Vitamin D up-regulated protein 1 (VDUP1) is a key mediator of oxidative stress on various cellular processes via downstream effects on apoptosis signaling kinase 1 (ASK1) and p38 mitogen-activated protein kinase (MAPK). Here, we report that VDUP1 expression is significantly increased in rat hearts following acute myocardial ischemia, suggesting it may have important regulatory effects on cardiac physiological processes during periods of oxidative stress. Transfection of H9C2 cardiomyoblasts with a sequence-specific VDUP1 DNA enzyme to down-regulate VDUP1 mRNA expression significantly reduced apoptosis and enhanced cell survival under conditions of H2O2 stress, and these effects involved inhibition of ASK1 activity. Direct intracardiac injection of the DNA enzyme at the time of acute myocardial infarction reduced myocardial VDUP1 mRNA expression and resulted in prolonged reduction in cardiomyocyte apoptosis and ASK1 activity. Moreover, down-regulation of VDUP1 was accompanied by significant reduction in cardiac expression of pro-collagen type I mRNA level, as well as marked reduction in myocardial scar formation. These features were accompanied by significant improvement in cardiac function. Together, these results suggest a direct role for VDUP1 in the adverse effects of ischemia and oxidative stress on cardiomyocyte survival, left ventricular collagen deposition, and cardiac function. Strategies to inhibit VDUP1 expression and/or function during acute ischemic events may be beneficial to cardiac functional recovery and prevention of left ventricular remodeling.

The thioredoxin (Trx) and glutathione systems are the two major thiol-reducing pathways by which living cells regulate redox responses to stress signals (1, 2). The disulfide reductase Trx is a highly conserved and ubiquitously expressed small protein that participates widely in thiol-dependent cellular reductive processes, including signal transduction and regulation of the activity of transcription factors such as TFIIIC, NF-κB, and glucocorticoid receptors (3–5). In addition to its direct effects on electron transfer, Trx functions to maintain intracellular redox homeostasis through its action as a physiological inhibitor of the apoptosis signal-regulated kinase-1 (ASK1), a pivotal component in cellular apoptotic responses to cytokine- and stress-induced signals (6–8). Trx directly binds to ASK1, inducing its ubiquitination and degradation and resulting in inhibition of cellular apoptosis (9). These intrinsic properties of Trx are of central importance to the maintenance of healthy mammalian tissue function, including the cardiovascular system (10).

A recently identified negative regulator of Trx activity and expression is thioredoxin-binding protein 2 or thioredoxin-interacting protein (11). This protein, also termed “vitamin D3 up-regulated protein 1” (VDUP1) because of its induction in HL-60 cells differentiated by vitamin D3 (12), has been found to play important roles in diverse cellular processes, including the regulation of cellular redox balance, apoptosis, proliferation, and differentiation (13–18). Recent in vitro studies have shown that VDUP1 overexpression results in inhibition of proliferation of several cell types, including induction of cardiomyocyte apoptosis (16). Although deficits in antioxidants and increased oxidative stress accompanying myocardial infarction have been directly implicated in the pathogenesis of post-infarct heart failure, a possible role for VDUP1 in the adverse outcomes accompanying myocardial ischemia has not yet been investigated.

To develop a clinically relevant approach to inhibit VDUP1 expression, we focused on the use of a new generation of catalytic nucleic acids containing DNA molecules with catalytic activity for specific RNA sequences (19, 20). These DNA enzymes exhibit greater catalytic efficiency than hammerhead ribozymes, offer greater substrate specificity, are more resistant to chemical and enzymatic degradation, and are far cheaper to synthesize. Using a sequence-specific DNA enzyme to down-regulate VDUP1 mRNA in vitro and in vivo, we evaluated the role of VDUP1 in cardiomyocyte survival, cardiac functional recovery, and myocardial remodeling after oxidative stress and acute myocardial ischemia. Our data demonstrate that VDUP1 is a critical mediator of cardiomyocyte apoptosis and myocardial scar formation after oxidative stress and acute ischemia and suggest that therapeutic approaches to reduce myocardial VDUP1 expression may be of benefit in inducing cardiac functional recovery and preventing adverse myocardial remodeling after acute ischemia.

