o.i. have a reduction in cell viability (see Fig. 2), whereas there was no cell death observed in the other cell types when infected at either 100 m.o.i. (Fig. 4B) or at 200 m.o.i. (data not shown). These results suggest that the apoptotic effects of Id1 may have different thresholds in different cell types.

One significant difference between cardiac myocytes and the other cell types tested is their limited capacity for cell division. We thought it possible, therefore, that Id1 may induce apoptosis only in nondividing cells. To explore this possibility, we

infected confluent growth-arrested endothelial cells. Under these conditions, there is little if any endothelial cell division, and there is no effect of Id1 on cell viability (Fig. 4C).

Previous experiments have demonstrated that ROS may participate in apoptosis (for review, see Ref. 30). In particular, expression of wild-type p53 results in the initiation of a redox-dependent apoptotic program in certain cell types (31, 32). Given these previous observations, we next sought to assess whether Id1 expression altered the redox state of cells. Using the peroxide-sensitive fluorophore DCF, levels of intracellular hydrogen peroxide were assessed. Two days after infection, cardiac myocytes infected with Ad.Id1 demonstrated a 2–4-fold increase in the level of DCF fluorescence compared with cells infected with the control virus (Fig. 5A and B). In contrast, in endothelial cells, which do not undergo apoptosis after Ad.Id1 infection, there was no comparable increase in DCF fluorescence after Id1 expression (Fig. 5C).

We next sought to determine the source of Id1-induced ROS production. A variety of evidence has suggested that under basal conditions the mitochondria are the predominant source of ROS and that these organelles are also responsible for the increase in ROS seen during apoptotic conditions (for review, see Ref. 33). Consistent with the mitochondria being the source of ROS, when Id1-infected cells were treated with rotenone, a specific inhibitor of mitochondrial electron transport, the in-

