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Journal of Cellular Biochemistry 112:1337–1347 (2011)

Journal of Cellular Biochemistry

Synthetic Small Interfering RNA Down-Regulates Caspase-3 and Affects Apoptosis, IL-1β, and Viability of Porcine Proximal Tubular Cells

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ABSTRACT

Proximal tubular cells are most vulnerable to ischemia reperfusion injury (IRI) in renal transplantation. Caspase-3 can be up-regulated by IRI due to a variety of pathogenic processes such as oxidative damage. This study utilized synthetic small interfering RNA (siRNA) to posttranscriptionally silence target gene, caspase-3, may represent a feasible approach to produce transient effects, but avoid side actions caused by viral vectors. The porcine proximal tubular cells (LLC-PK1), with or without the stimulation of hydrogen peroxide (H_2O_2, an oxidizer), were transfected with synthetic caspase-3 siRNA using a cationic lipid-based transfection regent. The expression of caspase-3 at mRNA and protein level was assessed at different times posttransfection and its downstream biological events were also monitored. The caspase-3 mRNA was posttranscriptionally silenced by its siRNA up to 50% after 24 h. The active caspase-3 protein was increased by transfection reagent alone and H_2O_2 in a dose- and time-dependent manner. Both the precursor and active protein of caspase-3 were decreased by siRNA after 48 h and maintained up to 96 h at least, with a consistent change in its activity. Consequently, apoptotic cells and active IL-1β protein expression was reduced by caspase-3 siRNA; cell viability, especially with H_2O_2 treatment, was also improved. Taken together, caspase-3 and apoptosis are sensitive markers for cellular injury; using synthetic siRNA silencing caspase-3 may provide not only a valid approach for underlying mechanisms of diseases, but also a potential therapeutic intervention for a wide range of acute clinical disorders including IRI in renal transplantation. J. Cell. Biochem. 112: 1337–1347, 2011.

KEY WORDS: ISCHEMIA REPERFUSION INJURY; CASPASE-3; APOPTOSIS; INFLAMMATION; SMALL INTERFERING RNA AND PROXIMAL TUBULE CELLS

Ischemia reperfusion injury (IRI) is the most common cause of acute renal failure associated with renal transplantation. IRI has detrimental effects on long-term allograft function, of which tubular epithelial cells, particularly in the proximal tubules, are the most vulnerable. The causes of renal IRI are complex, and linked to oxidative stress, apoptosis, and inflammation [Chien et al., 2005; Yang et al., 2005, 2006]. Apoptosis is a cascade of events, which includes the up-regulation of caspases (cysteine proteases). Caspase-3, an effector caspase, can be up-regulated by IRI [Daemen et al., 1999], providing a converging point for a number of apoptotic pathways. Caspase-3 also plays a crucial role in inflammation and in T-cell-mediated immunological responses [Daemen et al., 1999].

Pan caspase inhibitors and specific caspase-1 and caspase-3 inhibitors have been shown to manipulate IRI-induced inflammation, apoptosis, and necrosis in a number of studies [Daemen et al., 1999; Chatterjee et al., 2004]. However, the potential toxicities of these inhibitors have thus far limited their clinical use in patients. The development of specific caspase-3 inhibitors has also been hampered by the high degree of homology shared by different caspases in the family [Kerr et al., 2004]. Nevertheless, caspase-3 knockout (Casp3−/−) mice have been shown to be protected from developing diabetes due to their resistance to apoptosis and the absence of lymphocyte infiltration in pancreatic islets [Liadis et al., 2005]. Transgenic mice overexpressing bcl-2 are similarly protected from IRI in the renal tubular epithelia, and from subsequent

All the authors declared no conflict of interest.

Grant sponsor: Research & Development Directorate of the University Hospitals of Leicester NHS Trust; Grant sponsor: Royal College of Surgeons of England.

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Received 26 November 2010; Accepted 20 January 2011 • DOI 10.1002/jcb.23050 • © 2011 Wiley-Liss, Inc.
Published online 14 February 2011 in Wiley Online Library (wileyonlinelibrary.com).

interstitial injury due to inhibition of tubular apoptosis [Suzuki et al., 2008]. Therefore, targeted and specific inhibition of caspase-3 could be a promising strategy for the protection of tubular cells against inflammation and/or apoptosis-mediated injury.

The emergence of RNA interference (RNAi), one type of posttranscriptional gene silencing, has provided one such strategy for the targeted inhibition of caspase-3 [Racz and Hamar, 2008]. RNAi using a 21-nucleotide small interfering RNA (siRNA) is an approach, in which the introduction of a double-stranded RNA elicits the selective degradation of homologous mRNA transcripts. The exogenous administration of siRNA is capable of blocking gene expression in mammalian cells [Elbashir et al., 2001], and has proved to be a very potent and specific method of gene silencing (specific to one nucleotide mismatch) [Celotto and Graveley, 2002; Grunweller et al., 2003]. The transfection efficacy of siRNA in vitro is high with consistent expression, and remains active over multiple cell cycles [Grishok et al., 2000; Hill et al., 2003]. Administration of siRNA in vivo is relatively safe and can simultaneously target multiple genes [Yang et al., 2002; Contreras et al., 2004; Zheng et al., 2006].

Delivery of caspase-8 and caspase-3 siRNAs has been shown to successfully decrease mice liver IRI [Contreras et al., 2004]. Treatment with Fas siRNA protects mice against renal IRI [Hamar et al., 2004]; and rapid delivery of p53 siRNA to proximal tubule cells leads to the attenuation of apoptotic signaling, yielding therapeutic benefit for ischemic kidney injury in mice [Molitoris et al., 2009]. The siRNAs of complement 3, caspase-3, caspase-8, and complement 5a receptors prevent mice renal IRI [Zhang et al., 2006; Zheng et al., 2006, 2008]. In these studies, siRNAs were systemically delivered in either their naked synthetic version [Contreras et al., 2004; Hamar et al., 2004; Molitoris et al., 2009] or by non-viral plasmid vectors [Zhang et al., 2006; Zheng et al., 2006, 2008] by hydrodynamic or a simple bolus intravenous injection. Delivery of siRNA in vectors can lead to a relatively stable and long-term silencing, but it takes time to construct vectors, and the side effects of vectors such as inflammation and immune modification, especially using viral vectors, are inevitable. More recently, University of Wisconsin (UW) solution containing siRNAs targeting TNF-α, complement 3, and Fas genes knocked down the expression of targeted genes at mRNA and protein levels, improved histology, and retained strong beating up in mice heart grafts preserved at 4°C for 48 h and subsequently transplanted into syngeneic recipients for more than 100 days. Therefore, incorporation of siRNA into organ storage solution is a feasible approach to attenuate heart IRI, protect cardiac function and prolong graft survival [Zheng et al., 2009].