**EXPERIMENTAL PROCEDURES**

**cDNA Subtractive Hybridization**—This technique enabled comparison of the pattern of gene expression between hearts from normal rats and rats who underwent left anterior descending (LAD) coronary artery ligation 48 h earlier. Briefly, messenger RNA was isolated from each heart, and 1 μg was used for first-strand cDNA synthesis with random
primers. The subtractive hybridization was performed with the PCR-select cDNA subtraction kit (Clontech), following the manufacturer’s recommendations. After second strand synthesis, the two cDNA libraries were digested with Rsal. Digestion products of the “tester” library were ligated to a specific adapter (T7 promoter), then hybridized with a 30-fold excess of the “driver” library for subtraction. After hybridization, the remaining products were further amplified by PCR. In the forward subtraction, which determines the genes that are overexpressed in the ischemic sample, the ischemic tissue is the tester and the normal tissue is the driver. In the reverse subtraction, the tester and the driver are switched to determine the genes that are down-regulated in the ischemic sample.

DNA Enzyme Synthesis and in Vitro Cleavage—DNA enzymes with inverted thymidine (leading to a 3’−3’ linkage) were synthesized by Integrated DNA Technologies (Coralville, IA) and purified by RNase-free high-performance liquid chromatography. The short RNA substrates corresponding to target DNA enzyme sequences were chemically synthesized followed by RNase-free PAGE purification. Synthetic RNA substrate was end-labeled with 32P using T4 polynucleotide kinase. In vitro cleavage reaction system included 60 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 150 mM NaCl, 0.5 mM 32P-labeled RNA oligonucleotide, 0.05−5 μM DNA enzyme. Reactions were allowed to proceed at 37 °C and were quenched by transfer of aliquots to tubes containing 90% formamide, 20 mM EDTA, and loading dye. Samples were separated by electrophoresis on Tris-borate-EDTA-urea denaturing polyacrylamide gels (15% gel).

Cell Culture and DNA Enzyme Transfection—Rat fetal cardiomyocyte H9C2 cells were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO2. For transfection with DNA enzyme, rat cells were plated into each well of 6-well plates (~1 × 105 cells/well). Subconfluent (70−80%) cells were washed twice with 1 ml of HEPES buffer (pH 7.4) and transfected using 0.5 ml of serum-free Dulbecco’s modified Eagle’s medium containing 1 μM test molecule (VDUP1 DNA enzyme E4 or scrambled control E0) and 7 μl/ml SuperFect (Qiagen) for 4 h. After completion of transfection, transfected cells were lysed to isolate RNA using TRIzol reagent. For in vitro expression of collagen mRNA: the rat fibroblast cell line Rat 2 (from American Type Culture Collection) cultured in Dulbecco’s modified Eagle’s medium was seeded into 6-well plates and transfected with 1 μM of VDUP1 DNA enzyme and scrambled control. 24 h after completion of transfection, total RNA was extracted using TRIzol reagent and collagen 1α2 and 3α1 mRNA levels were analyzed by semiquantitative RT-PCR.

Semiquantitative RT-PCR—1-μg RNA samples were used to perform reverse transcription in a 20-μl reaction system, and 2-μl products were then used as templates to amplify rat VDUP1 or GAPDH in a 50-μl PCR system (5 μl of 10 × buffer, 1 μl of dNTP, 0.25 μl of Taq polymerase, 1 μl of forward and reverse primer, 38 μl of H2O, 2 μl of [32P]dCTP (3000 Ci/mmol and 10 μCi/μl). The reaction conditions were: 94 °C for 40 s, 56 °C for 40 s, and 72 °C for 1 min for 30 cycles (25 cycles for GAPDH). Rat GAPDH was used as internal control to quantify VDUP1. Primers were as follows: VDUP1 (5’-ACCCTTTATTTTCGTTGACTCCT-3’ for forward, 5’-TGCCTCACATCTGTTAGGGCATC-3’ for reverse, PCR size of 358 bp); GAPDH (5’-CTCTACCCACGGCAAGTTTCAAA-3’/5’GGGATGACCTTGGCCACAGC-3’, 515 bp). For PCR primers used for measurement of mRNA level in tissue in vivo, sequences and cycles were as follows: RPL32 (ribosomal protein L32 gene, CACACTGCTGGCTGTGGAAGGC/GAACACGACACG-CACACAAGCCATC, 27 cycles); VDUP1 (CATTGGAGGGTGAGGGGTTAGGG/CAGGTTTCTCCGGTGAATGTAGATG, 27 cycles); collagen 1α2 (TTCTTGGTGGTCCTCAATTCTGGGGCTCTCCCTTGATCCTGGAATACC, 25 cycles); collagen 3α1 (GAGGAGATGATCTCTCAGCTAATGTGGAGTACCCGG/CCACCATGTCTAGAGGTTGGGATCTATCTAGT, 25 cycles). PCR conditions for in vivo tissue are 94 °C for 30 s for denaturing and 40 cycles for annealing and extension.

