Functional roles of tissue transglutaminase in apoptotic mechanisms of cardiomyocytes

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Functional roles of tissue transglutaminase in apoptotic mechanisms of cardiomyocytes

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I dedicate the dissertation to my parents and my loving family.

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Tissue transglutaminase (tTG) is ubiquitously expressed in mammalian tissues; found both extracellularly at the cell surface in association with the extracellular matrix and intracellularly in membrane-associated as well as cytosolic forms. The physiological role of tTG remains unclear in the cellular function. The majority of studies support the notion that transamidation by tTG can both facilitate and inhibit apoptosis, while the GTP-bound form of the enzyme generally protects cells against death. In that, many previous studies indicated Janus face of tTG in the apoptotic program. In this study, we hypothesize that tTG plays a pivotal role in neonatal rat ventricular cardiomyocyte under apoptotic condition. Neonatal rat cardiomyocytes were treated with 500 μM H_2O_2 for 2 hours or 100 μM retinoic acids for 24 hours. Immunocytochemistry and flow cytometry was performed to determine more

increased expression of tTG after apoptotic stimulation. Caspase-3 assay demonstrated considerable correlation between tTG expression and apoptosis. Immunoblot assay analysis demonstrated increased tTG expression significantly inhibit survival or anti-apoptotic signal molecules such as p-ERK1, 2, p-Akt and Bcl-2 but enhance activation of caspase-8, cytochrome c and Bax. tTG expression also regulated intracellular Ca^{2+} overload because cardiomyocytes transfected with tTG under treatment of 500 μM H_2O_2 induced reduction of PLC-δ1 expression and PKC activation. Furthermore, our data showed that tTG expression and tTG-related apoptotic signal molecules were controlled by calreticulin (CRT) expression level. Therefore, the results from this study show that tTG expression level modulates apoptotic signal molecule and undergo on intracellular calcium level and signal.

Key Words: Cardiomyocytes, tissue traansglutaminase (tTG), Apoptosis, Hydrogen Peroxide, Retinoic Acids

Functional roles of tissue transglutaminase

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I. INTRODUCTION

A novel GTPase [termed as $G\alpha_h$ and tissue transglutaminase (tTG)], observed in rat liver plasma membrane as a mediator of transmembrane signaling, also has transglutaminase (TGase) activity.[1] Because tTG binds and hydrolyzes GTP with an affinity and catalytic rate similar to the α subunits of large heterotrimeric G proteins and small Ras-type G proteins, the GTPase reaction of tTG transduces signals from receptors to effectors, and the subsequent association of GDP-bound tTG with calreticulin (CRT) completes one cycle of signaling.[2-4] tTG couples α_{1b-} and α_{1d-} adrenoreceptors, thromboxane and oxytocin receptors to phospholipase C (PLCδ1), mediating inositol phosphate production in response to agonist activation. Gαh, also known as tTG (tissue Tgase), is a member of the TGase family (thiol- and

Ca^{2+}-dependent acyl transferases family) that catalyzes the catalyze the formation of a covalent bond between the γ-carboxamide groups of peptide-bound glutamine residues and various primary amines, including the ε-amino group of lysine in certain proteins.

In general, tTG is ubiquitously expressed in mammalian tissues, found both extracellularly at the cell surface in association with the extracellular matrix and intracellularly in membrane-associated as well as cytosolic forms.[5, 6] The physiological role of the TGase function of Gαh remains unclear, and the regulation of isopeptide formation is completely unknown. The formation of irreversible isopepetides in a number of proteins has been observed during diverse cellular processes, including cell differentiation, cell adhesion, cross-linking of interacting extracellular matrix, and induction of apoptosis.[7-10] The GTPase activity of $G\alpha_h$ has been shown to be involved in regulation of cell cycle progression.[11]

tTG is often upregulated in cells undergoing apoptosis. In cultured cells tTG may exert both pro- and anti-apoptotic effects depending upon the type of cell, the kind of death stimuli, the intracellular localization of the enzyme and the type of its activities switched on. The majority of studies support the notion that transamidation by tTG can both facilitate and inhibit apoptosis,

while the GTP-bound form of the enzyme generally protects cells against death. But many previous studies indicated Janus face of tTG in the apoptotic program.

