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Involvement of death receptor signaling in mechanical stretch-induced cardiomyocyte apoptosis

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Abstract

Recent evidences suggest that mechanical overload associated with abnormal blood pressure causes apoptosis in cardiovascular system. Still, the intracellular signaling leading to cardiomyocyte apoptosis has not been fully defined. Previous reports ascribed stretch-induced cardiomyocyte apoptosis to rennin-angiotensin-system (RAS) signaling and/or mitochondria-dependent apoptosis pathway. The present study shows the involvement of death receptor signaling in mechanical stretch-induced cardiomyocyte apoptosis. By employing a well-described in vitro stretch model, we studied stretch-induced apoptosis and found that the death receptor-mediated apoptotic signaling was activated in stretch-induced apoptosis in neonatal rat cardiomyocytes. The major finding are as following: (1) The mechanical stretch activated death receptor-mediated apoptotic signaling in cardiomyocytes, including activation of caspases 8, 9 and 3, up-regulation of Fas, FasL expression and cell surface trafficking of death ligands (FasL and TRAIL); (2) That exogenous death ligand (TRAIL) enhanced, while soluble death receptor (sDR5) neutralized, stretch-induced apoptosis; (3) Adenovirus-delivered dominant negative FADD (FADD-DN) significantly reduced apoptosis, caspases 8, 9, and 3 activation, and stretch-induced cyt c release from mitochondria. These data clearly suggested mechanical stretch activated death receptor-mediated apoptotic signaling in cardiomyocytes. In conclusion, our data suggest that the FADD-linked death receptor signaling may

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contribute to stretch-induced cardiomyocyte apoptosis, probably through activating mitochondria-dependent apoptotic signaling.

Keywords: Mechanical stretch; Death receptor; FADD; Apoptosis; Cardiomyocyte

Introduction

Abnormal mechanical load, as seen in hypertension, is an important pathological factor leading to heart remodeling and heart failure. Accumulating evidences suggest that mechanical overload causes apoptosis in the cardiovascular system (Anversa et al., 1990; Teiger et al., 1996). Given that the resultant cardiomyocyte loss through apoptosis may lead to loss of circulation power and eventually result in the transition from hypertrophy to heart failure, understanding the signaling pathways that regulate cardiomyocyte apoptosis may have important implications to clinical therapy of cardiovascular diseases.

Apoptosis is a tightly-controlled cell suicide process, which is characterized by cytomembrane overturn, DNA fragmentation, nuclear condensation and cellular shrink (Hengartner, 2000). Generally, there are two types of apoptosis pathways: the mitochondria-dependent (intrinsic) and the death receptor-dependent (extrinsic) apoptotic pathways. Both pathways have been found in cardiomyocyte apoptosis (Bishopric et al., 2001).

In the intrinsic pathway, the death machinery is mainly dependent on mitochondria, which release apoptotic factors, such as cytochrome c (cyt c), apoptosis inducing factor (AIF), Smac/DIABLO, and endonuclease G, into cytosol when stimulated by an "intrinsic" death signal. Once released into cytosol, cyt c rapidly activate caspase 9, which leads to activation of caspase 3 and succedent execution of apoptosis (Green and Reed, 1998; Wang, 2001). Bcl-2 family proteins are considered responsible in regulating this process (Gross et al., 1999).

In the extrinsic pathway, apoptosis occurs after the death receptors (DR) are bound by corresponding ligands. The best-characterized death receptors are Fas (also called CD95 or Apo1) and tumor necrosis factor receptor 1(TNFR1). Additional death receptors are DR3 (Apo3), DR4 and DR5 (Apo2/TRAIL-R2). The ligands that activate these DRs are structurally related molecules that belong to TNF superfamily. FasL binds to Fas, TNF-α binds to TNFR1, Apo3L to binds DR3, and TRAIL (TNF-related apoptosis-inducing ligand, also called Apo2L) binds to DR4 and DR5, respectively. Binding of ligand results in trimerization of DR, recruitment and activation of pro-caspase 8 through specific adaptor molecules, such as FADD (Fas-associated protein with death domain). Active caspase 8 can subsequently cleave downstream caspases as well as other pro-apoptotic factors to initiate intracellular process of apoptosis (Ashkenazi and Dixit, 1998).

There is cross-talk between the two apoptotic pathways. For example, DR-activated caspase 8 can cleave a pro-apoptotic member of Bcl-2 proteins, Bid, enabling it to insert into mitochondrial membrane to induce cyt c release (Li et al., 1998; Luo et al., 1998). Hence, it may servers as an amplification step of DR-mediated apoptotic signaling and it is required in some DR-mediated apoptosis (Tang et al., 2000).

FADD is the most important adaptor of DR signaling, which binds DRs (TNFR1, Fas, DR3) at a conserve death domain (DD) through its own DD. FADD also contains a death effecter domain (DED), a specific form of the caspases recruitment domain (CARD), to recruit pro-caspase 8. DRs can utilize FADD to recruit pro-caspse 8 directly (Fas pathway) or through another adaptor TRADD (TNFR1-

associated death domain protein) to bind FADD indirectly (TNFR1 pathway) (Ashkenazi and Dixit, 1998). Although it is unclear whether FADD is required in TRAIL/DR4/DR5 induced apoptosis, FADD is required in Fas-induced apoptosis and in most circumstance of TNF-induced apoptosis (Yeh et al., 1998; Schneider et al., 1997; Wajant, 2002). A dominant negative form of FADD (FADD-DN), that contains DD but lacks DED, will bind to the DR but will not activate caspase 8. Thus FADD-DN can block the DR apoptotic signaling, providing a way to manipulate DR-mediated apoptosis signaling (Wajant et al., 1998).

