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Involvement of cytochrome *c* release and caspase-3 activation in the oxidative stress-induced apoptosis in human tendon fibroblasts

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Abstract

Our previous studies have demonstrated that oxidative stress and apoptosis are involved in human tendon degeneration. The objectives of our current study were to investigate the effect of oxidative stress on human tendon cell apoptosis, and to explore pathways by which tendon cell apoptosis was induced. In vitro oxidative stress was created by exposure of cultured human rotator cuff tendon cells to H_2O_2. Apoptotic cells were assessed by Annexin V-FITC staining and necrotic cells by propidium iodide (PI) staining using flow cytometry. Cytochrome *c* and caspase-3 protein expression were detected by Western blotting. A mini-dialysis unit was employed to increase the protein concentration of the cytosolic fraction. Caspase-3 activity was determined by a colorimetric assay. Tendon cell apoptosis induced by H_2O_2 was both dose and time dependent. Addition of H_2O_2 resulted in the release of cytochrome *c* to the cytosol, and an increase of caspase-3 activity and the expression of caspase-3 subunit. The data suggest that oxidative stress-induced apoptosis in human tendon fibroblasts is mediated via pathway(s) that includes release of cytochrome *c* from mitochondria to the cytosol and activation of caspase-3.

Keywords: Apoptosis; Oxidative stress; Pathway; Hydrogen peroxide; Caspase-3; Cytochrome *c*

1. Introduction

Tendon degeneration is a common pathophysiological process contributing to significant morbidity. Fibroblasts or tenocytes are the major cell types found in tendon tissues [1,2]. Although the cellular and molecular bases of tendon degeneration remain largely unclear, we have shown that oxidative stress [3] and apoptosis [4,5] are implicated in tendon tissue degeneration and tendon overuse.

Apoptosis, or programmed cell death, is an important physiological process involved in cell deletion during organogenesis and in the control of cell proliferation and differentiation in adult tissues. It is characterized by distinct biochemical features, in which activation of catabolic processes and enzymes occurs prior to cytolysis, thereby facilitating cell morphological changes, such as phosphatidylserine (PS) externalization to the cell surface, mitochondrial alterations, membrane blebbing, cell shrinkage, and nuclear condensation/fragmentation [6]. Apoptosis can be triggered by numerous mediators including receptor-mediated signals, withdrawal of growth factors, and environmental agents [7–9]. Direct exposure of cells to reactive oxygen species (ROS) via hydrogen peroxide (H_2O_2) or superoxide ($O_2^{\cdot -}$) generating agents can induce apoptosis [8,10,11]. However, the mechanisms whereby ROS induce the apoptotic pathways remain largely unknown.

There are numerous factors involved in apoptotic cascades. The activation of caspases has attracted particular attention because there is evidence that caspases are believed to be common executors for apoptosis [12–14]. Caspases are expressed as inactive proenzymes in living cells and become activated when cells receive an apoptosis-inducing signal. Once activated, they cleave a number of key substrates, resulting in their activation or inactivation. Activation of caspases leads to the morphological and biochemical features of apoptosis [15–18]. Two caspase-activating cascades that regulate apoptosis have been

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described: one is initiated from the cell surface death receptor and the other is triggered by changes in mitochondria integrity [19,20]. It has been reported that oxidative stress induces the release of mitochondrial cytochrome *c* and other apoptogenic proteins (e.g. apoptosis-inducing factor) from the mitochondrial intermembrane space into the cytosol. These proteins then bind to Apaf-1 and activate pro-caspase-3, which leads to mitochondrial dependent apoptosis [21–24]. Microinjection of cytochrome *c* to cells is sufficient to induce apoptosis in some but not all cells [25].

Although the caspases cascade has been identified in the apoptotic process, evidence is now emerging for a caspase-3-independent form of apoptosis. Recent reports demonstrate that both caspase-3-dependent and -independent pathways are involved in oxidative stress-induced apoptosis in a cell- and stimuli-specific manner [26]. The involvement of caspases in oxidative stress-induced apoptosis in human tendon cells has not been evaluated.

In the present study, we exposed primary cultured human rotator cuff tendon fibroblasts to hydrogen peroxide (H_2O_2) to create an in vitro oxidative stress situation, aiming to investigate the effect of oxidative stress on human tendon cell apoptosis, and to explore pathways by which tendon cell apoptosis was induced.