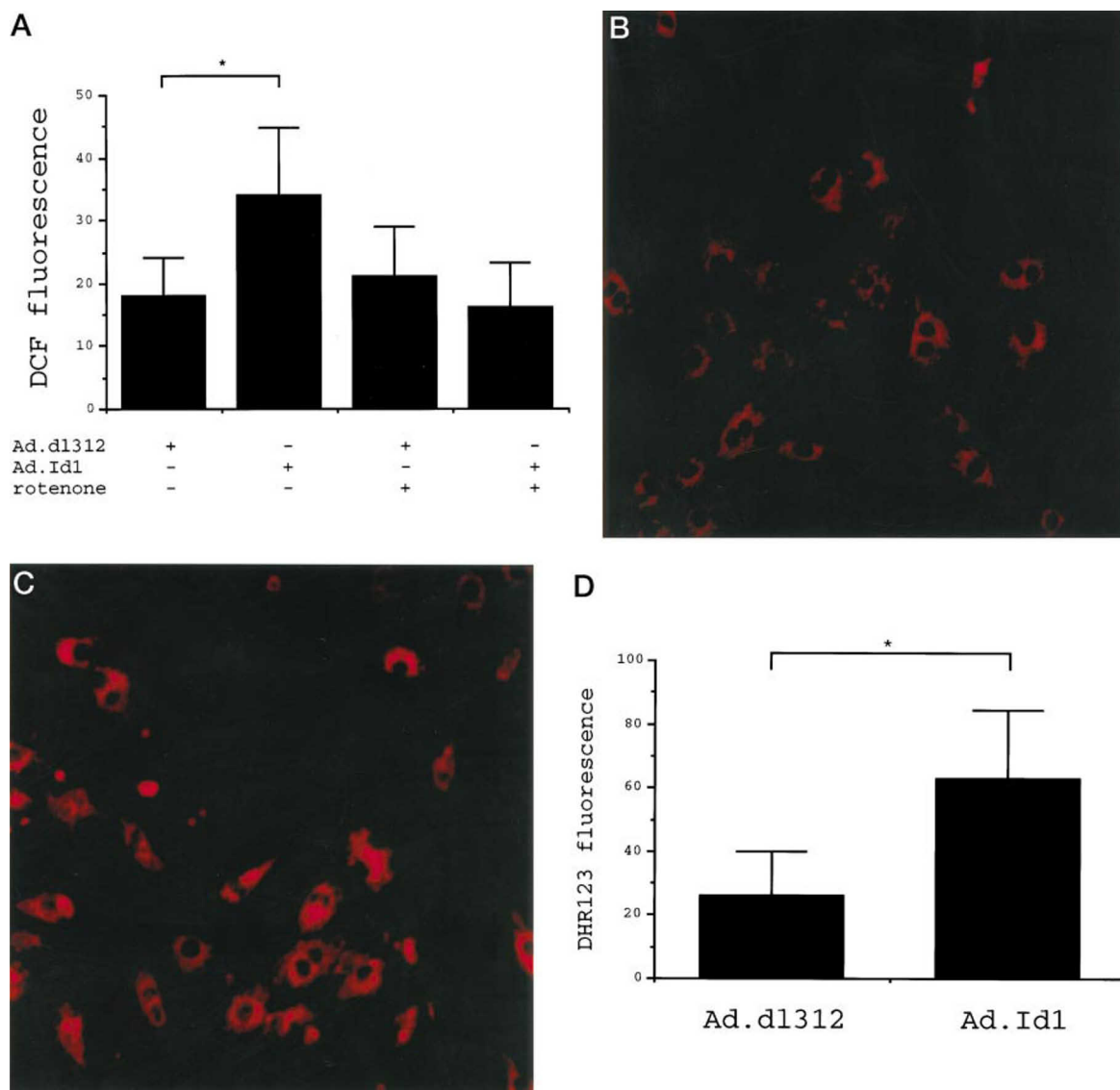


FIG. 6. **Id1 increases the level of mitochondrial ROS.** *Panel A*, levels of DCF fluorescence in the presence or absence of the mitochondrial electron transport inhibitor rotenone (1 μ M). *Panel B*, level of DHR123 fluorescence in Ad.d1312-infected cells or (*panel C*) Ad.Id1-infected cells. *Panel D*, quantification of DHR123 fluorescence (arbitrary units) from approximately 60 random cells.

crease in cytosolic DCF fluorescence was abolished (Fig. 6A). We next assessed the mitochondrial ROS level directly using the fluorophore DHR123, which is taken up selectively by mitochondria and converts to a fluorescent moiety by mitochondrial ROS. As seen in Fig. 6, *B* and *C*, compared with control cells, Ad.Id1-infected cells had higher levels of DHR123 fluorescence. Quantification of DHR123 from control and Id1-expressing cells demonstrated an approximate 2–3-fold rise in DHR123 fluorescence after Ad.Id1 infection (Fig. 6D).

To understand the significance of the rise in ROS after Ad.Id1 infection of cardiac myocytes, we made use of the observation that the formation of ROS is a function of ambient oxygen tension (34, 35). If the rise in ROS was essential for Id1-induced apoptosis, then lowering the ambient oxygen concentration should protect the cells. Therefore, cells infected with Ad.Id1 or a control adenovirus were grown in a 1%, 5%, or the standard 20% oxygen environment. Consistent with the observation that Id1 results in an increase in oxidative stress, measurement of lipid peroxide levels demonstrated a significant rise after Ad.Id1 infection (Fig. 7). Lowering the level of ambient oxygen, consistent with the known ability of oxygen to regulate ROS levels, resulted in a decreasing amount of lipid

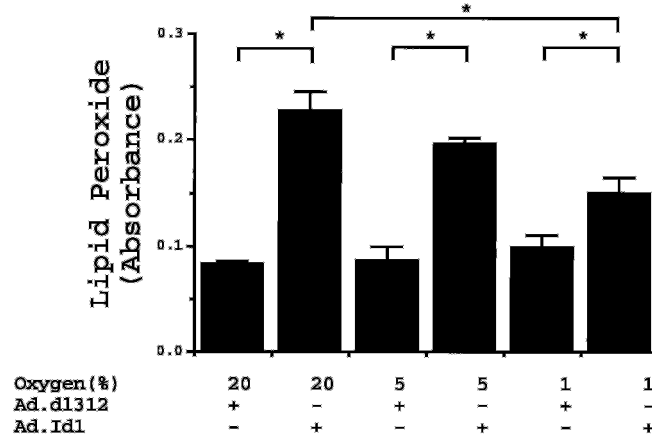


FIG. 7. **Id1 increases the level of lipid peroxides.** Levels of lipid peroxides were measured in neonatal myocytes infected with either Ad.d1312 or Ad.Id1 that had been incubated in the indicated level of ambient oxygen.