Apoptosis of cardiomyocytes has been recently recognized as a major mechanism in the development of a number of cardiovascular diseases, including mechanical overload-related heart remodeling (Haunstetter and Izumo, 1998). However, little is known about the intracellular signaling pathways, through which mechanical signals induce cardiomyocyte apoptosis. Based on in vitro studies, Leri et al reported sustained stretch induced apoptosis in adult cardiomyocytes via enhancement of angiotensin II secretion and consequential p53-related Bax expression (Leri et al., 1998), while another report ascribed cardiomyocyte apoptosis to the production of reactive oxygen species (ROS) during periodic stretch (Pimentel et al., 2001). Given that Bax and ROS both can impact on mitochondria, these reports suggest the roles of mitochondria-dependent pathway in stretch-induced cardiomyocyte apoptosis. Indeed, we recently provided evidences demonstrating release of cyt c and loss of mitochondria membrane potential during stretch-induced apoptosis in neonatal cardiomyocytes (Liao et al., 2004).

Despite the known mechanisms mentioned above, a possibility still exists for the involvement of DR-dependent pathways in stretch-induced cardiomyocyte apoptosis. DR pathways (TNF-α, FasL, TRAIL, etc) have been found to regulate post-ischemic cardiomyocyte apoptosis as well as cardiomyocyte apoptosis induced by other treatments (Meldrum, 1998; Jeremias et al., 2000; Nakamura et al., 2000). Several other lines of evidences seem to favor this hypothesis. First, dearth receptors and death ligands (such as Fas and FasL, etc) are constitutively expressed in cardiomyocyte (Chao et al., 2002). Second, heart is a TNF-producing organ because both the myocardial macrophages and cardiomyocyte themselves synthesize TNF (Meldrum, 1998). Third, moreover, diastolic wall stress due to volume-overload induced increase of Fas expression in cardiomyocyte, although no enhancement of cardiomyocyte apoptosis was detected in that setting (Wollert et al., 2000).

In the present study, by using an in vitro cellular sustained stretch system, we investigated the involvement of death receptors signaling in mechanical stretch-induced apoptosis in neonatal rat cardiomyocyte. To address roles of death receptors signaling, recombinant adenovirus carrying a dominant negative FADD gene (Ad-FADD-DN) was employed to block FADD-mediated DR apoptotic pathway. Recombinant TRAIL and soluble DR5 (sDR5) peptide were also used to stimulate or block DR signaling, respectively. With these approaches, we demonstrate that FADD-linked death receptors signaling is involved in regulation of cardiomyocyte apoptosis induced by mechanical stretch.

Materials and methods

Materials

Fetal bovine serum (FBS) was purchased from HyClone (UT, USA) and all other cell culture materials were products of Invitrogen (CA, USA). The caspases inhibitor zVAD-fuoromethylketone

(zVAD-fmk) and all other protease inhibitors were from Calbiochem (CA, USA). DEAD-pNA, LEHD-pNA, and IETD-pNA were products of BIOMOL (PA, USA). β-actin antibody, BrdU (5-bromo-2′-deoxyuridine), PI (propidium iodide), DAPI (4,6-diamidino-2-phenyindole) were from Sigma (CA, USA). Anti-Fas (610197), anti-FasL (610410) and Annexin V apoptosis detection kit were from BD (NJ, USA). Anti-TRAIL (sc-7877) and anti-DR5 antibodies were from Santa Cruz (CA, USA). The silicone elastic membrane was purchased from the Specialty MFG (MN, USA). The horseradish peroxidase (HRP)-labeled secondary antibodies were from KPL (MD, USA) and the PE-labeled fluorescent secondary antibodies were from BD. The enhanced chemiluminescent (ECL) Western Blotting detection kit was purchased from Pierce (IL, USA). Recombined TRAIL (1–170) and soluble DR5 (sDR5) proteins were expressed and purified by using a standard GST-aided *E. coli* gene expression system (Invitrogen).

Cell preparation

Ventricular myocytes isolated from the hearts of neonatal Wistar rats (1–3 days) were cultured as described previously (Liao et al., 2004). Briefly, hearts were removed, minced and trypsinized at 37 °C with gently stirring in Saline A (in mmol/L: 137 NaCl, 5.4 KCl, 4.2 $NaHCO_3$, 5.5 glucose, 1.0 HEPES, pH 7.4) containing 0.1% trypsin. Then cells were centrifuged and resuspended in Dulbecco's modified Eagle medium/F-12 containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. After incubation at 37 °C for 90 min, the unattached cardiomyocytes were seeded at a density of 5×10^4 cells/cm^2 into a cell stretch system described below. Then, 0.1 mmol/L BrdU were added in the culture for at least first 36 h to inhibit proliferation of non-myocytes. Over 95% of these cells were cardiomyocytes, as identified by anti-α-sarcomeric actin antibody immunostaining (data not shown). To apply mechanical stretch in vitro, cells were cultured on a sheet of silicone elastic membrane fine coated with rat-tail collagen (0.1 mg/ml, in 0.01% acetic acid) in stretch chambers described previously (Lee et al., 1996), in which cells were grown in DMEM/F-12 containing 10% FBS for 72 h and reached 80% confluence. After 24 h serum starvation, the medium was changed with fresh medium, and then the cells were subjected to 20% sustained stretch. Cells without stretch were cultured under identical conditions as controls.