So far, most studies using synthetic siRNA have been carried out in murine models. These models yield important results and are cost effective, but may fail to mirror the human diseases faithfully and may miss out findings that can emerge only in longitudinal studies. Thus, large animal models of human disease complement murine studies because they have greater similarity with humans, particularly with respect to porcine models which in common with humans have a diverse genetic backgrounds [Casal and Haskins, 2006; Blagbrough and Zara, 2009; Hosgood et al., 2010]. siRNA-mediated suppression of anti-apoptotic RNA-stabilizing protein HuR increases apoptosis during energy depletion in porcine proximal tubular cells (LLC-PK1) [Ayupova et al., 2009]. However, the effect of synthetic caspase-3 siRNA on LLC-PK1 cells has not been well defined. In this study, we investigated the effect of down-regulating caspase-3 gene expression by synthetic siRNA in LLC-PK1 cells with or without the concomitant hydrogen peroxide (H_2O_2, an oxidizer) treatment. The level of caspse-3 mRNA, protein, and activity was monitored; and in particular its downstream biological events such as apoptosis, IL-1β maturation, and cell viability were evaluated.

MATERIALS AND METHODS

CELL CULTURE

The LLC-PK1 cell line was a gift from Dr. Alan Bevington, University of Leicester [Burton et al., 1994]. LLC-PK1 cells were cultured in DMEM F-12 medium (Invitrogen, Paisley, UK) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (Sigma, Dorset, UK), at 37°C under a humidified atmosphere of 95% air/5% CO_2.

Three pairs of double-stranded caspase-3 siRNA (c3siRNA), targeting porcine caspase-3 mRNA (NCBI CoreNucleotide Accession No. AB029345), were designed and constructed by Applied Biosystems/Ambion (Warrington, UK). These were designated: c3siRNA73, sense, 5′-CCUCCGUGGAUUCAAAAUCtt-3′, and antisense, 5′-GAUUUUGAAUCCACGGAGGtt-3′; c3siRNA74, sense, 5′-GGGAGACCUUCACAAACUUtt-3′, and antisense, 5′-AAGUUUGUGAAGGUCUCCCtg-3′; and c3siRNA75, sense, 5′-CCUGUUGAUCUGAAAAAAUtt-3′, and antisense, 5′-AUUUUUUCAGAUCAACAGGtc-3′. The effective dose and time responses of these sequences were determined and compared to control cells (no treatment), or to cells treated with INTERFERin™ alone, a new generation of cationic lipid-based siRNA transfection reagent (TR, Polyplus Transfection, France).

Dose and time response of H_2O_2 (Sigma) on caspase-3 activity and apoptosis were examined over a 25–400 μM range for 6–24 h incubation. Cells were seeded in 12-well plates at 2×10^5 cells per well and reached about 50–70% confluence a day before transfection. Cells were incubated with TR and siRNA for 6 h, then changed into fresh medium, 100 μM H_2O_2 was added at the same time and further incubated for 24 h. The cells were then changed into fresh medium and cultured up to144 h. After treatment, attached cells were harvested by scraping, unless otherwise specified, mixed with the ''floating'' cells recovered from the media, and used for further analyses. Individual experiments were repeated at least three times; three wells per group were used each time.

REAL-TIME QUANTITATIVE RT-PCR (qPCR)

To detect caspase-3 mRNA, a set of qPCR were performed using the primers and probes of caspase-3 and housekeeping gene β-actin, designed from the published GenBank database (http://www.ncbi.nlm.nih.gov/) using the Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and labeled by 6-carboxy-fluorescein (FAM) as the following: caspase-3 (Accession No. AB029345), forward primer, 5′-TCTAAGCCATGGTGAAGAAGGAAAAA-3′, probe, 5′-CCCCTCTGAAGAAACT-3′, reverse primer, 5′-GGGTTTGCCAGTTAGAGTTCTACAG-3′; β-actin, forward primer,

5′-CCGAGGCGCTCTTCCA-3′, probe, 5′-CCCTCCTTCCTGGGCATG-3′, reverse primer, 5′-GTGGATGCCGCAGGATTC-3′.

LLC-PK1 cells, transfected with c3siRNA73, c3siRNA74 and c3siRNA75 for 6 h and cultured with fresh medium for a further 24 h, were collected by adding lysis solution with DNase inhibitor from a "TaqMan Gene Expression Cells to CT Kit" (Ambion). The lysis reaction was mixed and incubated for 10 min at room temperature; then "Stop Solution" was added and incubated for 2 min. For cDNA synthesis using reverse transcription (RT), 10 μl lysate from each sample was gently mixed with 40 μl RT Master Mix containing 25 μl 2× Buffer, 2.5 μl 20× Enzyme Mix, and 12.5 μl Nuclease-free Water and incubated for 60 min at 42 and 95°C for 5 min. Ten microliters cDNA from RT reaction was added to 40 μl PCR cocktail containing 25 μl 2× Master Mix, 2.5 μl 20× Primers and Probe, and 12.5 μl Nuclease-free Water and amplified at 95°C for 10 min followed by 50 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s using an Mx4000™ Multiplex qPCR System (Stratagene, Foster City, UK). Additional reactions were performed on known dilutions of porcine cDNA to construct a standard curve and validate amplification efficiencies. The accuracy and specificity of qPCR were verified by the absence of signals in non-template control samples before 40 cycles. Caspase-3 mRNA expression was calculated against β-actin using threshold cycle (C_t) values and expressed as a percentage of control.

MEASUREMENT OF CASPASE-3 AND IL-1 β PROTEINS

Cells were scraped off, lysed in Tris/Acete buffer, homogenated, and centrifuged at 12,000*g* 4°C for 10 min. Twenty micrograms of supernatant protein was separated on a 15% (w/v) acrylamide gel and electro-blotted onto Hybond-C membrane (Amersham Life Science, Buckinghamshire, UK). The membrane was blocked with 5% (w/v) milk and probed by polyclonal anti-full-length caspase-3 (1:1,000 dilution, Santa Cruz Biochemicals, CA), IL-1β (1:1,000 dilution, NIBSC, Potters Bar, UK), and monoclonal anti-β-actin antibodies (1:10,000 dilution, Insight Biotechnology, Middlesex, UK) separately for 1 h at room temperature. The antibody binding was revealed by a peroxidase-conjugated secondary antibody at 1:10 dilution from a DAKO ChemMate EnVision™ Detection Kit (DAKO, Glostrup, Denmark) using an ECL detection system (Amersham Life Science). Developed films were semi-quantitatively analyzed by volume density using a Bio-Rad GS-800 densitometer (Bio-Rad Laboratories Ltd, Hertfordshire, UK). The optical volume density of detected protein was corrected for loading, using β-actin [Yang et al., 2001, 2003].