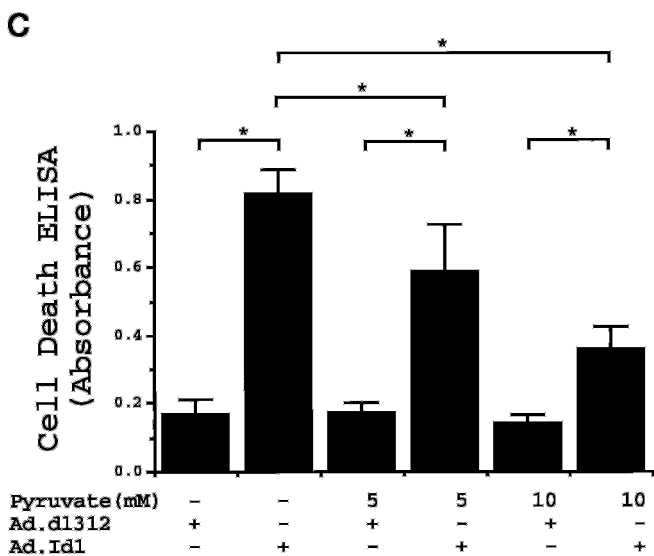
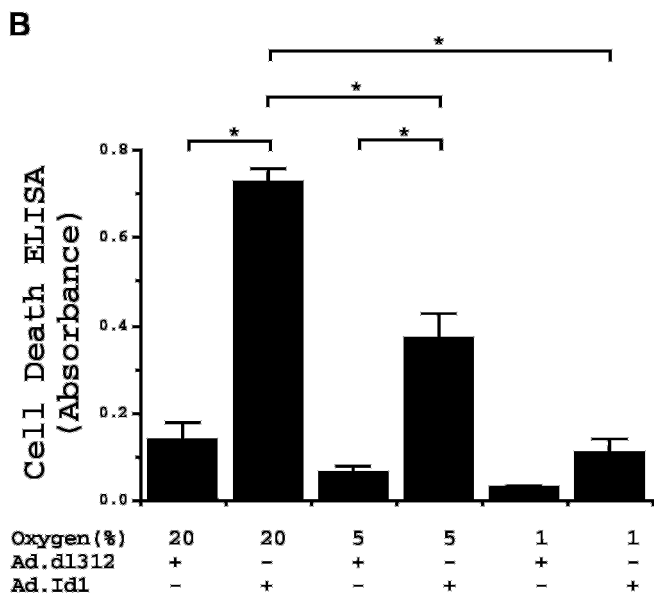
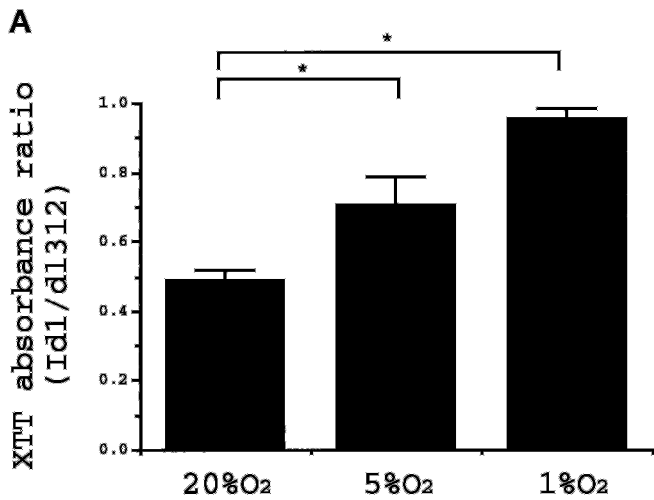


FIG. 8. The apoptotic effects of Id1 are inhibited by lowering ROS levels. Panel A, cultures of cardiac myocytes were infected with Ad.d1312 or Ad.Id1 and transferred to a 1%, 5%, or 20% O₂ environment. Cell viability was assessed 3 days after infection. Panel B, levels of apoptosis measured by the cell death ELISA (arbitrary units). Panel C, levels of apoptosis as measured by the cell death ELISA in the presence or absence of the H₂O₂ scavenger pyruvate. Results are from

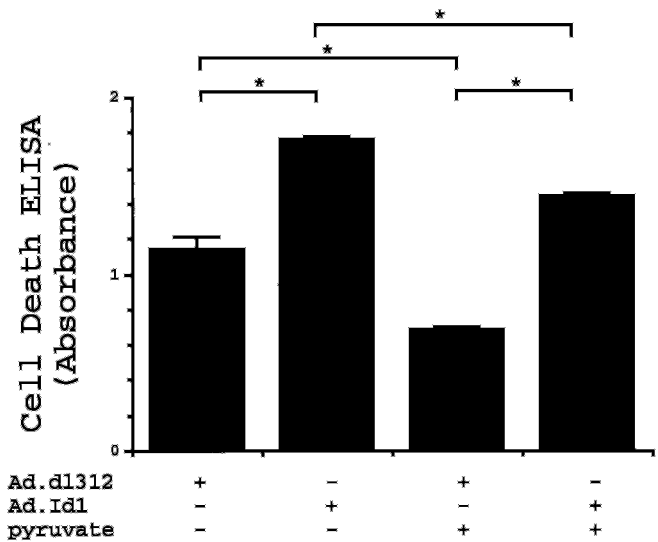


FIG. 9. Id1 expression increases adult cardiac myocyte cell death. Shown are levels of apoptosis using the cell death ELISA after Ad.d1312 or Ad.Id1 infection of adult cardiac myocytes in the presence or absence of the H₂O₂ scavenger pyruvate.

peroxide generation after Id1 expression.