Recombination of adenovirus carrying dominant negative FADD gene

A dominant negative mutant of the human FADD gene, FADD (80–208), fused with green fluorescent protein (GFP) gene (GFP-FADD-DN) (Wajant et al., 1998), was cloned into a recombinant adenovirus using the AdEasy adenovirus system as described by He et al (He et al., 1998) and then transferred into neonatal rat cardiomyocyte 12 h after seeding. The total infection period lasted for 24 h before stretch was applied. Adenovirus carrying GFP as well as Lac Z gene (Ad-Lac Z) was used as vector control.

Apoptosis assays

Apoptosis was determined by detecting nuclear condensation and fragmentation using DAPI staining (Liao et al., 2003). Cells were washed with PBS (phosphate buffer saline, pH 7.4) and stained by 10 ng/ml DAPI before being visualized with fluorescent microscopy. At least 200 cells were counted in each

experiment. Apoptosis was also assayed by detection of phosphatidylserine exposure on cell membrane (Liao et al., 2003), using a commercial Annexin V kit, following manufacturer's instruction. Comparative experiments were performed at the same time by bivariate flow cytometry using a FACScan (BD) and analyzed with the CellQuest software.

The caspases activity was determined as previously described (Liao et al., 2004), using the p-nitroanilide-derived chlorometric substrate (DEAD-pNA, LEHD-pNA, and IETD-pNA for Caspase3, 9 and 8, respectively). Enzyme-catalyzed release of pNA was monitored at 405 nm in a 96-well microtiter plate reader. Caspase activity was expressed as relative absorbance at 405 nm (A405).

Western blotting

Western blotting was performed as described (Liao et al., 2004) . Cells were prepared and lysed in lysis buffer (in mmol/L: 25 HEPES, pH7.4, 5 EDTA, 8.0 EGTA, 1.0 Na3VO5, 0.25 NaF, 0.1 phenylmethylsulfonyl fluoride, 1.0 dithiothreitol, 1% NP-40, 5 μg/mL aprotinin, 100 μg/ml leupeptin, 50 μg/ml trypsin inhibitor.). Cellular protein (20 μg) was loaded and separated on sodium dodecyl sulfate polyacrylamide gel (BioRad mini gel) and transferred to a nitrocellulose membrane by standard electric transfer protocol. The membrane was blocked at room temperature with PBS containing 0.1% Tween-20 (PBST) plus 5% non-fat milk for 2 h and then probed with primary antibody overnight at 4 °C, followed by wash with PBST and incubation with HRP-labeled secondary antibody for 2 h at room temperature. The membrane was then exposed to chemiluminescent detection system to visualize immuno-reactive bands.

Cell fractionation

Cell fractionation was done routinely as previously described (Liao et al., 2004). Briefly, 1 $\times 10^6$ cells were washed twice with ice-cold PBS, and resuspended with five volumes of buffer A (in mmol/L: 20 HEPES-KOH, pH7.2, 10 KCl, 1.5 $MgCl_2$, 1.0 EDTA, 1.0 EGTA, 250 sucrose, 1.0 dithiothreitol, 0.1 phenylmethylsulfonyl fluoride, 10 aprotinin, leupeptin and pepstatin.), then kept on ice for 30 min. The cell suspension was gently homogenized with a Dounce homogenizer (5–10 strokes) and cell lysis was checked by trypan blue staining. The homogenate was centrifuged at 750 g for 5 min at 4 °C, and the supernatant subjected to further centrifugation at 10,000 g for 10 min at 4 °C. This pellet, containing mitochondria, was designated P10. The supernatant, designated S10, was subjected to further ultracentrifugation at 100,000 g for 45 min at 4 °C. The resulting pellet and supernatant were designated P100 and S100. Cytochrome C (cyt c) was not detectable in the P100 fraction.

Immuno-flowcytometry

Cells (10^6–10^7) were harvested after treatment, gently washed twice with serum free medium and blocked in 100 μl FBS for 1 h on ice. Primary antibody was added and incubated at 4 °C for another 1 h. Then cells were washed with 10 ml PBST containing 10% FBS (PBST/FBS) and resuspended in 100 μl FBS to incubate with secondary antibody (PE-2Ab) for 1 h. Finally, cells were washed with 10 ml PBST/FBS and resuspended in 500 μl PBST/FBS for detection with a FACScan (BD).

Statistical analysis

All results were expressed as mean ± SD of at least 3 independent experiments unless stated otherwise. Significance was evaluated with Student's 2-tailed, unpaired *t* test. Difference between groups were considered significant at a value of $p < 0.05$.

Result

Mechanical stretch induced cardiomyocyte apoptosis

We employed a sustained stretch model to mimic the in vivo diastolic volume stress that occurs in many cardiovascular diseases. Isolated neonatal rat cardiomyocytes (CM) were cultured on silicone membrane coated with rat-tail collagen and subjected to 20% sustained mechanical stretch up to 24 h.

Apoptosis was assessed both by examining nuclear condensation revealed by DAPI-based fluorescent microscopy, and phosphatidylserine exposure, by Annexin V-based flowcytometry. DAPI staining showed that a significant portion of cells had condensed and fragmented nuclei in 24 h stretched CM, which were smaller and/or brighter than normal ones (Fig. 1A). Flowcytometric data revealed appearance of Annexin V positive (PI negative) population after 24 h stretch (Fig. 1B, C). There was also significant increase of DEVD cleavage activity following the stretch as revealed by pNA-based assay, indicating caspase 3 activation (Fig. 1D). This DEVD cleavage activity could be completely inhibited by zVAD-fmk, a caspase inhibitor. These data demonstrated that excessive 20% sustained mechanical stretch successfully induced apoptosis and caspase 3 activation in CM.