MEASUREMENT OF CASPASE-3 ACTIVITY

Caspase-3 activity was detected by a modified Fluorometric CaspACE™ Assay System (Promega, Southampton, UK) [Yang et al., 2004]. One hundred micrograms supernatant protein, prepared in the same way as for Western blotting was used for analysis. The assay is based on the ability of caspase-3 to cleave the fluorogenic substrate Ac-DEVD-7-amino-4-methyl coumarin (AMC). The specificity of the assay was determined using the caspase-3 inhibitor Ac-DEVD-CHO. The fluorescence of this reaction was monitored at 360 nm excitation and 460 nm emission using an Mx4000™ Multiplex qPCR System. Fluorescence intensity was calibrated against standard concentrations of AMC and caspase-3 activity was expressed as pmol AMC liberated/min/μg protein at 30°C.

IN SITU END-LABELING (ISEL) APOPTOTIC CELLS

Cells were grown on glass coverslips and fixed in 1% (w/v) paraformadehyde, postfixed in ethanol/acetic acid (1:1) and used to label the fragmented DNA in situ using an ApopTag™ peroxidase kit (Appligene Oncor, Illkirch, France) [Yang et al., 2004]. Briefly, sections were incubated with digoxigenin–deoxyuridine and terminal deoxynucleotidyl transferase (TdT) at 37°C for 60 min and transferred to "stop" buffer for 30 min. After adding anti-digoxigenin–peroxidase complex and following 30 min of incubation, the sections were developed with 3′-amino-9-ethylcarbazole substrate to obtain dark red positive staining. Negative control sections were incubated with the omission of TdT enzyme. For each sample, apoptotic cells were examined and counted at 400× magnification in up to 20 fields.

DETECTION OF APOPTOSIS AND NECROSIS BY FLOW CYTOMETRY

Apoptosis was assessed in the combination of "floating" cells and trypsinized adhesion cells by fluorescein-labeled Annexin-V (AV) binding to exposed phosphatidylserine on cell surface and necrosis by nuclear staining with propidium iodide (PI) using an Annexin-V-FLUOS staining kit (Roche Diagnostics GmbH, Mannheim, Germany) [Yang et al., 2004]. Cells were acquired using Cell Quest™ (BDB Oxford England) software on a Becton Dickinson (BDB Oxford England), FACSCalibur flow cytometer equipped with a 488 nm Argon Laser and 530/30 nm bandpass filter for fluorescein (AV) detection and 585/42 nm bandpass filter for PI detection. Ten thousand gated events were acquired, which allowed discrimination of cells from debris. Quadrant markers were used to discriminate

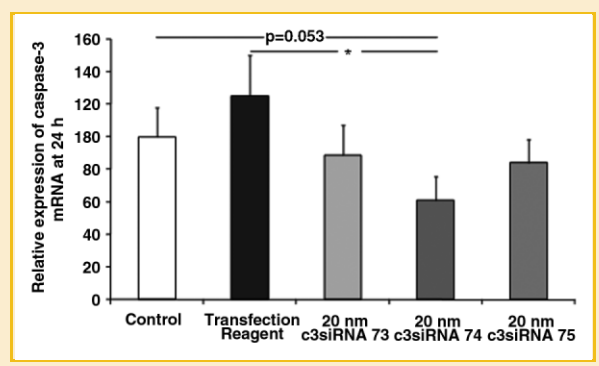

Fig. 1. Caspase-3 mRNA was detected by qPCR. In the cells transfected with c3siRNA74 at 20 nM for 24 h, the expression of caspase-3 mRNA was significantly reduced by up to 50% compared to the cells treated by TR alone; and marginally reduced in comparison to the control cells. All data are expressed as the percentage of control (mean ± SEM), n = 5. siRNA, small interfering RNA; TR, transfection reagent; $^*P < 0.05$.

three cell populations: AV−/PI− (living cells), AV+/PI− (apoptosis), and AV±/PI+ (necrosis); results are expressed as a percentage of gated events.

CELL SURVIVAL

Both "floating" cells and trypsinized adhesion cells were stained with trypan blue (Sigma) and counted on a hemocytometer. The majority of attached cells excluded trypan blue appearing "phase bright" or colorless, thus designated as living cells; while most floating cells took up the stain and were considered to be dying or dead. Cell survival is expressed as mean percentage of living cells compared to the total number of counted cells for each experimental condition.

STATISTICAL ANALYSIS

Results are expressed as mean ± standard error of the mean (SEM). Statistical differences in two or multiple groups were analyzed by unpaired *t*-test or one-way analysis of variance (ANOVA) with a Tukey–Kramer test, as the data were normally distributed, using GraphPad InStat version 3 (GraphPad Software, Inc., San Diego). *P* values <0.05 were considered to be statistically significant.