Survival and Apoptotic Assay of In Vitro Cultured Cells—H9C2 cells were seeded into 96-well plate were transfected at 70% confluence with 1 μM VDUP1 DNA enzymes or control scrambled DNA enzymes complexed with 80 μl of SuperFect/5.8 ml for 3 h. After further culture for 20 h, cells were stimulated with different concentrations of H2O2. For the apoptotic assay, cells were subsequently lysed with 200 μl of lysis buffer (Roche Applied Science, cell death enzyme-linked immunosorbent assay) according to the instruction manual of the company, and 20 μl of lysate supernatant was used in enzyme-linked immunosorbent assay reaction to measure A405 nm to A630 nm. For survival assay, after completion of stimulation, cells were cultured in 100 μl of medium for an addition 4 h and then 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (catalog no. G3582, Promega) was added to each well. Two hours later, the absorbance values at 490 nm were measured.

ASK1 Kinase Activity Assay—At 70% confluence, rat fetal H9C2 cardiomyocytes seeded in 100-mm dishes were transfected for 4 h with transfection solution containing 1 μM VDUP1 DNA enzyme or scrambled DNA enzyme and 8 μl/ml SuperFect. After removal of transfection solution, cells were cultured for another 4 h in normal medium and then stimulated for 20 h by 37.50 μM H2O2. 24 h after completion of transfection, cells were lysed on ice in 0.6 ml of 1× ice-cold cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM Na2EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and underwent brief sonication. The cell extracts were clarified by centrifugation, and protein concentrations were determined with a Bio-Rad kit. Cell lysate supernatant containing 400 μg of protein were incubated with 5 μg of primary antibody against ASK1 (Santa Cruz Biotechnology), gently rocking overnight at 4 °C, then protein A-Sepharose beads (Sigma, 30 μl of 50% bead slurry) were added, and the mixture was incubated with gently rocking for 3 h at 4 °C. Protein beads were collected at 14,000 rpm for 2 min, washed two times with 0.5 ml of lysis buffer and two times with 0.5 ml of kinase buffer (25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2, and 2 mM MnCl2) and subjected to the kinase assay. To measure kinase activity, the bead pellet was suspended in 40 μl of kinase buffer, and 1 μg of myelin basic protein (1 μg/ml) and 1 μl of [γ−32P]ATP (10 μCi/μl) were added. After the kinase assay was performed at 37 °C for 20 min, the reaction was terminated by the addition of 4× Laemmli sample buffer. The samples were resolved by SDS-PAGE (12% polyacrylamide), and the phosphorylated myelin basic protein was visualized by autoradiography and analyzed by an image analyzer.

Thioredoxin-reducing Activity Assay (Insulin-reducing Assay)—Cells were lysed in lysis buffer (20 mM HEPES (pH 7.9), 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 0.1% Nonidet P-40 plus protein inhibitors). Cell extracts (20 μg) were preincubated at 37 °C for 20 min with 2 μl of dithiothreitol activation buffer composed of 50 mM HEPES (pH 7.6), 1 mM EDTA, 1 mg/ml bovine serum albumin, and 2 mM dithiothreitol in a total volume of 70 μl to reduce Trx, then 40 μl of reaction mixture containing 200 μl of 1 M HEPES (pH 7.6), 40 μl of 0.2 M EDTA, 40 μl of...
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NADPH (40 mg/ml), and 500 μl of insulin (10 mg/ml) was added. The reaction began with the addition of 10 μl of rat Trx reductase (400 μg/360 μl, specific activity of 12.5 units/mg, American Diagnostics), and incubation continued for 20 min at 37 °C. The reaction was stopped by the addition of 0.5 ml 6 M guanidine-HCl and 1 mM 3-carboxy-4-nitrophenyl disulfide (Sigma). The absorbance at 412 nm was measured.