2. Materials and methods

2.1. Reagents

N-[2-hydroxyethyl] piperazine-*N*′-[2 ethanesulfonic acid] (HEPES), antibiotic–antimycotic, Hank's balanced salt solution, phosphate-buffered saline (PBS), and Dulbecco's modified Eagle medium (DMEM) were purchased from Invitrogen (Melbourne, Australia). PolyScreen PVDF transfer membrane was obtained from NEN Life Science Products (Boston, MA).

Monoclonal anti-human cytochrome *c* antibody and Annexin V-fluorescein isothiocyanate (FITC) were purchased from BD PharMingen (San Diego, CA). Monoclonal anti-human caspase-3 (CPP-32) and anti-human actin antibodies were from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase conjugated anti-rabbit and anti-mouse IgG were obtained from Chemicon (Melbourne, Australia). Slide-A-lyzer concentrating solution and mini-dialysis unit, enhanced chemiluminescence (ECL) reagents, Restore Western Blot Stripping Buffer, and MicroBCA protein assay kit were from Pierce (Rockford, IL).

The source of all other materials was Sigma-Aldrich, unless otherwise noted.

2.2. Human tendon cell culture

Human tissue collection was approved by the South Eastern Sydney Area Health Service Ethics Committee, Australia. Human rotator cuff tendon tissue was aseptically collected from patients undergoing rotator cuff repair surgery. Tendon samples were digested with 0.25 mg/ml type IA collagenase in Hank's balanced salt solution containing 2% (v/v) 1 M HEPES and 2% (v/v) antibiotic–antimycotic at 37 °C overnight. Tendon fibroblasts were harvested, resuspended in DMEM with 10% (v/v) fetal calf serum (FCS), and maintained in culture at 37 °C in a 5% CO_2 humidified incubator. Only two to three passaged cells were used in our experiments. H_2O_2 was used as an inducer of oxidative stress.

2.3. Cell viability assay

To assess the cytotoxicity of H_2O_2 challenge, tendon cell viability was determined using CellTiter 96®AQ_{ueous} One solution reagent (Promega, Madison, WI) following the manufacturer's instructions. Briefly, 1×10^4 cells were seeded onto 96-well plates overnight to reach an 80% confluency. Cells were treated with or without H_2O_2 in DMEM with 10% FCS. Four hours after the addition of H_2O_2, 20 μl/well of CellTiter 96®AQ_{ueous} One solution reagent was added to each well, followed by a further incubation for 4 h at 37 °C in 5% CO_2. The absorbance at 490 nm was read using a spectrophotometer (SPECTRAmax™ 250, Molecular Devices, Sunnyvale, CA).

2.4. Apoptosis assay

Apoptotic cells were detected based on the principle of Annexin V binding to translocated plasma membrane PS. During the apoptotic process, PS translocates from the inner membrane to the outer membrane of the cells [27]. FITC-labeled Annexin-V was added to cultured cells and bound to exposed PS. FITC signals were detected by flow cytometry. PI was added to cultured cells to identify the loss of integrity of the cell membrane, which is specific for necrotic cells. Briefly, cells were washed, trypsinized, and pelleted. Cells (1×10^5) were suspended in 100 μl staining buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) containing 1 μg/ml PI and 5 μl Annexin V-FITC, incubated for 15 min at room temperature, and then subjected to analysis by a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA). The green fluorescence (FITC) and red fluorescence (PI) were detected by filtration through a 530- and a 585-nm band pass filter, respectively. Spectral overlap was electronically compensated using single-color controls including cells alone, FITC alone, and PI alone in separate tubes. A minimum of 3×10^4 events was collected on each sample. Analysis of the multivariate data was performed with CELLQuest™ software (Becton Dickinson). $FITC^-/PI^-$, $FITC^+/PI^-$ or $FITC^+/PI^+$ represented viable (intact) cells, apoptotic cells, or necrotic cells, respectively (Fig 1).