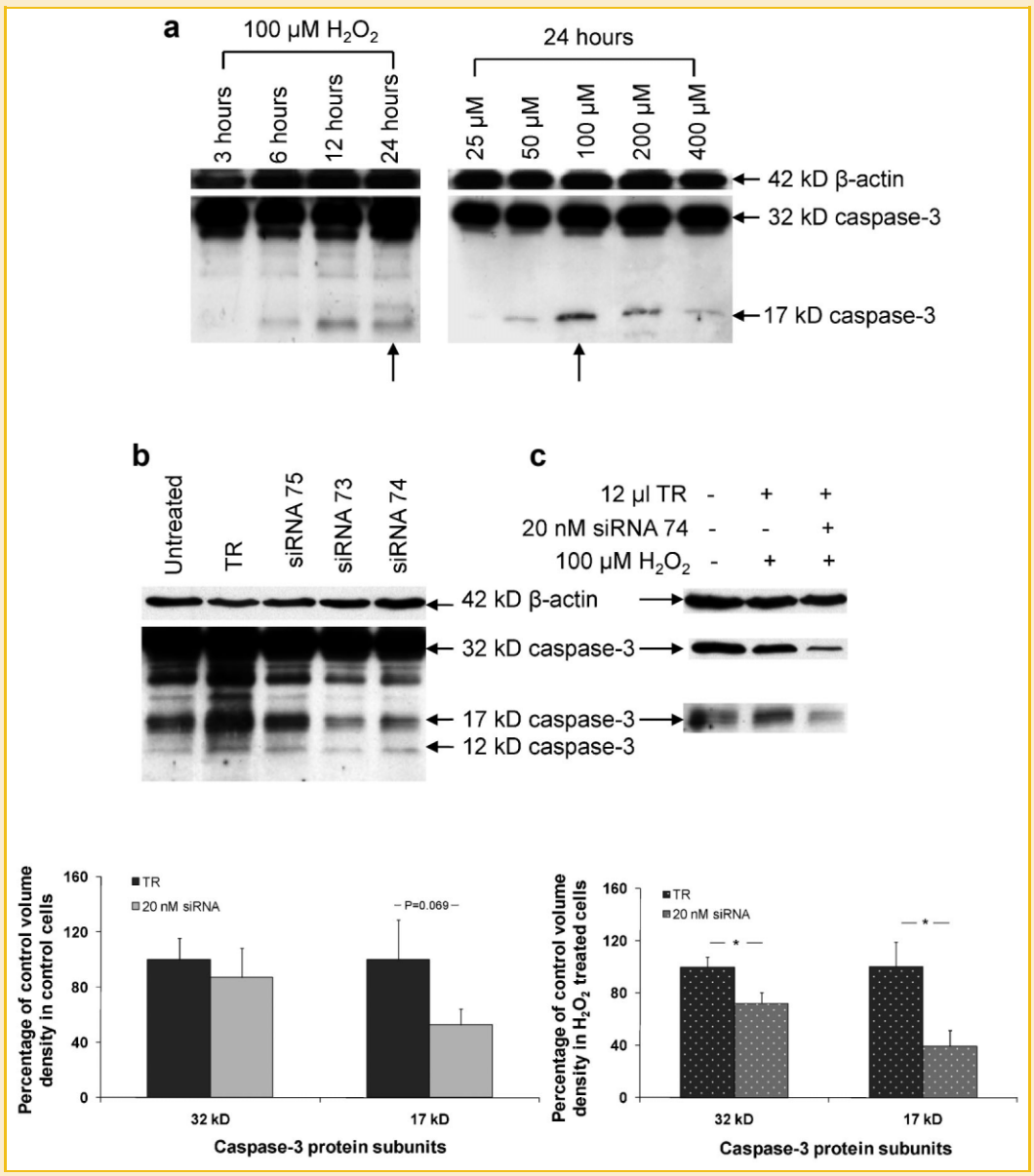

Fig. 2. Expression of caspase-3 protein was measured by Western blotting. The 32 kDa precursor and 17 kDa active subunit of caspase-3 were predominantly revealed. The active caspase-3 was increased by H_2O_2 in a time- and dose-dependent manner, with the maximum effect at 100 μM, 24 h (A). The c3siRNA74 sequence marginally decreased active caspase-3 in control cells compared to TR alone, but did not change its precursor (B). In H_2O_2 treated cells, both caspase-3 active subunit and precursor were decrease by 20 nM c3siRNA74 at 96 h (C). Data are expressed as the percentage of control volume density (mean ± SEM), n = 6. siRNA, small interfering RNA; TR, transfection reagent; $^*P < 0.05$.

RESULTS

CASPASE-3 siRNA DOWN-REGULATED ITS mRNA EXPRESSION

In the cells transfected with c3siRNA74, the expression of caspase-3 mRNA was significantly reduced by more than 50% compared to the cells treated by TR alone ($P < 0.05$); when compared to the control cells, it was only marginally reduced ($P = 0.053$, Fig. 1). The sequences of c3siRNA73 and c3siRNA75 did not cause any statistical differences. In this case, c3siRNA73 and c3siRNA75 were used for not only selecting effective sequences, but also acting as a relative "negative" control.

CASPASE-3 siRNA REDUCED ITS PROTEIN EXPRESSION

The expression of caspase-3 protein was detected by Western blotting. H_2O_2 increased 17 kDa active caspase-3 in a dose- and time-dependant manner in LLC-PK1 cells, with a maximized effect at 100 μM at 24 h incubation (Fig. 2A). All three sequences of caspase-3 siRNA at 20 nM caused a noticeable reduction in both active and precursor caspase-3 in comparison to cells treated with TR alone for 96 h. The c3siRNA74 sequence was consistently found to be the most effective in silencing caspase-3 protein. The c3siRNA74 sequence did not significantly change caspase-3 precursor levels, but marginally decreased the 17 kDa active caspase-3 in the control cells compared to the TR treated cells ($P = 0.069$, Fig. 2B). Furthermore, treatment of cells with TR alone increased the level of active caspase-3 compared to the H_2O_2 treated cells at 96 h; the addition of c3siRNA74 significantly decreased the level of active caspase-3 and its precursor (both $P < 0.05$, Fig. 2C). The c3siRNA73 and c3siRNA75 sequences did not significantly affect caspase-3 proteins (data not shown).

CASPASE-3 siRNA DECREASED ITS ACTIVITY

The activity of caspase-3 in LLC-PK1 cells with or without H_2O_2 treatment was analyzed by enzyme cleavage assay. TR alone caused a significant increase in caspase-3 activity compared with the control cells ($140.2 \pm 3.1\%$ vs. $100.0 \pm 12.6\%$, $P < 0.05$, Fig. 3A). In cells transfected with 2 and 20 nM c3siRNA74 for 72 h, caspase-3 activity was significantly decreased compared with the cells treated by TR alone ($113.6 \pm 13.3\%$, $P < 0.05$; 49.2 ± 10.1, $P < 0.01$, Fig. 3A). However, when compared with the control cells, caspase-3 activity was only significantly reduced by c3siRNA74 at a higher concentration ($P < 0.05$, Fig. 3A). In addition, in the cells transfected with c3siRNA74 at 2 and 20 nM for 6 h, then concomitantly treated by H_2O_2 for 24 h, and further cultured by up to a total of 72 h, caspase-3 activity was significantly reduced in comparison to the cells treated by TR ($76.1 \pm 25.4\%$ and $86.3 \pm 35.6\%$ vs. $175.5 \pm 16.4\%$, $P < 0.01$ or 0.05, Fig. 3B), while additional TR marginally increased the level of caspase-3 activity compared to the cells treated by H_2O_2 ($P = 0.066$).

CASPASE-3 siRNA DIMINISHED APOPTOSIS, A DOWNSTREAM EVENT OF CASPASE-3

LLC-PK1 cells grown on glass coverslips were examined for apoptosis by ISEL fragmented DNAs. The number of apoptotic cells was affected by the density of initially seeded cells and the period of observation. The denser the cells and the longer the culture, the higher the fraction of apoptotic cells. Apoptotic LLC-PK1 cells (per high power field) were significantly increased by TR treatment (2.6 ± 0.3) compared with the control cells (0.4 ± 0.1, $P < 0.01$). However, the increase in the fraction of apoptotic cells was significantly reduced by c3siRNA74 at 2 and 20 nM (0.5 ± 0.3; 1.3 ± 0.6, both $P < 0.01$, Fig. 4A). In addition, the fraction of apoptotic LLC-PK1 cells was greatly increased by H_2O_2 treatment (11.8 ± 2.0) and further increased by TR (15.6 ± 2.0). Transfection of c3siRNA74 at 2 nM (3.0 ± 0.9) and 20 nM (4.8 ± 3.9) significantly decreased the fraction of apoptotic LLC-PK1 cells after 48 h (Fig. 4B).