Animals, Surgical Procedures, and DNA Enzyme Administration—Rowett (nu/nu) athymic nude rats (body weight of 220–250 g, Harlan Sprague-Dawley, Indianapolis, IN) were used in studies approved by the Columbia University Institute for Animal Care and Use Committee. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (4 mg/ml and 10 μl/g of body weight). A midline cervical skin incision was made along with reflection of the muscles overlaying the trachea. Room air was provided through the endotracheal tube connected to a rodent ventilator (Harvard, South Natick, MA). Normal chest expansion was noted similar to that found in conscious rats. The heart was exposed through the left thoracotomy incision. The left auricle was slightly retracted exposing the left anterior descending (LAD) coronary artery, which was ligated with a 7-0 silk suture 1–3 mm from tip of the normally positioned left auricle. Following LAD ligation or sham procedure, rats were divided into groups that received three intracardiac injections of E4 DNA enzyme (200 μM, 50 μM, 10 μM), three intracardiac injections of E0 scrambled control, or three intracardiac injections of saline. Each group consisted of 6–8 rats. The baseline echo prior to heart attack and echo at 48 h following heart attack were measured, but all animals for histological and echo analysis were excluded if they did not survive beyond the 48-h baseline assessment.

In Situ Hybridization—VDUP1 mRNA in paraffin-embedded heart sections was detected by in situ hybridization using digoxigenin-labeled antisense probe and visualized as blue by combination of NBT/BCIP and alkaline phosphatase. The hybridization procedure follows the manual of universal RISH & AP Detection kit (catalog no. HKD30, Zymed Laboratories, CA) except for the following modifications: hybridization for 30 h at 37 °C and antibody incubation for 12 h at 37 °C. The digoxigenin end-labeled antisense probe was 5’-gagacctgtttgctgcccacttgaaagttggcggcccaca-3’, and control digoxigenin sense probe was 5’-tgtggccgccagcactttccaggggccagaagtc-3’ (synthesized by Integrated DNA Technologies).

Quantitation of Cardiomyocyte Apoptosis—For in situ detection of apoptosis at the single cell level we used the TUNEL method of DNA end-labeling mediated by deoxynucleotidyl transferase (TdT) (Roche Applied Science). Rat myocardial tissue sections were obtained from LAD-ligated rats at 2 weeks after injection of either saline or DNA enzymes, and from healthy rats as negative controls. The TUNEL stain was visualized with a substrate system in which nuclei with DNA fragmentation stained blue (BCIP/NBT substrate system, DAKO, Carpentaria, CA). To determine the proportion of blue-staining apoptotic nuclei within myocytes, tissue was counterstained with a monoclonal antibody specific for Troponin I (Sigma) and avidin-biotin complex (Vector Laboratories, Burlingame, CA). The myocytes were visualized brown following exposure in 3.3’-diaminobenzidine tetrahydrochloride solution mixture (Sigma). Tissue sections were examined microscopically at 40× magnification, and at least 100 cells were counted in a minimum of 8 high power fields.

Immunohistochemical Staining of ASK1 and Phospho-ASK1—Immunohistochemical staining was performed using the Histostain-SP kit (catalog no. 95-9551, Zymed Laboratories, CA) according to the manufacturer’s recommendations. The slides were incubated with 1 μg/ml polyclonal rabbit anti-rat ASK antibody (Santa Cruz Biotechnology) or rabbit anti-Phospho-ASK1 at Thr-845 (Cell Signaling, Beverly, MA) overnight at 4 °C. ASK1 and Phospho-ASK1 positively staining cells are visualized as brown and sections counterstained in hematoxylin solution.

Histology and Measurement of Infarct Size—Following excision at 2 weeks, left ventricles from each experimental animal were sliced at 10–15 transverse sections from apex to base. Representative sections were fixed in formalin and stained for routine histology to determine cellularity of the myocardium, expressed as cell number per high power field (600×). A Masson’s trichrome stain was performed, which labels collagen blue and myocardium red, to evaluate collagen content on a semiquantitative scale (0 to 3+), with 1+ light blue, 2+ light blue and patches of dark blue, and 3+ dark blue staining. This enabled measurement of the size of the myocardial scar using a digital image analyzer. The lengths of the infarcted surfaces, involving both epicardial and endocardial regions, were measured with a Planimeter digital image analyzer and expressed as a percentage of the total ventricular circumference. All studies were performed by a blinded pathologist. Infarct size was expressed as percentage of total left ventricular area.

Analyses of Myocardial Function—Echocardiograph studies were performed using a high frequency liner array transducer (SONOS 5500, Hewlett Packard, Andover, MA). Two-dimensional images were obtained at mid-papillary and apical levels. End-diastolic (EDV) and end-systolic (ESV) left ventricular volumes were obtained by area-length method, and percent left ventricular ejection fraction was calculated as [(EDV – ESV)/EDV] × 100.

Statistical Analysis—Data are presented as mean ± S.E. or S.D. Comparisons between groups were made by Student’s t test. Values of p < 0.05 were considered significant.