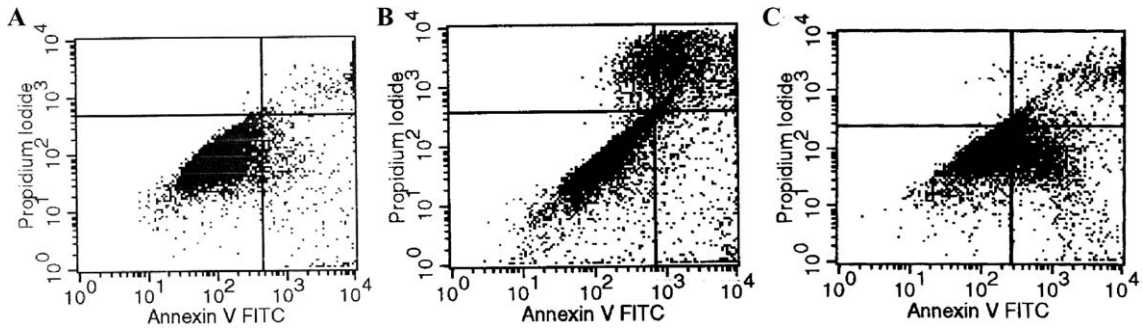

Fig. 1. Representative flow cytometric analysis of H_2O_2-induced cell death. Dot plot diagrams represent typical apoptotic and necrotic cell population detected by Annexin V-FITC and PI staining. (A) Untreated tendon cells. (B) Tendon cells were treated with 1 mM H_2O_2 for 4 h. (C) Tendon cells were treated with 50 μM H_2O_2 for 24 h. The lower left quadrants of each panels show the viable (intact) cells, which exclude PI and are negative for Annexin V-FITC binding ($FITC^-/PI^-$). The upper right quadrants contain the nonviable, necrotic cells, positive for Annexin V-FITC binding and for PI uptake ($FITC^+/PI^+$). The lower right quadrants represent the apoptotic cells, positive for Annexin V-FITC and negative for PI ($FITC^+/PI^-$).

2.5. Preparation of cytosolic fraction of human tendon cells

The preparation of cytosolic fraction was carried out using the methods described by Fujimura et al. [28] and Liu et al. [29]. Cells were washed with PBS and then resuspended in ice-cold lysis buffer (5×10^6 cells/ml) containing 20 mM HEPES/KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT) and protease inhibitors (0.1 mM PMSF, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 25 μg/ml *N*-Acetyl-Leu-Leu-Norleu-Al). Cell homogenization was performed on ice in an ice-cold Dounce tissue grinder. After sitting on ice for 10 min, the homogenate was centrifuged at $600 \times g$ for 5 min at 4 °C. The supernatant was further centrifuged at $20{,}000 \times g$ for 60 min at 4 °C. The resulting supernatant, the cytosolic fraction of tendon cells, was concentrated against Slide-A-lyzer concentrating solution using a Slide-A-lyzer mini-dialysis unit (10 K MWCO). The sample volume was reduced from 200 μl starting volume to 50 μl after 4–6 h dialysis. Proteins with molecular weight greater than 10 kDa were retained and enriched in the cytosolic fraction using this method. Protein concentration of samples was detected by MicroBCA protein assay following the manufacturer's instruction.

2.6. Western blot analysis for cytochrome c

Proteins in the cytosolic fraction of cell homogenates were denatured by boiling for 5 min in Laemmli sample buffer and fractionated by electrophoresis on 12.5% (w/v) SDS-polyarcylamide gel. The fractionated proteins were electrophoretically transferred to a PVDF membrane, and the blots were blocked with 5% (w/v) nonfat dry milk TTBS solution containing 25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% (w/v) Tween 20. After washing in TTBS, the blots were incubated for 1 h with an anti-cytochrome *c* monoclonal antibody at 1:200 dilution, followed by horseradish peroxidase-conjugated anti-mouse IgG at 1:1000 dilution. Immunoreactive bands were detected by ECL reagents. All membranes were stripped with Restore Western Blot Stripping Buffer and reprobed using a rabbit anti-human actin antibody as a housekeeping control.

2.7. Caspase-3 activation and activity assay

Human tendon cells were seeded onto 75-cm^2 flasks and cultured overnight to reach an 80% confluency. Cells were treated with or without H_2O_2 in DMEM with 10% FCS for various time. After being washed with cold PBS, cells were harvested and then centrifuged at $800 \times g$ for 5 min. The cell pellet was resuspended in ice-cold hypotonic lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, 2 mM phenylmethyl sulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin) at a concentration of 1×10^8 cells/ml. After four cycles of freezing and thawing, cell lysate was incubated on ice for 15 min and centrifuged at $15{,}000 \times g$ for 20 min at 4 °C. The supernatant was collected for assays on caspase-3 activity and its precursor and subunit.