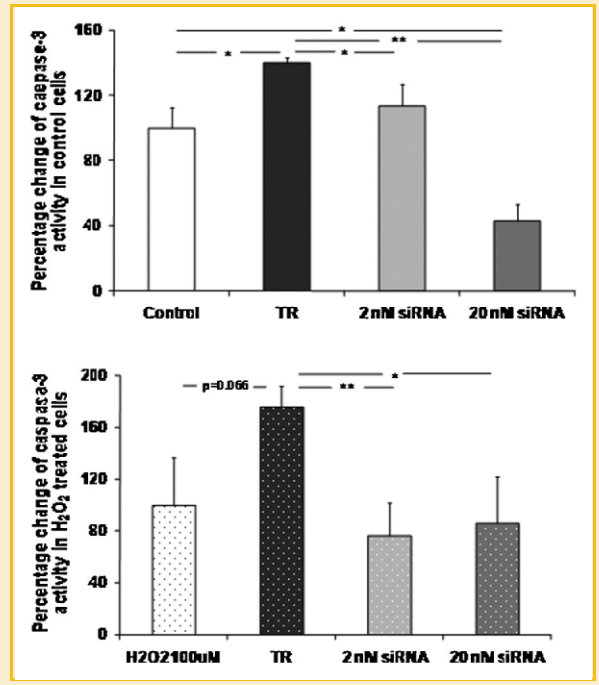

Fig. 3. Caspase-3 activity was detected by enzyme cleavage assay. In LLC-PK1 cells, caspase-3 activity was increased by TR alone and decreased by 2 and 20 nM c3siRNA74 at 3 days (A). In H_2O_2 treated cells, the increased caspase-3 activity by TR alone was also reduced by c3siRNA74 at both 2 and 20 nM concentrations (B). All data are expressed as the percentage of control (mean ± SEM), n = 5. siRNA, small interfering RNA; TR, transfection reagent; $^{*}P < 0.05$; $^{**}P < 0.01$.

CASPASE-3 siRNA INHIBITED IL-1 β MATURATION, ANOTHER DOWNSTREAM EVENT OF CASPASE-3

The expression of IL-1β protein was detected by Western blotting. The 35 kDa precursor and 17 kDa active IL-1β protein were revealed (Fig. 5). There was no significant change in IL-1β precursor between groups. TR appeared to increase active IL-1β protein, but it did not reach statistical significance when compared to the control cells ($154.2 \pm 29.3\%$ vs. $100.0 \pm 32.9\%$, Fig. 5A) or H_2O_2 treated LLC-PK1 cells ($145.7 \pm 32.5\%$ vs. $100.0 \pm 19.1\%$, Fig. 5B). The expression of active IL-1β was significantly decreased to nearly half in the 2 nM c3siRNA74 treated cells ($87.5 \pm 13.6\%$, $P < 0.05$, Fig. 5A) compared to TR treated control cells, and by two-thirds in the 20 nM

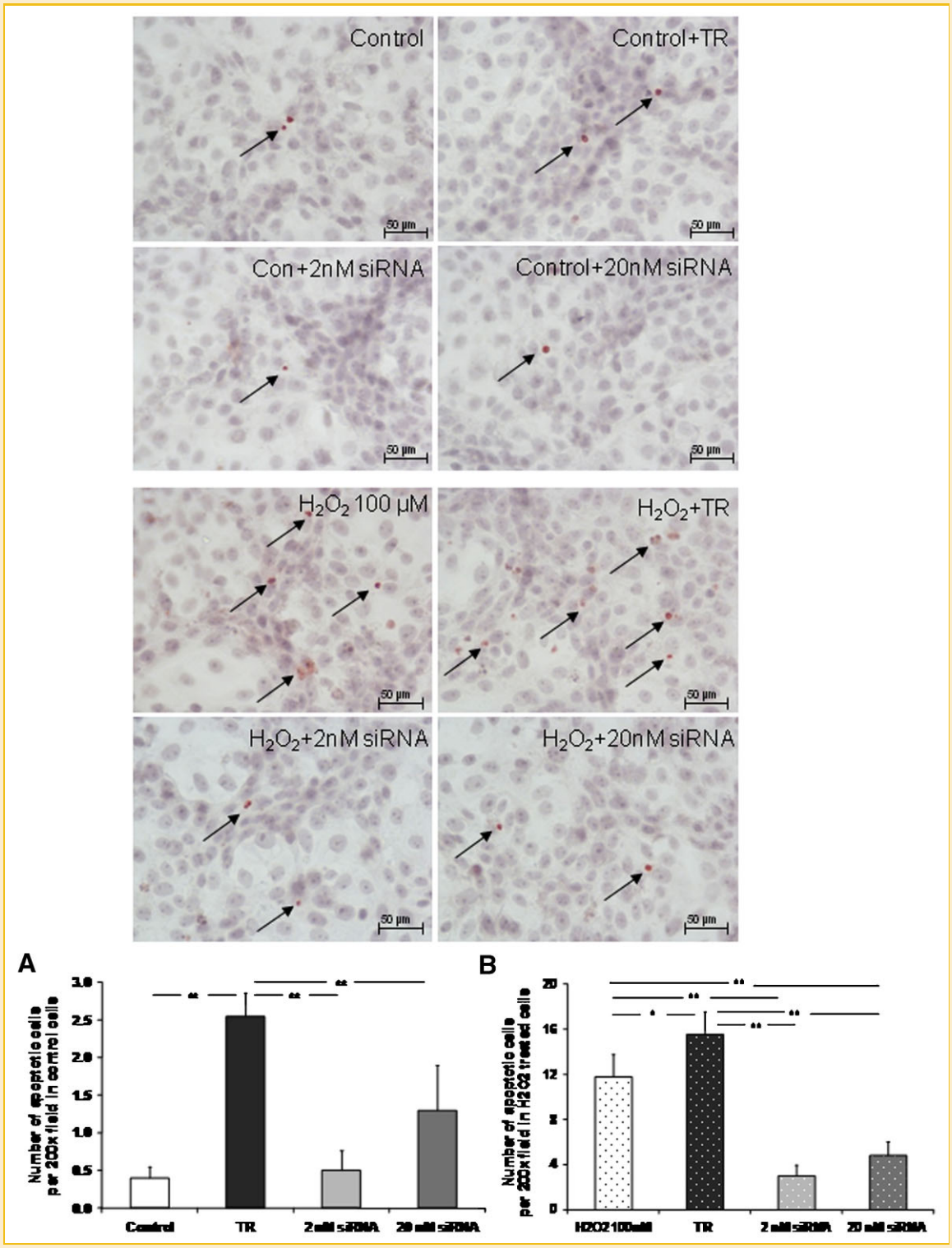

Fig. 4. The change of apoptosis detected by in situ end labeling of fragmented DNA. The number of apoptotic cells was increased by TR and reduced by siRNA at both 2 and 20 nM concentrations in control LLC-PK1 cells (A). The number of apoptotic cells were greatly enhanced by H_2O_2, further increased by TR, but decreased by 2 and 20 nM c3siRNA74 at 3 days (B). Data are expressed as mean number in the high power field of each group (mean ± SEM), n = 8. siRNA, small interfering RNA; TR, transfection reagent; $^{*}P < 0.05$; $^{**}P < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