Mechanical stretch activated death receptors signaling

In order to understand if death receptor-mediated signaling could be involved in mechanical-induced apoptosis in CM, we checked the effects of stretch on the expression of death receptor-mediated signaling molecules. Western blotting showed that stretch rapidly induced Fas and FasL expression in CM but had little influence on TRAIL or DR5 expression (Fig. 2A). Interestingly, Fas and FasL only expressed in CM but not in cardiac fibroblasts (non-cardiomyocyte). This is consistent with our previous finding that cardiac fibroblasts are resistant to stretch induced apoptosis. Since FasL/Fas and TRAIL/DR5 were not functional before they translocated to the cell membrane, we next examined the levels of FasL and TRAIL on the CM membrane by immuno-flowcytometry. After 4 h stretch, more FasL (1.9 ± 0.4 folds v.s. Control) (Fig. 2B) and TRAIL (2.0 ± 0.6 folds v.s. Control) (Fig. 2C) were found on cell surface. Since FasL expression was induced, it is easy to understand that cytomembrane accumulation of FasL increased too after stretch. However, although expression of TRAIL was not changed, stretch still resulted in cytomembrane accumulation of TRAIL. Previously, Bennett et al reported that p53 activation rapidly increased surface Fas expression by transport from the Golgi complex (surface trafficking) in human vascular smooth muscle cells (Bennett et al., 1998). It is possible that stretch may activate cell surface trafficking of these cytomembrane-bonded death ligands even in the absence of increase of their expression.

To confirm TRAIL is indeed involved in stretch induced apoptosis in CM, we utilized exogenous death ligand, TRAIL (1–170), to see if it could enhance apoptosis in CM. Our results showed that,

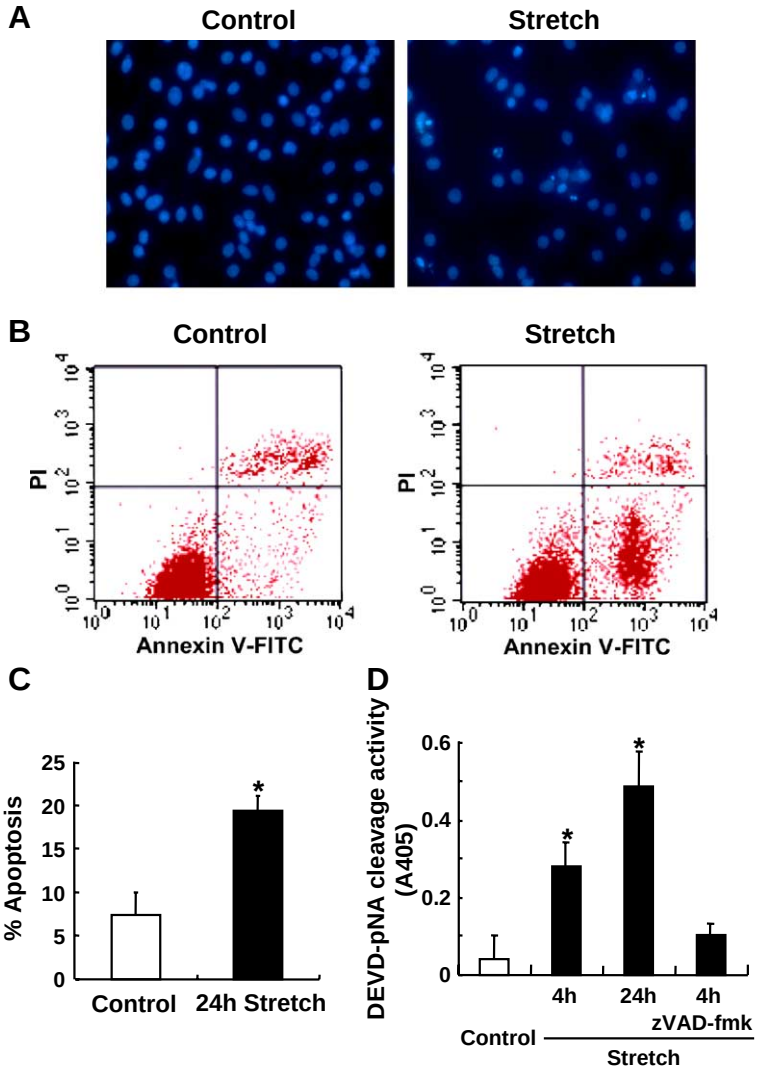

Fig. 1. Mechanical stretch induced typical apoptosis and caspase 3 activation in neonatal rat CM. Neonatal rat cardiomyocytes (CM) were cultured on silicone membrane and set to 20% sustain stretch up to 24 h as described in "material and methods". Apoptosis of CM was determined with DAPI-based fluorescent microscopy (A) and Annexin V-based flowcytometry (B). Data shown were representatives of these assays. The percent of Annexin V+/PI-cells were calculated with CellQuest software from 3 independent experiments and shown as apoptosis ratio (C). Caspase activation in CM was measured by DEVD-pNA-based assay, which showed that z-VAD-fmk (50 mmol/L) inhibit-able cleavage activities, indicating that stretch induced caspase 3 activation (D). Data were obtained from 4 independent experiments. *$p < 0.05$ *v.s.* control.

although 24 h treatment of TRAIL alone did not induce cardiomyocyte apoptosis, it significantly enhanced stretch-induced apoptosis in CM (stretch alone: 16 ± 4% v.s. Stretch plus TRAIL: 24 ± 9%) (Fig. 3), Meanwhile, soluble DR5 (sDR5), which should competitively block endogenous DR5, significantly decreased stretch-induced CM apoptosis (Stretch plus sDR5: 8 ± 3%;) (Fig. 3). These data,