Positive and negative controls for caspase-3 activity assay were generated from human Jurkat cell line. The cells were grown in DMEM medium containing 10% (v/v) FCS and 1% penicillin–streptomycin in a humidified, 5% CO_2 incubator at 37 °C. H_2O_2 was added to the Jurkat cell (1×10^6 cells/ml) culture to a final concentration of 50 μM followed by incubation for 4 h. The cell extract from the H_2O_2-treated Jurkat cells was used as a positive control, and the extract from untreated Jurkat cells served as a negative control. Cell permeable pan-caspase-3 inhibitor Z-VAD-FMK was included in each experiment as an inhibitory control.

Caspase-3 activity was determined using a colorimetric assay according to the manufacturer's instructions (CaspACE assay system, Promega). In the assay system, the colorimetric substrate (Ac-DEVD-pNA) is labeled with the chromophore *p*-nitroaniline (pNA). pNA is released from the substrate upon cleavage by caspase-3. Free pNA produces a yellow color that is monitored by a plate reader at 405 nm. The

amount of yellow color produced upon cleavage is proportional to the amount of caspase-3 activity present in the sample [30]. Blank (no cell extract), negative control (extract from untreated Jurkat cells), positive control (extract from treated Jurkat cells), and tendon cell samples were included in each assay. Data were normalized by protein content of each sample. Results of caspase-3 activity were expressed as OD value per microgram of protein.

The expressions of caspase-3 precursor and subunit were detected by Western blotting using a monoclonal anti-caspase-3 antibody (CPP-32). The blot was reprobed with an anti-actin antibody for protein normalization. Protein concentration of cell extracts was detected by MicroBCA assay kit.

2.8. Statistical analysis

All values in the text and figures are expressed as mean ± S.E. of n observations. Statistical comparison between experimental group and control was performed using unpaired two-tailed Student's t-tests. The confidence limit was predetermined at an α level of 0.05.

3. Results

3.1. Apoptosis induced by H_2O_2 was both dose and time dependent

In vitro oxidative stress was created by exposing primary cultured human tendon cells to hydrogen peroxide (H_2O_2). Oxidants are known to induce both apoptosis and necrosis in cells, with the concentrations required dependent on the cell type being investigated [31,32]. We conducted a cell death analysis in order to determine an optimal concentration of H_2O_2 at which tendon cell apoptosis was induced while cell necrosis was minimal.

Fig. 1 shows a typical flow cytometric profile of tendon cell apoptosis and necrosis. Human tendon cells were exposed to H_2O_2 at various concentrations for 4 h and analyzed for both Annexin V-FITC and PI staining by flow cytometry. There was a dose-dependent increase in necrotic cells after H_2O_2 treatment (Fig. 2A). At 1 mM H_2O_2 challenge, although apoptotic cell numbers reached 20%, the necrotic cell population increased to 42%. The increased necrotic cell death was further confirmed by cell viability assay. As shown in Fig. 2B, exposure of cells to increasing concentration of H_2O_2 for 4 h resulted in a dose-dependent decrease in cell viability. Induction of apoptosis by H_2O_2 was also time dependent (Fig. 2C). With a 50 μM H_2O_2 challenge for 12 h, a significant increase in apoptotic cell (8%) was detected. Twenty-four hours after H_2O_2 stimulation, the apoptotic cell population reached 26% while the proportion of necrotic cells was under 10%. Based on the above findings, we chose 50 μM H_2O_2 as an apoptotic inducer for the study of apoptotic pathway in tendon cells.