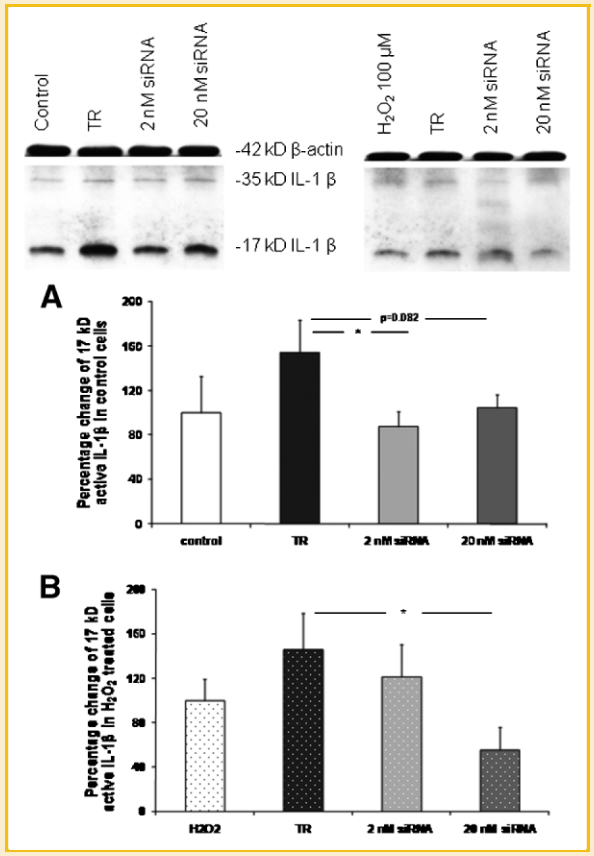

Fig. 5. Expression of IL-1β protein detected by Western blots in LLC-PK1 cells. The 35 and 17 kDa bands, representing IL-1β precursor and active subunit, were revealed. The expression of active IL-1β was significantly decreased by about 50% in 2 nM c3siRNA74 treated cells compared to TR treated control cells (A), and by two-thirds in 20 nM c3siRNA74 compared to TR and H_2O_2 treated cells (B). Data are expressed as mean percentage volume density compare to the mean control value (mean ± SEM), n = 4. siRNA, small interfering RNA; TR, transfection reagent; $^*P < 0.05$.

c3siRNA74 treated cells (55.7 ± 20.4%, $P < 0.05$, Fig. 5B) compared to TR and H_2O_2 treated cells.

LESS APOPTOSIS CONFIRMED BY FLOW CYTOMETRY

Using the Annexin-V fluorescein staining system on combined floating and attached LLC-PK1 cells, three cell clusters representing viable (AV−/PI−), apoptotic (AV+/PI−), and nectroic (AV±/PI+) cells (Fig. 6A–C) were clearly identified. The cells within each cluster were then counted and represented as a percentage of the gated cells. H_2O_2 significantly increased the number of apoptotic cells by 2.9-fold compared to the control. This was significantly decreased by 56% in the presence of 20 nM c3siRNA74 (Fig. 6D). However, there was no significant statistical difference between groups in any other of the different cell populations (Fig. 6E,F).

CASPASE-3 siRNA AMELIORATED CELL MORPHOLOGY AND VIABILITY

The control LLC-PK1 cells did not change noticeably by TR, 2 and 20 nM c3siRNA74 treatment for 24 h. The spindle shape change, cellular shrinkage, and chromatin condensation were caused by H_2O_2 at 24 h and enhanced further by the additional presence of TR. However, these changes were alleviated by c3siRNA74 at both 2 and 20 nM (Fig. 7A). These phenomena were less clearly visible following replacement of the culture medium and when the culture was prolonged to 144 h, the cells acquired a more squashed appearance with the formation of domes (Fig. 7B). The domes are fluid-filled localized raisings of the cell monolayer and characteristic structures of epithelial cell cultures [Rotoli et al. 2002].

Cell viability was assessed by counting trypan blue excluding cells using a hemocytometer. There was no significant difference between the cell groups without H_2O_2 treatment. After incubation with H_2O_2 for 24 h, cell viability was reduced compared to the control cells (81.0 ± 0.9% vs. 92.5 ± 1.2%, $P < 0.01$, Fig. 7C). However, the addition of either 2 or 20 nM c3siRNA74 increased the level of cell viability to 84.7 ± 0.4% and 86.6 ± 1.8% compared to H_2O_2 or TR alone treated cells (78.8 ± 1.1%, all $P < 0.05$, Fig. 7C).

DISCUSSION

The data presented in this study indicate that caspase-3 does play a crucial role in transplant-related proximal tubular injury; synthetic siRNA can reduce the expression of caspase-3 mRNA and protein. This targeted inhibition of caspase-3 expression, can provide a therapeutic benefit in biological disorders arising from the up-regulated expression of caspase-3 in conditions such as IRI in renal transplantation. This conclusion is based on the experimental evidence that siRNA-mediated silencing of caspase-3 could be efficiently achieved within 24 h of addition of caspase-3 siRNA to target cells in culture. The efficiency of this inhibition could be demonstrated by the decreased presence of active caspase-3 protein within 48 h of siRNA administration and maintained for at least 96 h. Furthermore, the data obtained shows the effect of siRNA-mediated inhibition of caspase-3 on downstream events such as apoptosis and IL-1β maturation in the H_2O_2 treated cells.

No single genetic modification is likely to be optimal for all gene therapeutic applications. "Perfect" regulators of gene expression will be administered by a non-invasive delivery route, will target specifically to the desired cells within specific tissues, and will cause the overexpression or silencing of the target gene/s [Kay et al., 2001; Fenjves et al., 2008]. There are two fundamentally different gene delivery systems, viral and non-viral. Non-viral gene therapy such as cationic lipid-based transfection of synthetic siRNA offers several advantages over approaches that rely on viral vectors. These include the comparative simplicity and reduced costs, lower immunogenicity and toxicity; as well as the usually transient nature of alterations in target gene expression following the use of non-viral vectors. This latter property may be highly desirable in certain clinical settings, for instance in acute ischemic reperfusion injury. However, synthetic siRNA is more susceptible to degradation and therefore the possibility of reduced efficacy [Daemen et al., 1999; Chatterjee et al., 2004; Yang et al., 2005, 2006].