RESULTS

Myocardial VDUP1 mRNA Expression in Vivo Is Increased following Acute Ischemia—During a series of experiments employing cDNA subtractive hybridization to identify patterns of changes in gene expression between normal and ischemic rat hearts, we identified VDUP1 mRNA as being overexpressed in rat myocardium 48 h after LAD ligation. By RT-PCR, increased myocardial VDUP1 mRNA expression was shown to have 20% higher mean levels of VDUP1 mRNA in hearts from LAD-ligated rats than those from normal rats (Fig. 1a, p < 0.01). Because VDUP1 is a key mediator of physiological cell processes triggered by oxidative stress (12–18), our next series of experiments were aimed at investigating the role of VDUP1 on cardiomyocyte survival in vitro and on cardiac outcomes following oxidative stress and acute ischemia in vivo.

A Sequence-specific VDUP1 DNA Enzyme Cleaves a Synthetic VDUP1 mRNA Oligonucleotide and Reduces Cellular VDUP1 mRNA Expression—To regulate expression of VDUP1 mRNA in vitro and in vivo, we designed an mRNA-cleaving DNA enzyme containing the 10–23 motif (19, 20), which specifically targeted one sequence of VDUP1 mRNA near its translation start codon (Fig. 1b, GenBankTM accession number U30789, position 967–985). Fig. 1c shows that the VDUP1-specific DNA enzyme E4 cleaved a synthetic VDUP1 mRNA oligonucleotide in a dose- and time-dependent manner. In contrast, a control DNA enzyme E0 containing a scrambled sequence had no cleavage activity even at high concentration (5 μM) and with incubation of 2 h. To confirm the cleavage activity of VDUP1 DNA enzyme when present intracellularly, H9C2 cardiomyoblast cells were transfected with E4 or E0 DNA enzymes complexed with the transfection reagent SuperFect. The relative amount of VDUP1 mRNA in treated cells was measured by RT-PCR at various time points and normalized to GAPDH mRNA. As shown in Fig. 1D, VDUP1 mRNA levels were significantly
FIGURE 1. Up-regulation of rat myocardial VDUP-1 mRNA following myocardium infarction and in vitro cleavage of VDUP1 mRNA by VDUP1 DNA enzyme. a, results of RT-PCR, showing increased expression of VDUP-1 mRNA in rat myocardium 48 h after acute ischemia compared with normal rat myocardium (n = 6, p < 0.01). L32 ribosomal protein gene (RPL32) mRNA were used as internal control of PCR. b, Watson-Crick base-pairing of VDUP1 DNA enzyme with its RNA target site. The catalytic core of the enzyme corresponds to the selected 10–23 motif [19] and last base at 3′-end was inverted to increase stability of the DNA enzyme. The arrow indicates the site of substrate cleavage. c, in vitro cleavage of synthetic RNA by VDUP1 DNA enzyme E4 in a time- and dose-dependent manner. As a control, scrambled DNA enzyme E0 showed no cleavage activity. The concentration of RNA substrate was 0.5 μM. d, reduced VDUP1 mRNA level in H9c2 cells after transfection with VDUP1 DNA enzyme E4. Representatives of four separate experiments and data are expressed as mean ± S.E. (n = 4, p < 0.01).
reduced in E4-treated cells, to a maximum of 70% below those in E0-treated controls (p < 0.01).

VDUP1 DNA Enzyme Transfection Reduces Apoptosis of H9C2 Cells and Increases Their Survival in Response to H2O2 Stress—We next tested the ability of the VDUP1 DNA enzyme to inhibit apoptosis and promote cell survival of H9C2 cardiomyoblast cells cultured with different concentrations of H2O2. Cell lysate supernatants were used in cell death detection enzyme-linked immunosorbent assay to determine cytoplasmic mono- and oligonucleosome release. As shown in Fig. 2a, the VDUP1 DNA enzyme E4 used at 1 μM decreased apoptosis of H9C2...
cells by means of 34 and 41% in the presence of 50 and 100 μM H₂O₂ stimulation, respectively, compared with control conditions where H9C2 cells were transfected with the scrambled DNA enzyme E0 (both p < 0.05). Moreover, consistent with the observed reduction in apoptosis, H9C2 cells transfected with E4 demonstrated 113 and 149% higher mean survival rates than E0-transfected cells (Fig. 2b) on culture with 50 and 100 μM H₂O₂ (p < 0.05 and p < 0.01, respectively). The effect of VDUP1 down-regulation was dose-dependent, with an increase in DNA enzyme concentration to 5 μM resulting in 46% reduction in H9C2 apoptosis and increased survival to 201% of control following culture in 100 μM H₂O₂ for 50 min (Fig. 2c). Taken together, these results demonstrated that down-regulation of VDUP1 by the DNA enzyme E4 augmented resistance of H9C2 cells to H₂O₂-mediated stress injury and promoted their survival.