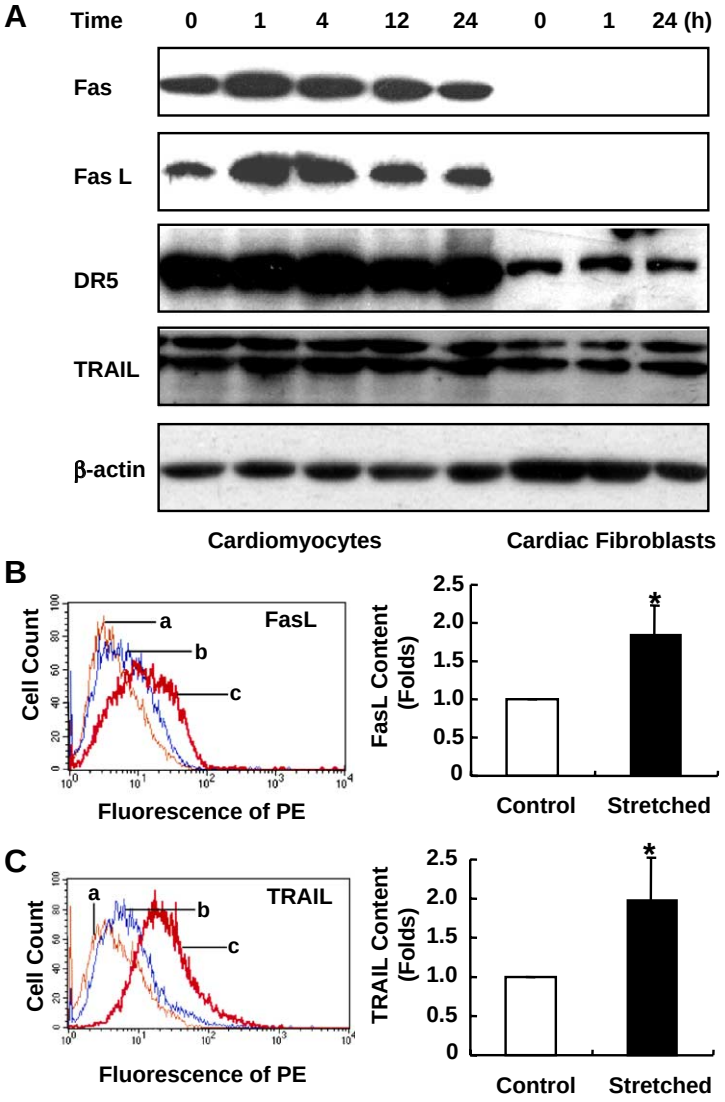

Fig. 2. Mechanical stretch activated death receptor signaling in CM. Fas, FasL, TRAIL and DR5 proteins expression was examined by Western blotting with corresponding antibodies as described in "material and methods", demonstrating that stretch induced rapid up-regulation of Fas and FasL expression in CM while had little effect on TRAIL or DR5 expression. Moreover, there were no detectable Fas or FasL expressed in non-myocyte from the same hearts (A). To further prove the activation of death receptor signaling, exposure of FasL and TRAIL on CM cytomembrane was examined with immno-flowcytometry. After 4 h stretch, cells were harvested, finely washed and incubated with anti-FasL or anti-TRAIL antibodies (same to Ab used in Western blotting), respectively. Cells without stretch were used as control. Binding of primary antibody was revealed with red fluorescent PE-labeled secondary antibody. To eliminate background binding of secondary antibody, blank sample was prepared with cells dealt with each incubation but without primary antibody. The results demonstrated that 4 h stretch induced significant increase of these death ligands trans-located to cytomembrane, indicating DR signaling activation (B, C). FasL: 1.9 ± 0.4 folds v.s. Control (n = 3) (B); TRAIL: 2.0 ± 0.6 folds v.s. Control (n = 3) (C). Lines in left panel: blank cells (a); control cells (b) and stretched cells (c). *$p < 0.05$ *v.s.* control.