It has been widely demonstrated that caspase-3 plays central roles in the execution of apoptosis and the maturation of inflammatory

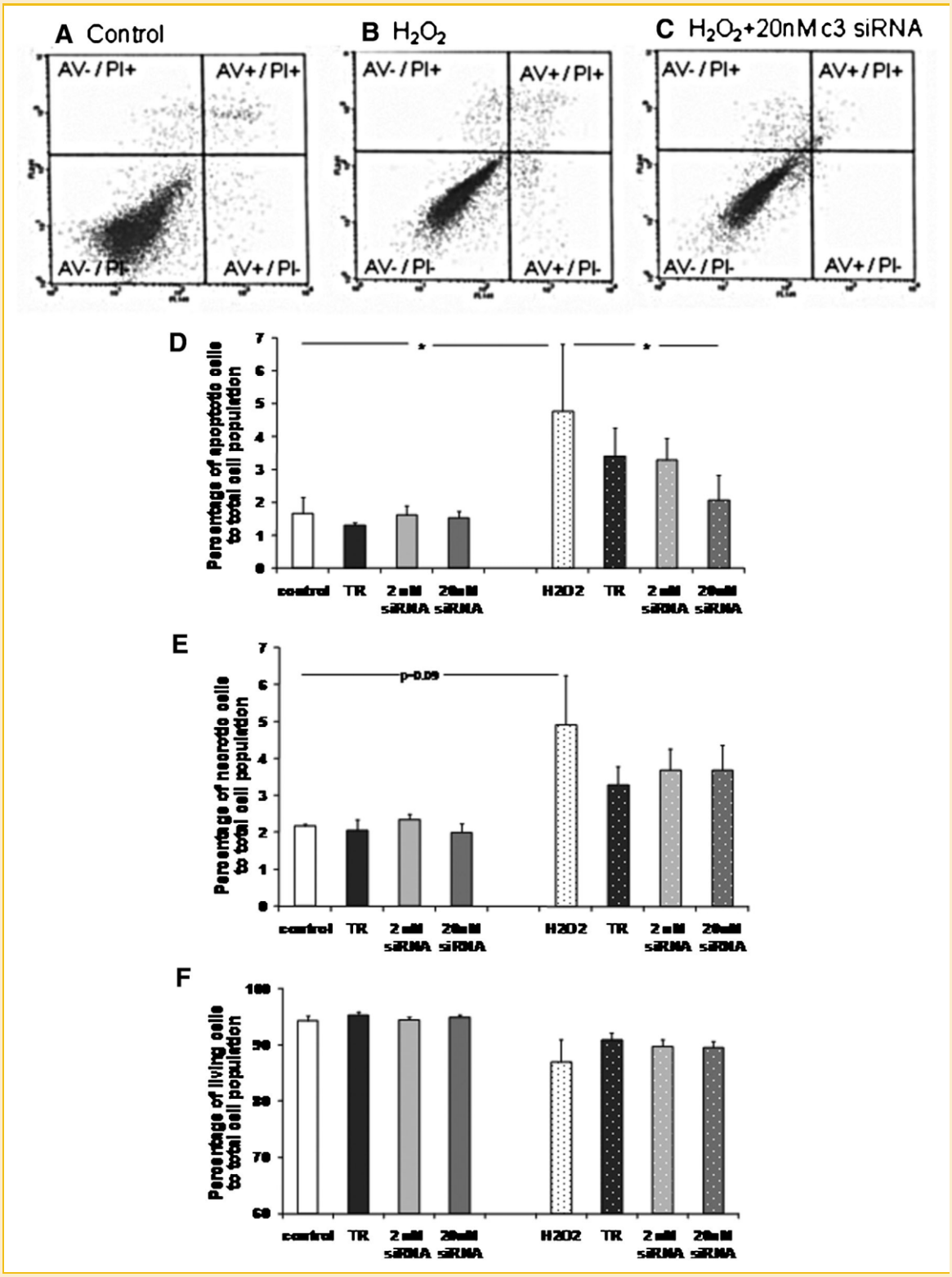

Fig. 6. Flow cytometric analysis showing typical cell cluster diagram following staining with FITC-labeled Annexin-V (*x*-axis) and propidium iodide (*y*-axis). Living cells are shown in the lower left quarter (AV−/PI−), apoptotic cells in the lower right (AV+/PI−), and necrotic cells in upper left and right quarters (AV±/PI+, A–C). The number of apoptotic cells was significantly increased by about 2.9-fold following H_2O_2 treatment compared to the control cells, but decreased 56% by 20 nM c3siRNA74 (D). There was no significant change in the necrotic and living cell population (F). Data represents the percentage of apoptosis, necrosis (E), and living cell population (mean ± SEM), n = 4. siRNA, small interfering RNA; TR, transfection reagent; $^{*}P < 0.05$.