VDUP1 DNA Enzyme Transfection Reduces ASK1, but Not Thioredoxin, Activity in H9C2 Cells and Inhibits Expression of Collagen Transcript in Fibroblasts—VDUP1 augments cellular apoptosis following oxidative stress by binding to the thiol-reducing agent thioredoxin (Trx) and preventing its physical association with the apoptosis signal regulating kinase 1 (ASK1) (11), an interaction that otherwise functions to negatively regulate ASK1 activity (6). Unbound ASK1 results in sustained activation of JNK/p38 and cellular apoptosis accompanying oxidative stress (8). We measured ASK1 kinase activity in H9C2 cells transfected with VDUP1 DNA enzyme to examine whether this pathway was being affected. As shown in Fig. 2d, upper panel, immune complex kinase assay showed that VDUP1 DNA enzyme transfection resulted in 37% lower mean ASK1 activity compared with E0-transfected controls in the presence of H₂O₂ stimulation (p < 0.05). We also examined whether VDUP1 down-regulation affected the Trx-reducing activity in H9C2 cells, a possible alternative mechanism to explain the reduced level of cellular apoptosis. As shown in Fig. 2e, no differences were observed between Trx activities in H9C2 cells transfected with VDUP1 DNA enzyme or the E0-scrambled DNA enzyme in the presence or absence of H₂O₂ stimulation. These results suggest that VDUP1 down-regulation resulted in protection of H9C2 cells against apoptosis by augmenting the binding of Trx to ASK1, rather than by direct augmentation of Trx reducing activity.

Because ASK1 has been shown to exist in fibroblasts (8) and collagen gene transcription in cardiac fibroblasts is mediated by the p38 MAPK pathway (21), which is a downstream target of ASK1, we investigated whether down-regulation of VDUP1 expression in cultured rat 2 fibroblasts might affect collagen gene expression. The results of RT-PCR analysis showed that VDUP1 mRNA enzyme transfection results in significant reduction of procollagen type I α2 and type III α1 mRNA levels in fibroblasts (Fig. 2f).

Down-regulation of Myocardial VDUP1 Expression in Vivo Protects against Cardiomyocyte Apoptosis following Acute Ischemia—Next we examined the in vivo effects of VDUP1 down-regulation in the acutely ischemic myocardium. Immediately following LAD ligation, VDUP1 DNA enzyme (200 μM, 50 μM, and 10 μM) or scrambled DNA enzyme control were directly injected into the peri-infarct region of the rat left ventricular muscle. Animals receiving either 200 μM or 50 μM VDUP1 DNA enzyme demonstrated significantly greater post-infarct mortality (in each group six of 8 died within 3 days of LAD ligation) compared with those receiving the scrambled control (2 of 8), p < 0.05. In contrast, intramyocardial injection of the same dosages of VDUP1 DNA enzymes had no effect on survival of sham-operated rats. These results suggest that high doses (50–200 μM) of VDUP1 DNA enzyme are likely to inhibit certain cellular functions critical for cardiac survival and recovery after acute ischemia (also see relevant discussion). In contrast, only 1 of 7 animals receiving 10 μM VDUP1 DNA enzyme developed early post-infarct mortality. Outcome of this group was compared with controls treated with the scrambled DNA enzyme. Fig. 3a (left panel) shows that left ventricular tissue of rats injected with 10 μM E4 VDUP1 DNA enzyme at the time of acute ischemia demonstrated significantly reduced levels of VDUP1 mRNA 2 weeks later in comparison with ani-
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In animals treated with the scrambled E0 control DNA enzyme (p < 0.01). To demonstrate the down-regulation of VDUP1 mRNA in left ventricular tissue following intramyocardial injection of VDUP1 DNA enzymes, in situ hybridization was also performed with the digoxigenin-labeled oligonucleotide-DNA probe, followed by an incubation with diluted antidigoxigenin antibody solution (anti-digoxigenin Fab fragment alkaline phosphatase-conjugate) and a colorimetric detection of NBT/BCIP. In situ hybridization result showed no detectable signal for VDUP1 mRNA in VDUP1 DNA enzyme-treated tissue section. However, strong signals were observed in scrambled DNA enzyme-treated tissue section (Fig. 3a, right panel). Above results further verified reduced VDUP1 mRNA level after intramyocardial injection of 10 μM VDUP1 DNA enzyme. In parallel, left ventricular tissue of rats injected with the E4 VDUP1 DNA enzyme demonstrated a mean of 20% lower numbers of apoptotic cardiomyocytes, as determined by concomitant staining for the cardiomyocyte-specific marker troponin I and TUNEL (Fig. 3b, p < 0.05).