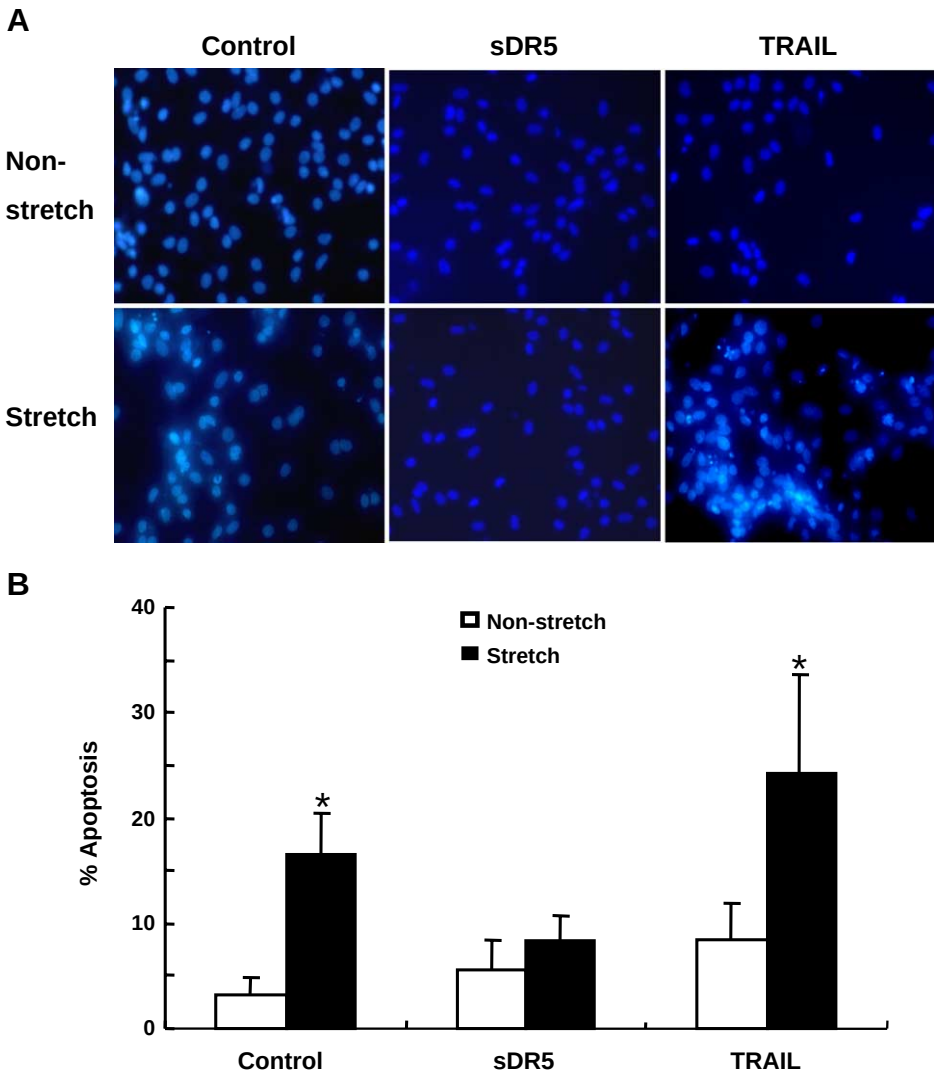

Fig. 3. Death ligand enhanced while soluble DR attenuated stretch-induced apoptosis in CM. Cultured neonatal rat cardiomyocytes were exposed to 1 μg/ml TRAIL (1–170) or 10 μg/ml soluble DR5 (sDR5) for 24 h in present or absent of stretch. Apoptosis was examined with DAPI assay (A). TRAIL or sDR5 treatment pe si had little impact on CM apoptosis. While treating with stretch, TRAIL markedly enhanced apoptosis but contrarily sDR5 attenuated apoptosis. Three experiments were performed in each group and data were shown as histogram (B). *$p < 0.05$ *v.s.* non-stretch control.

together with caspase 8 activation (described below), indicated that mechanical stretch induced by mechanical stretch utilized death receptors-mediated apoptotic signaling in CM.

FADD-DN attenuated both stretch-induced apoptosis and intrinsic apoptotic signals

To delineate the functional contribution of DR signaling in this model, we constructed recombinant adenoviral vectors carrying GFP-fused dominant negative form of FADD (Ad-GFP-FADD-DN), and

infected CM were subjected to stretch. Adenoviral gene transfer-delivered expression of GFP-FADD-DN fusion protein in CM was indicated by strong green fluorescence of GFP and further confirmed by PCR (Fig. 4A). Apoptosis assay showed that stretch resulted in a 5.2 fold increase in apoptosis of CM while FADD-DN transfer reduced apoptosis by 2.4 fold (Fig. 4B). Transfer of Ad-GFP-FADD-DN alone or control virus Ad-Lac Z, had little impact on apoptosis (Fig. 4B). FADD-DN also significantly inhibited stretch-induced activation of caspase 8 (Fig. 5A). These data suggested that stretch activated DR-mediated apoptotic pathway through FADD signaling.

We and other have shown that mechanical stretch activated mitochondria-dependent (intrinsic) apoptotic signals in CM, such as cyt c release and caspase 9 activation (Liao et al., 2004). We further investigated whether FADD-DN could impact on these intrinsic apoptotic signals. As shown in Fig. 5, besides inhibition of caspase 8, FADD-DN transfection also attenuated stretch-induced cyt c release (Fig. 5B) and activation of caspase 9 and 3 (Fig. 5C, D). These data suggested possible