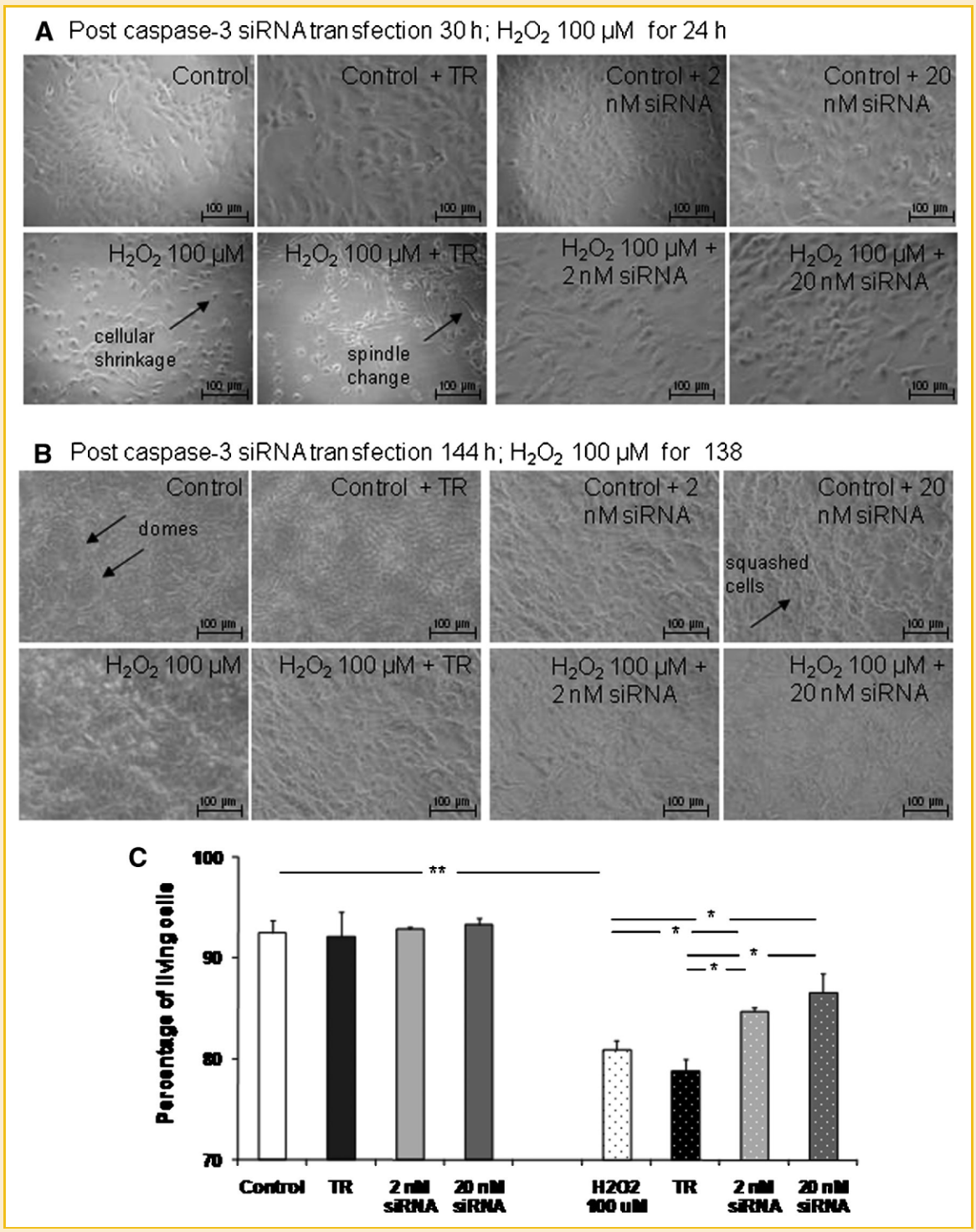

Fig. 7. Cell viability was determined by trypan blue exclusion in LLC-PK1 cells. The morphological changes such as spindle shape, cellular shrinkage, and chromatin condensation were caused by H_2O_2 at 24 h and enhanced by TR, but alleviated by c3siRNA74 (A). These changes were reduced by the addition of fresh culture medium. Following prolonged culture up to 144 h cells acquires a squashed appearance with the formation of domes (B). Cell viability was unchanged by TR alone or siRNA in control cells, decreased by H_2O_2 and improved by c3siRNA74 (C). Data represents percentage of living cell population (mean ± SEM), n = 4. siRNA, small interfering RNA; TR, transfection reagent; $^{*}P < 0.05$; $^{**}P < 0.01$.

mediators in different biological systems including renal IRI models [Daemen et al., 1999; Chatterjee et al., 2004; Yang et al., 2005, 2006]. In our study, the silencing of caspase-3 gene by synthetic siRNA significantly inhibited the expression of caspase-3 mRNA, protein and activity, and affected its downstream biological events including reduced apoptosis and decreased levels of IL-1β subunit in the LLC-PK1 cells with or without H_2O_2 treatment. These results imply the critical importance of caspase-3 in the apoptotic and inflammatory cascades and show that synthetic caspase-3 siRNA may provide a potential therapeutic strategy for injuries associated with increase levels of caspase-3 activity. Previous studies have already shown that, in human kidney proximal tubule (HK-2) cells, RNAi-mediated silencing of apoptosis antagonizing transcription factor (AATF) could exacerbate IRI-induced mitochondrial dysfunction, lipid peroxidation, caspase-3 activation, and apoptotic cell death, identifying AATF as a novel cytoprotective factor [Xie and Guo, 2006]. Therefore, the use of synthetic siRNA to posttranscriptionally silence a target gene provides not only a valid approach for the study and identification of disease mechanisms, but also for the subsequent therapeutic intervention.

With improvements in the understanding design and delivery of siRNA, its silencing potency is enhanced and its off-target activity is blocked more effectively. In our study, a low concentration of 2 nM siRNA was able to show effects directly at the level of caspase-3 activity, but also at the level of its downstream events such as apoptosis, and expression of IL-1β subunit. The design of siRNAs can be improved with the aid of bioinformatics to identify miRNA seed regions and toxic sequence motifs, and by incorporation of strategic chemical modifications [Bergauer et al., 2009; Bramsen et al., 2009]. As a result, these siRNAs can provide remarkable specificity and thus more consistent phenotypic data. The reduction of caspase-3 mRNA was detectable as early as 24 h by real-time RT-PCR. The changes in caspase-3 protein levels occurred at 48 h. Western blotting using a full-length antibody was found to be the most informative as it reveals the changes not only in active subunits, but also in the precursor, while the enzyme activity assay of caspase-3 provided a further confirmation of the change in its active protein. Caspase-3 activity is more closely linked to the downstream biological events.

The duration of silencing of caspase-3 expression by synthetic siRNA will be important in clinical applications. Transfection of insulinoma cells and islets with siRNA reduced caspase-3 mRNA transcripts by 50–67% and 50%, respectively, with corresponding levels of inhibition of apoptosis. However, the achieved reduction in caspase-3 protein level did not last beyond 2 days after transfection with the naked siRNA. Relatively higher levels and longer duration of gene silencing beyond 5 days were achieved by infection of target cells with an adenoviral vector encoding caspase-3-shRNA [Cheng et al., 2008]. In our study, caspase-3 mRNA was decreased after 24 h transfection with a synthetic siRNA. This resulted in reduced levels of caspase-3 protein at 48 h, with the inhibition remaining at detectable levels for at least 96 h. This prolonged reduction in caspase-3 protein may be due to the characteristics of LLC-PK1 cells or the specific sequence of c3siRNA74 used in this study. Nevertheless, the period of down-regulation revealed here provides valuable time window in the acute clinical settings of up-regulated caspase-3 expression after IRI.

The safety, specificity, and potency of therapeutic siRNAs have still been widely investigated, although the first phase I clinic trial was reported in 2005 using siRNAs to target VEGFR-1, a receptor in the pathway that mediates blood vessel growth, for age-related macular degeneration [Whelan, 2005]. siRNAs have been used in many fields and recently applied to renal IRI mouse models [Contreras et al., 2004; Hamar et al., 2004; Zheng et al., 2008]. In particular, the systemic administration of caspase-3, -8 siRNA plasmid vectors inhibited caspase-3, -8 and prevented apoptotic damage in mouse renal IRI [Zhang et al., 2006; Zheng et al., 2006]. Recent studies have shown that siRNA solutions containing a mixture of naked siRNAs targeting apoptosis, inflammation, and complement associated damage could promote organ preservation for 48 h and to prevent cold ischemic damage, and to improve both graft survival and cardiac function posttransplant [Zheng et al., 2009]. Utilizing synthetic siRNA represents a novel non-viral approach and provides a transient effect that could adequately prevent or treat acute injuries in different organ systems. Porcine kidneys have similar size, anatomical, and physiological characteristics to adult human kidneys [Sachs, 1994]. Therefore, the efficacy of synthetic caspase-3 siRNA was verified here in porcine proximal tubular cells, which should facilitate further tests in porcine kidney preservation and transplantation. These are well-established models and the necessary study procedures for preclinical studies of synthetic siRNA in renal transplant patients.

In conclusion, down-regulating caspase-3 gene by siRNA leads to reduction of caspase-3 protein and activity, resulting in decreased cellular apoptosis, suppression of IL-β maturation and hence reduced inflammatory responses, thus improved cell viability. This strategy provides a feasible approach for delineating mechanisms of diseases and a therapeutic intervention for a wide range of clinical disorders including IRI in renal transplantation.

ACKNOWLEDGMENTS

This study was supported by the Research & Development Directorate of the University Hospitals of Leicester NHS Trust. Joshua E. Elias was supported by the Royal College of Surgeons of England.

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