To understand whether the rise in ROS was important in the ability of Id1 to induce apoptosis, we assessed cell viability under different O₂ levels. As demonstrated in Fig. 8A, lowering the ambient O₂ level to 1% resulted in a significant rescue in cell viability of Id1-infected cells. Similarly, direct measurement of the level of apoptosis demonstrated that lowering the ambient oxygen lowered the rate of apoptosis (Fig. 8B).

To substantiate further the relationship between ROS production and apoptosis, we attempted to treat myocytes with cell-permeant antioxidants. We observed toxicity when neonatal myocytes were exposed to either *N*-acetylcysteine or pyrrolidine dithiocarbamate. A similar toxic effect has been observed previously in other cell types (36). In contrast, treatment with pyruvate, a known scavenger of hydrogen peroxide (37–40), resulted in significant protection from Id1-induced apoptosis (Fig. 8C).

Finally, we sought to understand if similar results were obtainable in adult myocytes. In contrast to neonatal myocytes the level of apoptosis under basal conditions was approximately 5-fold higher in adult myocytes. Nonetheless, as demonstrated in Fig. 9, expression of Id1 resulted in an increase in the level of apoptosis. Although the magnitude of this effect was smaller, as in neonatal cells, treatment with the H₂O₂ scavenger pyruvate had a protective effect on both control and Id1-expressing cells. This may suggest that ROS may also play a role in the elevated basal rate of apoptosis observed in adult myocytes.

DISCUSSION

Our results demonstrate that expression of the HLH protein Id1 results in the induction of apoptosis in cardiac myocytes. Previous experiments have demonstrated that in the myoblast cell line C₂C₁₂, it is difficult to express Id1 stably (10). These results suggest a potential growth-inhibitory or apoptotic effect of Id1 in these skeletal muscle cells. In contrast, other studies using different cell lines suggest that expression of Id1 can be achieved readily (9, 12, 41). Taken together, these results sug-

one of three similar experiments, each performed in triplicate. The asterisk (*) indicates *p* < 0.05.

gest that high level expression of Id1 can, in certain cell types, result in apoptosis. It should be noted that in the course of these experiments another report reached a similar conclusion, namely that overexpression of Id3 could induce apoptosis in serum-starved embryonic fibroblasts (42).

In many experimental situations, apoptosis is accompanied by a rise in intracellular ROS (30). Perhaps the system most relevant to the present results is the previous demonstration using adenovirus-mediated gene transfer of wild-type p53 to normal smooth muscle cells (31) or transformed epithelial cell lines (32). It has been demonstrated that human smooth muscle cells but not rat smooth muscle cells undergo apoptosis after p53 overexpression. The sensitivity to p53-mediated apoptosis correlated with the ability of p53 expression to increase the level of intracellular ROS (31). The mechanism by which p53 changes intracellular ROS levels appears to be related, at least in part, to its ability to alter the levels of gene products that function to regulate the redox state (32). Inhibiting the rise in ROS levels in each system dramatically inhibits the ability of p53 to induce apoptosis.

A rise in ROS preceding the onset of apoptosis appears to be a common but not universal (30, 34, 35) feature of cell death. It is conceivable that Id1 functions to alter the redox state of cells by changing the level of p53. However, Western blot analysis of Ad.Id1-infected myocytes demonstrated no change in the amount of p53.² Although apoptosis is accompanied by a rise in ROS levels, it is difficult to prove that such a rise is the cause rather than a consequence of cell death. Nonetheless, our experiments in low ambient O₂ conditions as well as with the peroxide scavenger pyruvate support the concept that ROS are important mediators of apoptosis.

Previous studies have demonstrated that in adult myocytes, levels of Id are low but can be stimulated significantly by maintaining adult myocytes in culture and even more rapidly by agents such as phenylephrine (14, 15). The reexpression of Id gene products may be one reason why we observed a higher level of apoptosis in adult rather than neonatal myocytes. Expression of Id in this *in vitro* situation correlates with the reexpression of neonatal gene products, such as prepro-atrial natriuretic protein and skeletal muscle α -actin. It is interesting to note that, clinically, heart failure is accompanied by a similar reexpression of fetal gene products. In addition, recent studies suggest that the failing heart is characterized by an increase in oxidative stress (43, 44) and in the numbers of apoptotic cells (45, 46). It is tempting to speculate that these three presumably unrelated findings, *i.e.* the expression of dedifferentiated gene products, oxidative stress, and apoptosis, may be potentially related through the reactivation of Id1 expression in the failing myocardium.

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² K. Tanaka and T. Finkel, unpublished observations.