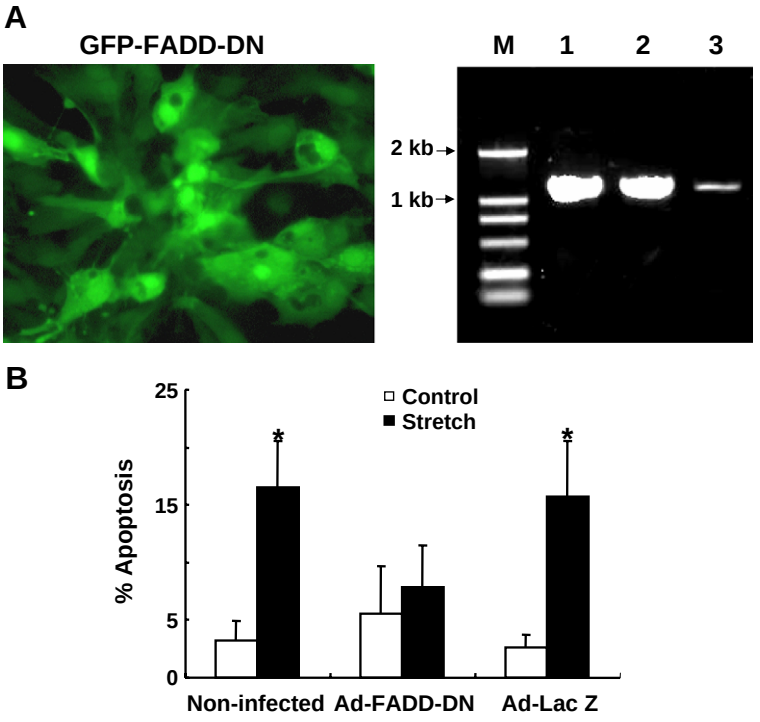

Fig. 4. FADD-DN attenuated stretch-induced apoptosis in CM. Adenovirus carrying GFP-fused dominant negative human FADD gene (Ad-GFP-FADD-DN) was recombined with the AdEasy Adenovirus system following the provider's protocol. After 24 h infection, green fluorescence of GFP was widely visible in CM, indicating success of gene transfer (A, left panel). To confirm co-existence of FADD-DN protein with GFP fluorescence, GFP-FADD-DN fusion gene was detected with PCR using the following primers: TGGTGAGCAAGGGCGAGGAG (sense, sequence from GFP) and TCAGGACGCTTCGGAGGTAG (anti-sense, sequence from human FADD) (A, right panel). Lanes: M: DNA ladder marker, *1*: pCDNA3.1-GFP-FADD-DN (original plasmid), *2*: Ad-GFP-FADD-DN plasmid, *3*: Ad-GFP-FADD-DN recombinant virus. PCR product was a 1.1 kb fragment. After changed into fresh medium, cells were set to 20% stretch for another 24 h. Adenovirus-delivered FADD-DN gene sufficiently decreased 24 h stretch-induced apoptosis in CM, as revealed by DAPI assay, while control virus had no such ability (B). $*p < 0.05$ *v.s.* control.

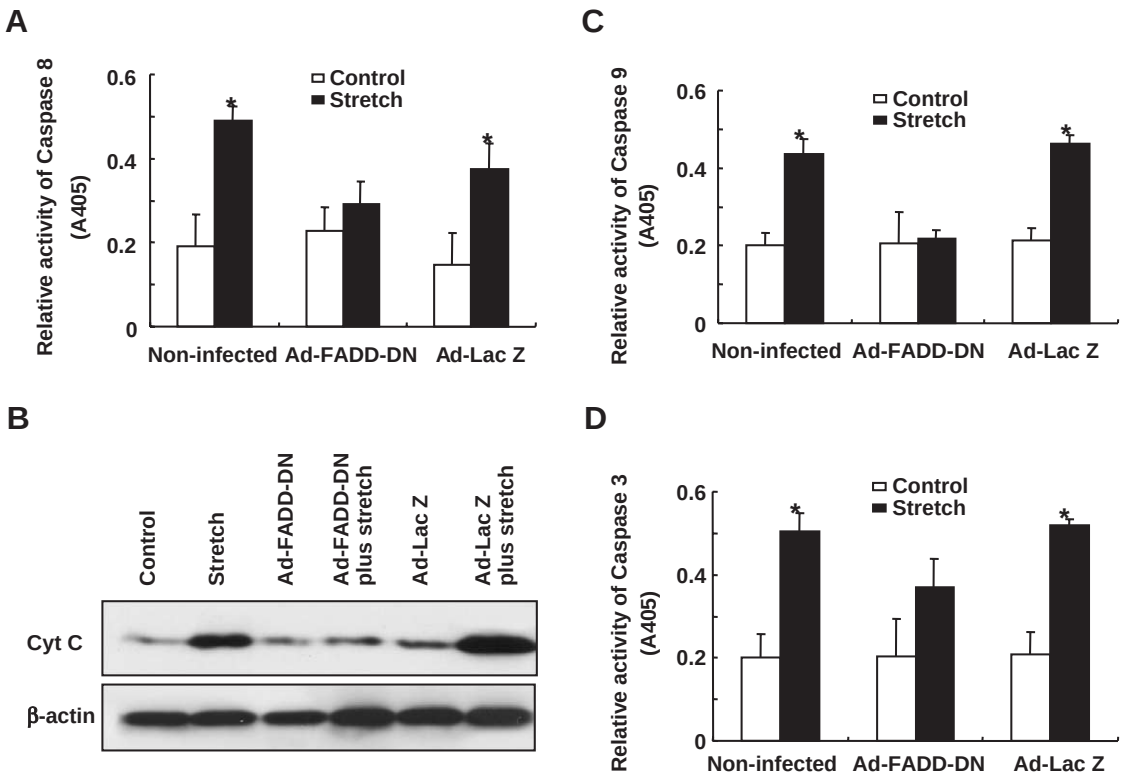

Fig. 5. FADD-DN attenuated stretch-induced caspases activation and cyt c release in CM. Cells were infected and stretch as described in Fig. 4. The result showed that 24 h stretch induced significant activation of caspase 8,9,3 in CM while FADD-DN transfer inhibited the caspases activation as demonstrated by pNA-based caspase assay (A, C, D). Cell fragmentation assay was performed to detect cyt c release from mitochondria. Western blotting showed that 24 h stretch induced profound cyt c release into cytosol in CM and FADD-DN remarkably attenuated this process (B). Data shown were from 3 independent experiments. *$p < 0.05$ *v.s.* non-infected control.

cross-talk between DR-dependent and mitochondria-dependent apoptosis pathways induced by stretch in CM.

Discussion

The present study was designed to investigate the role of death receptor (DR) signaling in mechanical stretch-induced cardiomyocyte apoptosis through manipulation of the critical adaptor molecule, FADD.