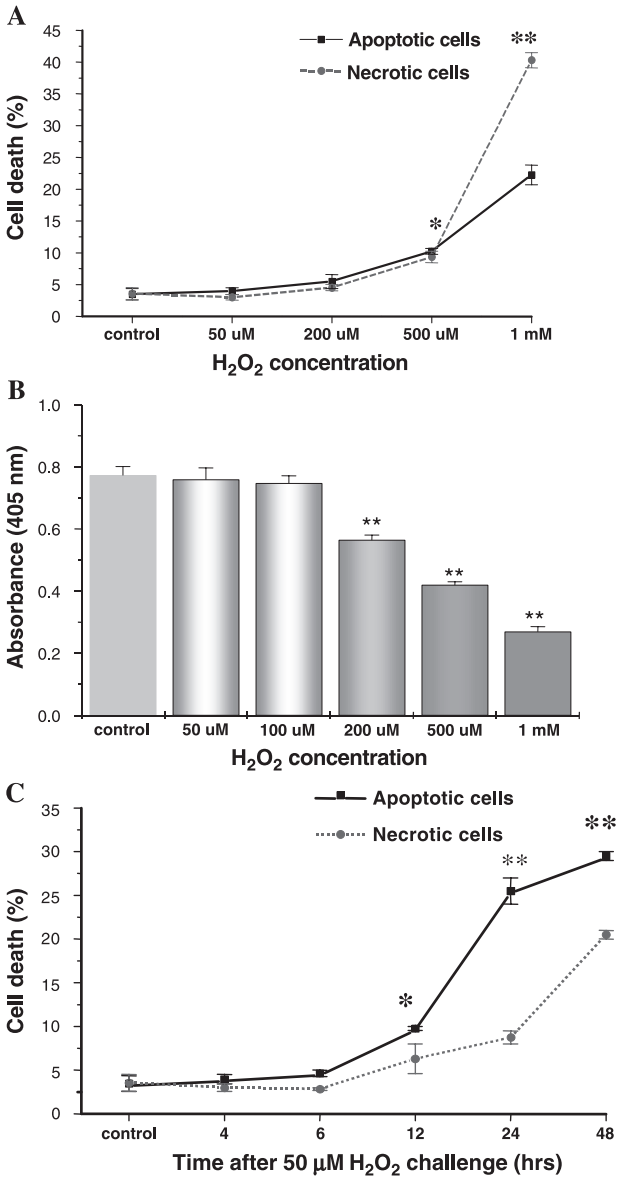

Fig. 2. Effect of H_2O_2 on human tendon cell death at the indicated time and dosage. (A) Human tendon cells were treated with H_2O_2 at the indicated concentration for 4 h. Cell death was measured by Annexin V-FITC and PI staining using flow cytometry. (B) Human tendon fibroblasts were cultured in the presence of the indicated concentration of H_2O_2 for 4 h. Cell viability was determined using CellTiter 96®AQ_{ueous} One solution reagent. (C) Human tendon cells were treated with 50 μM H_2O_2. Cell death was measured at different time by Annexin V-FITC and PI staining using flow cytometry. Data plotted are mean ± S.E. of three separate experiments. *$P<0.05$ when compared with untreated control cells and **$P<0.001$ when compared with untreated control cells using unpaired Student's t-tests.

3.2. Hydrogen peroxide-induced caspase-3 activation

To determine the involvement of caspase-3 in H_2O_2-induced apoptosis in human tendon fibroblasts, we examined the activation of caspase-3 by measuring caspase-3 activity and detecting caspase-3 precursor and subunit expression.

The activity of caspase-3 was measured by the colorimetric caspase-3 assay system. Exposure of human tendon cells to H_2O_2 (50 μM) for 6 h caused a threefold increase in caspase-3 activity compared to control (cells without treatment) (Fig. 3A). The caspase-3 inhibitor Z-VAD-FMK reversed the activation of caspase-3 (Fig. 3A). The activated caspase-3 enzyme expression was further confirmed by Western blotting analysis of caspase-3 precursor and subunit. Expression of caspase-3 subunit is an indicator of caspase-3 activation from its pro-caspase-3 enzyme. As shown in Fig. 3B, 6 h after exposure of human tendon fibroblasts to H_2O_2, there was a detectable caspase-3 subunit expression by Western blotting.