Down-regulation of Myocardial VDUP1 Expression in Vivo Inhibits Phosphorylation and Activity of ASK1—VDUP1 is known to regulate cellular ASK1 activity (14), which is required for tumor necrosis factor-and oxidative stress-induced sustained activations of JNK/p38 MAPK and apoptosis, thus we further examined involvement and modulation of ASK1 in the acutely ischemic myocardium injected with VDUP1 DNA enzyme. We performed immunohistochemical staining to observe the potential change in ASK1 and phosphorylated ASK1 levels. As shown in Fig. 4 (upper panel), in ischemic myocardium injected with saline or scrambled, there are ubiquitous expression of rat ASK1 in cardiomyocytes and other cardiac cells; moreover, ASK1 expression in some cardiomyocytes may be reduced by VDUP1 down-regulation. The localization of ASK1 in cardiomyocytes is completely consistent with He et al.’s (29) observation that ASK1 expression is localized in the cardiomyocyte sarcomere. More importantly, VDUP1 down-regulation also results in inhibition of phosphorylation of ASK1 at Thr-845 (Fig. 4, lower panel). Because the phospho-ASK1 level at Thr-845 is correlated with ASK1 kinase activity (30), our results indicate that ASK1 kinase activity in infarcted myocardium was reduced after transfection of VDUP1 DNA enzyme.

**Figure 4.** The expression and phosphorylation of ASK1 in infarcted left ventricular myocardium at 2 weeks post-myocardial infarction. Upper panel, immunohistochemical analysis of ASK1 expression in infarcted heart injected with saline or scrambled DNA enzyme, and inhibition of ASK1 expression in some cardiomyocytes of ischemic heart with injection of VDUP1 DNA enzyme. ASK1-positive stains are visualized brown (magnification, ×200). Lower panel, detection of phosphorylation of ASK1 at Thr-845 in ischemic heart with injection of VDUP1 DNA enzyme. Phospho-ASK1 positive stains are visualized brown (magnification, ×200).

**Figure 5.** In vivo effects of VDUP1 DNA enzyme on cardiac collagen gene expression, scar formation post-infarction. A and B, decreased expressions of myocardial pro-collagen type I α2 mRNA and type III α1 mRNA 48 h after LAD ligation and immediate intramyocardial injection of VDUP1 DNA enzyme as quantitated by RT-PCR (n = 6, p < 0.05). In C, left panel, reduced myocardial scar formation 2 weeks after LAD ligation and immediate intramyocardial injection of VDUP1 DNA enzyme, as measured by Masson’s trichrome stain (n = 6, p < 0.01). Right panel, representative trichrome-stained sections showing infarct scar area with collagen deposition (blue staining) in the VDUP1 DNA enzyme-treated group is smaller than in the scrambled DNA enzyme-treated control. Red staining shows the viable myocardium.
High levels of cellular ASK1 activity are induced by oxidative stress such as H2O2 activation (16). In the present study, we have shown that down-regulation of cellular VDUP1 mRNA expression by a DNA enzyme targeting a VDUP1-specific sequence resulted in reduced cellular apoptosis and enhanced cellular survival of H9C2 rat cardiomyoblasts in vitro and adult rat cardiomyocytes in vivo under conditions of oxidative stress, such as H2O2 activation and acute myocardial ischemia.