We provided several lines of evidences that suggested the involvement of FADD-linked DR signaling in regulation of mechanical stretch-induced apoptosis. First, the excessive 20% sustain stretch induced not only apoptosis in CM but also activation of caspases 8, 9 and 3, up-regulation of Fas, FasL expression and cytomembrane trafficking of death ligands (FasL and TRAIL) in CM. Second, exogenous death ligand (TRAIL) stimulation enhanced while soluble DR (sDR5) neutralization

attenuated stretch-induced apoptosis in CM. Third, adenovirus-delivered dominant negative FADD (FADD-DN) significantly decreased stretch-induced apoptosis and caspases 8, 9, and 3 activation in CM. Fourth, FADD-DN also attenuated stretch-induced cyt c release from mitochondria.

These data demonstrated the activation of death receptors signaling induced by mechanical stretch in CM and suggested that FADD-mediated activation of caspase 8 was partially responsible for cyt c release. Because FADD is a critical mediator of DR apoptotic signaling, the ability of FADD-DN to inhibit apoptosis suggests the involvement of the death receptors. To the best of our knowledge, this study is the first to demonstrate that death receptors-mediated apoptotic signaling is involved in cardiomyocyte apoptosis activated by stretch.

Consistent with the previous studies (Wollert et al., 2000; Cheng et al., 1995; Nelson et al., 2000), we showed mechanical stretch induced up-regulation of Fas and FasL expression. Although stretch showed little impact on TRAIL or DR5 expression, we showed that stretch induced increased levels of cell surface FasL and TRAIL, evidences for engagement of DR signaling (Bennett et al., 1998). In contrast to our results, Fas signaling has been reported to participate in regulation of hypertrophy rather than apoptosis (Wollert et al., 2000; Nelson et al., 2000). These contracting results may reflect differences between experimental conditions employed. For example, Nelson et al used FasL transgenic mice to study FasL-induced cardiomyocyte apoptosis (Nelson et al., 2000). Considering the complicated in vivo environment and the various dose of transgenic gene expression, it should be hard to verify the relationship between FasL and CM apoptosis in their study. In another in vitro study, Wollert et al reported that treating isolated CM with soluble recombinant FasL pe si resulted in no detectable apoptosis (Wollert et al., 2000). In the current study, we found similar results when treating CM with recombinant TRAIL (1–170) alone, while it enhanced cell death under stretch conditions (Fig. 3). In supporting this, Jeremias et al have have found that treatment with death ligands (TNF-α , FasL and TRAIL) did induce significant apoptosis in CM after stimulated ischemia (Jeremias et al., 2000) and they showed that some stress stimuli in CM cause marked increase in sensitivity to the effects of death ligands. In the present study, mechanical stretch may server as a stress stimulus, which sensitized CM to apoptosis stimuli, such as TRAIL treatment. Given that mechanical overload results in simultaneous hypertrophy and apoptosis in CM, events leading to heart remodeling, it is reasonable to propose that DR signaling has dual roles, hypertrophic as reported previously or apoptotic as seen in current study.

Our data also suggest possibility for cross-talk between the intrinsic and extrinsic apoptosis pathways induced by stretch in CM, since cty c release as well as caspase 9 activation, which are hallmarks of intrinsic apoptotic signaling, were partially dependent on FADD signaling (Fig. 5). It has been well demonstrated that Bid links caspase 8 to cyt c release (Luo et al., 1998; Li et al., 1998). Unfortunately, our efforts to identify Bid cleavage in this rat system have thus far been unsuccessful using available commercial antibodies against Bid. Although further evidence is required, the possibility exists for involvement of Bid in our model. Besides caspase 8, DR signaling may also employ other caspases that act on mitochondria to execute stretch-induced apoptosis in CM. Further investigation is required to elucidate the connections between the intrinsic and extrinsic pathways of apoptosis induced by mechanical stretch.

Then the question arises as to the degree of how mechanical stretch initiates death receptors signaling in cardiomyocyte. Since stretch was reported to induce angiotensin II secretion in CM (Leri et al., 1998), and there is evidence indicating angiotensin II signaling was required in Fas-induced apoptosis in epithelial cells (Wang et al., 1999), stretch-induced angiotensin II may also

participate in DR signaling in CM (Leri et al., 1998). Moreover, p53 has been shown to activated Fas apoptotic signaling in human vascular smooth muscle cells (Bennett et al., 1998). Given that stretch also activated p53 in CM (Leri et al., 1998; Liao et al., 2004), it would be reasonable to propose the involvement of p53 signaling in activation of death receptors in current study.

It has been reported that Fas/FasL system can function as a sensor for intrinsic apoptotic signals, such as p53 activation or DNA damage in some settings (Bennett et al., 1998; Hill et al., 1999). Based on this view, DR signaling may serve as a sensor of stress signals in many cells types, including cardiac cells. Considering that DRs and their ligands (Fas/FasL, TNFR1/TNF-α, TRAIL, etc.) are expressed constitutively in heart, DR signaling may play important roles in regulation of cellular activities in heart cells, including apoptosis. In this work, we did not identify which death receptor is dominant involved in signaling via FADD, which is worth of further investigation.

Conclusion

Signaling mechanism of mechanical overload-induced cardiomyocyte apoptosis is controversial. The results of the present study suggest that FADD-linked death receptor signaling is involved in regulation of cardiomyocyte apoptosis induced by stretch. Further study is required to identify the responsible DR that regulates stretch-induced cardiomyocyte apoptosis. Apoptosis has been recognized as a basic mechanism of cardiovascular diseases (Haunstetter and Izumo, 1998). However, it is just a beginning to understand its roles in mechanical overload-related heart diseases. Further delineation of the intracellular signaling pathways leading to cardiomyocyte apoptosis may offer novel promises for therapeutic interventions of cardiovascular diseases.

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