3.3. The release of mitochondrial cytochrome c to the cytosol after H_2O_2 stimulation

Cytochrome *c* immunoreactivity was evident as a band of molecular mass 15 kDa. Using Slide-A-lyzer concentrating solution and mini-dialysis unit to enrich protein in the cytosol, we were able to detect cytochrome *c* by Western blotting. As shown in Fig. 4, there was no detectable cytochrome *c* expression in the cytosolic fractions of control cultured human tendon fibroblasts, suggesting that release

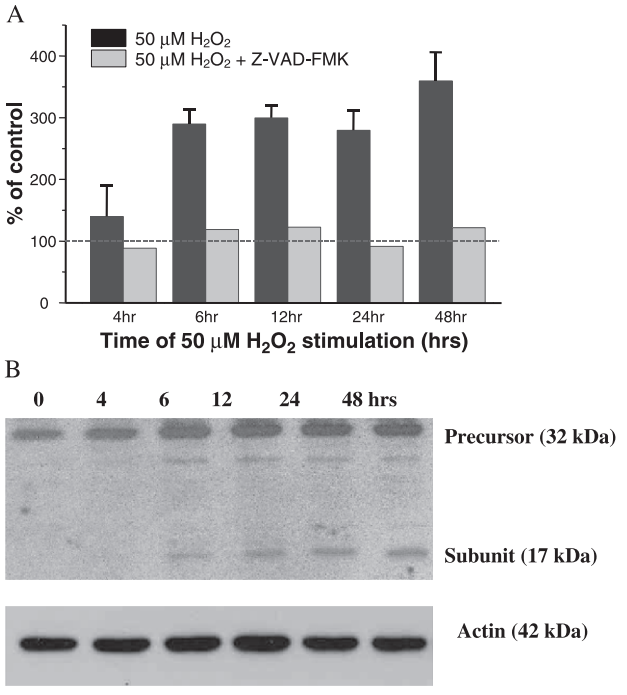

Fig. 3. Effect of H_2O_2 (50 μM) on activation of caspase-3 in human tendon fibroblasts. (A) Caspase-3 activity induced by H_2O_2 (50 μM) at the indicated time. Results were expressed as percentage of control (cells without treatment). Values are mean ± S.E. of three separate experiments. Caspase-3 specific inhibitor Z-VAD-FMK was included in each time point as an inhibitory control. (B) Caspase-3 precursor and subunit expression after H_2O_2 treatment at indicated time detected by Western blotting (upper panel). The anti-actin antibody was used to assure the equal amount of protein loaded (lower panel). This result is representative of three separate experiments.

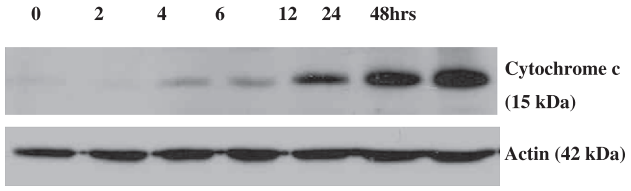

Fig. 4. Effects of H_2O_2 (50 μM) on cytochrome *c* release to cytosol in human tendon cells. Cytosolic fractions of tendon cell were separated from mitochondrial fractions and subjected to Western blotting with a monoclonal antibody to cytochrome *c* (upper panel). The blot was reprobed with an anti-actin antibody to assure the equal amount of protein loaded on each lane (lower panel). The panel is representative of three separate experiments.

of cytochrome *c* barely happened in untreated cells. However, 4 h after exposure to H_2O_2 (50 μM), released cytochrome *c* was found in the cytosolic fraction of tendon cells (Fig. 4). The release of cytochrome *c* accumulated and remained in the cytosolic extracts at 48 h after H_2O_2 stimulation.

4. Discussion

In the present study, we demonstrate a time- and dose-dependent induction of apoptosis with hydrogen peroxide (H_2O_2) in human tendon fibroblasts. A lower concentration of H_2O_2 (50 μM) led to tendon fibroblast apoptosis in a time dependent manner. However, with a higher concentration of H_2O_2, there was a significant increase in necrotic cell death. The oxidative stress-induced apoptosis in human tendon fibroblast is most likely through release of cytochrome *c* from mitochondria to cytosol and activation of caspase-3.

Hydrogen peroxide (H_2O_2) is known to modulate a variety of cell functions [33]. Its lower biological reactivity compared to many other ROS, combined with its capacity to cross membranes and diffuse away from the site of generation, makes it an ideal signaling molecule. Various levels of H_2O_2 can cause different outcomes [34–36]. It has been shown that a low level of H_2O_2 (1 μM) stimulated tendon fibroblasts proliferation [37]. At 10 μM–5 mM of concentration, H_2O_2 promotes apoptosis in Jurkat cells, cardiomyocytes, chondrocytes, and skin fibroblasts [31,38–40]. However, little information exists about the effects of H_2O_2 on human tendon cell apoptosis.