The mechanisms by which VDUP1 negatively regulates cell survival and proliferation are incompletely understood. Recent studies have shown that VDUP1 can induce cell cycle arrest at G0/G1 by forming a transcriptional repressor complex with various proteins, including histone deacetylase1, and can function as an inhibitor of tumor growth (17). A second mechanism by which VDUP1 modulates cellular survival involves regulatory effects on the activity of the mitogen-activated protein kinase kinase kinase (MAPKKK) apoptosis signaling kinase-1 (ASK1) (11, 14). High levels of cellular ASK1 activity are induced by various cellular stressors, including H2O2, Fas ligand, tumor necrosis factor, and anti-tumor agents, and result in activation of the JNK and p38 MAPK pathways and induction of cellular apoptosis (8, 22, 23). However, low and moderate levels of ASK1 activity appear to be important for induction and maintenance of cellular cycling and proliferation (24) and for differentiation of various cell types (25, 26), implying a need for strict regulation of cellular ASK1 activity levels. Cellular homeostasis of ASK1 activity levels is maintained by ubiquitination and degradation of ASK1, which occurs following direct binding of its N-terminal region to the reduced form of the redox-responsive protein thioredoxin (Trx) (9). Methods that enhance this interaction, such as overexpression of Trx, result in inhibition of ASK1-induced apoptosis (9). By contrast, VDUP1 overexpression inhibits the interaction between Trx and ASK1, and augments ASK1-induced cellular apoptosis (11, 14). As for the reason for high death rate in the LAD ligated rats with higher doses VDUP1 DNA enzyme, we think that certain levels of ASK1 activity might be critical to cell survival, and ASK1 activity is maintained in an exact balance. “ASK1 appears to act as a proapoptotic intermediate when cells receive strong stresses. In addition, ASK1 appears to possess various biological functions such as the control of differentiation status and the mediation of survival signals in order for cells to adapt or oppose various moderate stresses” (22, 25). It was recently reported that both proliferation and migration of vascular smooth muscle cells from ASK1 knock-out mice were significantly attenuated compared with vascular smooth muscle cells from wild-type mice (24). Although other potential reasons are likely to exist and remain to be investigated, the high death rate is most probably due to excessive inhibition of ASK1 caused by a high dose of VDUP1 DNA enzyme injection. In other massive experiments with the infarction model, even those that similarly involve the use of other types of DNA enzymes (high doses) targeting plasminogen activator inhibitor-1 and peroxiredoxins in the infarction model, we never observed a death rate so high. Because VDUP1 down-regulation by the VDUP1 DNA enzyme reduced intracellular ASK1 activity but had no effect on Trx activity, our results argue against a direct effect of VDUP1 on Trx transcription, nuclear translocation, or disulfide reducing activity (4, 27). Moreover, because treatment with high dose VDUP1 DNA enzyme resulted in increased post-infarct mortality, our results support the need to maintain a minimum level of intracellular ASK1 activity required for cellular survival and/or proliferation of cell types important in maintaining cardiac function, such as vascular smooth muscle (24).

In addition to enhanced cardiomyocyte survival, down-regulation of VDUP1 mRNA by intramyocardially administrated VDUP1 DNA enzyme was accompanied by significantly reduced myocardial collagen gene expression, collagen deposition, and scar formation in the left ventricular wall. In this regard, it is of note that VDUP1 overexpression has been reported to enhance transcription of collagen genes and to result in increased collagen accumulation in kidney mesangial cells and has been suggested as a possible molecular mediator/marker for fibrosis in diabetic nephropathy (28). Because transcription of collagen genes in cardiac fibroblasts is induced following activation of the p38 MAPK pathway (21), a downstream target of ASK1, our findings indicate that VDUP1 is a major mediator of stress-related activation and/or transcription of collagen genes in cardiac fibroblasts. Thus, methods that reduce myocardial VDUP1 expression, such as administration of sequence-specific catalytic DNA enzymes, may result in recovery of cardiac function after acute myocardial ischemia and prevention of post-infarct cardiac remodeling through effects on ASK1 activation, enhanced cardiomyocyte survival, and reduced collagen deposition.

**TABLE ONE**

| Echocardiographic parameters of left ventricular function | E0 | E4 |
|---------------------------------------------------------|----|----|
| LVEDD baseline (cm)*                                     | 0.60 ± 0.02 | 0.63 ± 0.01 |
| LVEDD post treatment (cm)                                | 0.67 ± 0.03 | 0.68 ± 0.06 |
| Change in LVEDD (cm)                                     | 0.076 ± 0.03 | 0.049 ± 0.06 |
| LVESD baseline (cm)                                     | 0.49 ± 0.03 | 0.57 ± 0.02 |
| LVESD post treatment (cm)                                | 0.52 ± 0.03 | 0.56 ± 0.03 |
| Change in LVESD (cm)                                     | 0.03 ± 0.05 | −0.01 ± 0.05 |
| EF baseline (%)                                          | 43.7 ± 5.2 | 29.7 ± 7.3 |
| EF post treatment (%)                                    | 50.1 ± 6.1 | 52.3 ± 4.3* |
| Change in EF (%)                                         | 6.4 ± 3.0 | 22.6 ± 3.2* |

*p = 0.019 versus baseline.

*p = 0.02 versus E0.

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