In the current study, we have demonstrated that H_2O_2 induces tendon cell apoptosis in a time- and dose-dependent manner. At a 50 μM concentration, H_2O_2 induced human tendon cell apoptosis while keeping cell necrosis at a minimum. Based on this finding, 50 μM H_2O_2 was added to cultured human tendon fibroblasts to evaluate apoptotic pathways. This concentration is higher than the intracellular physiological level [41], and likely to cause oxidative stress in tendon cells. Higher concentrations of H_2O_2 (1 mM) induced tendon cell necrosis. The mechanisms of H_2O_2-induced necrosis are still unknown. It was postulated that

instead of activation of capase-3, high concentrations of H_2O_2 caused oxidation of caspase-3 [42,43] or caused the failure of mitochondrial energy production [44,45], leading to fatal cell damage.

With the stimulation of 50 μM H_2O_2, enhanced caspase-3 activity was detected in human tendon fibroblasts, suggesting H_2O_2-induced tendon cell apoptosis was via through a caspase-3-dependent pathway. This is consistent with findings by other investigations in Jurkat cells, HL-60 cells, and endothelial cells [31,46–48]. The increased caspase-3 activity in H_2O_2-induced tendon cell apoptosis was further confirmed by Western blotting analysis, in which an increased caspase-3 subunit expression was detected, indicating an increase in the rate of conversion of caspase-3 from its inactive precursor to an active product.

Mitochondria are thought to play a central role in the activation of apoptosis induced by multiple stimuli. It is well known that mitochondria are both a target and source of ROS [49]. Results from our study demonstrate that H_2O_2 challenge caused the release of cytochrome *c* from mitochondrial to cytosol in human tendon fibroblasts. Cytochrome *c* is synthesized as an apoprotein on cytosolic ribosomes, which then translocates to mitochondrial intermembranous space. It is rarely detectable in cytosol. However, cytochrome *c* can be released from mitochondria to cytosol in apoptosis when the mitochondrial pathway is involved. In our study, we introduced a minidialysis unit to concentrate proteins in cytosolic fraction of tendon cells, and significantly increased the sensitivity of Western blotting analysis on cytochrome *c*. To completely lyse cell membranes and keep mitochondria intact, a high volume of homogenization buffer is normally used for cell lysis. This results in a lower protein concentration in the cytosolic fraction, affecting the detectability of cytochrome *c* in cytosol. To overcome this problem, immunoprecipitation was used to increase the sensitivity of cytochrome *c* detection [50,51]. Using the minidialysis method, we have achieved similar results with much simpler procedure compared to immunoprecipitation.

Vermes et al. [27] has reported that translocation of PS from the inner side of the plasma membrane to the out layer occurs in the early stage of apoptosis. Suzuki et al. [38] also demonstrated that PS translocation occurred at 4 h after H_2O_2 exposure in rat cardiomyocytes. In our tendon cell apoptosis study, we notice that cytochrome *c* release and caspase-3 activation were much earlier events (4–6 h) than the detectable PS translocation (12 h). These findings are consistent with observations that cytochrome *c* release into the cytosol precedes caspase activation and other cellular changes including PS exposure [52–54]. The released cytochrome *c* may activate the apoptosome-dependent death machinery by binding to Apaf-1, resulting in the activation of caspase and subsequent apoptotic changes as seen in Hela cells, 293 cells, and U937 cells [29]. The early release of cytochrome *c* and activation of caspase-3 observed in our study could be true events in H_2O_2-induced tendon cell apoptosis or results of difference in sensitivities of detection techniques in different cells [55,56]. Nevertheless, detected cytochrome *c* in the cytosol can be regarded as an early and sensitive apoptotic indicator in H_2O_2-induced tendon cell apoptosis.

In summary, we present evidence that oxidative stress by exogenous hydrogen peroxide promoted human tendon fibroblasts apoptosis. This apoptotic process is most likely through the mitochondrial cascade, in which cytochrome *c* is released from mitochondria to cytosol and the released cytoplasmic cytochrome *c*, in turn, activates caspase-3. Regulation of intracellular ROS and modification of apoptotic cascades may control apoptotic events and provide new strategies for the prevention and treatment of tendon degeneration.

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