Ischemia/reperfusion injury (I/R) is a common cardiac pathology resulting from resumption of blood flow in occluded coronary arteries. Myocardial ischemia initiates a range of cellular events, which are initially mild and become progressively damaging with increasing duration of ischemia. Although the meaning of reperfusion is a termination of ischemia and essential condition for cellular survival and restoration of normal function, it paradoxically causes damage to the cell. The major identified mediators of I/R injury are the reactive oxygen species (ROS) which include H_2O_2, the superoxide anion O_2^-, and OH^-.[12,13] Indeed, in recent years abundant reports have pointed to the important contribution of apoptosis to the underlying cardiac dysfunction in major heart pathologies, including ischemic heart diseases.[14,15] Most of the apoptotic damage occurs during the reperfusion phase, emphasizing the key role of ROS in the apoptotic damage in the ischemic/reperfused myocardium.

To investigate to what extent the change of tTG expression is a key biochemical event in the death program, we studied the effect on apoptosis of

H_2O_2-stimulated cardiomyocyte stably transfected with plasmids containing tTG cDNA or siRNA for tTG. Myocardial apoptosis involved in tTG in neonatal rat ventricular cardiomyocyte was characterized further by examining the change of protein related to apoptotic and calcium signals.

II. MATERIALS AND METHODS

1. Isolation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were isolated and purified by previously described methods. Briefly, hearts of 1-2 day-old Sprague Dawley rat pups were dissected and the ventricles washed with Dulbecco's phosphate-buffered saline solution (PBS) (pH 7.4, Gibco BRL) lacking Ca^{2+} and Mg^{2+}. Using micro-dissecting scissors, hearts were minced until the pieces were approximately 1 mm^3 and treated with 10ml of collagenase I (0.8 mg/ml, 262 units/mg, Gibco BRL) for 15 minutes at 37°C. The supernatant was then removed and the tissue treated with fresh collagenase I solution for an additional 15 minutes. The cells in the supernatant were transferred to a tube containing cell culture medium (α-MEM containing 10% fetal bovine serum,

Gibco BRL). The tubes were centrifuged at 1200 rpm for 4 minutes at room temperature, and the cell pellet was resuspended in 5 ml of cell culture medium. The above procedures were repeated 7-9 times until little tissue remained. Cell suspensions were collected and incubated in 100mm tissue culture dishes for 1-3 hours to reduce fibroblast contamination. The non-adherent cells were collected and seeded to achieve a final concentration of 5 x 10^5 cells/ml. After incubation for 4-6 hours, the cells were rinsed twice with cell culture medium and 0.1 μM BrdU was added. Cells were then cultured with cell culture medium in a CO_2 incubator at 37°C.

2. Immunocytochemistry

Cells were grown on 4-well plastic dishes (SonicSeal Slide, Nalge Nunc, Rochester, NY, USA). Following incubation, the cells were washed twice with PBS and then fixed with 4% paraformaldehyde in 0.5 ml PBS for 30 min at room temperature. The cells were washed again with PBS and then permeabilized for 30 min in PBS containing 0.2% triton. The cells were then blocked in PBS containing 10% goat serum and then incubated for 1 hr with rabbit polyclonal TG2 antibody. The cells were rewashed three times for 10 min with PBS and incubated with FITC-conjugated goat anti-rabbit antibody

as the secondary antibody for 1 hr. Photographs of cells were taken under fluorescence by immunofluorescence microscopy (Olympus, Melville, NY, USA). All images were made by using an excitation filter under reflected light fluorescence microscopy and transferred to a computer equipped with MetaMorph software ver. 4.6 (Universal Imaging Corp.). All images were made by using an excitation filter under reflected light fluorescence microscopy and transferred to a computer equipped with MetaMorph software ver. 4.6 (Universal Imaging Corp.).

3. Flow cytometry

Cardiomyocytes were retrieved with a standard trypsinization technique. Cells were washed in phosphate buffered saline (PBS) and fixed in 70% ethanol at 4 °C for 30 min with agitation. Cells were washed twice in PBS and resuspended at 2×106 cells/ml in blocking buffer (1% BSA, 0.1% FBS) containing anti-TG2 rabbit antibody diluted at 1: 200. The labeling reaction mixture was agitated for 20 min at room temperature. Cells were washed twice and then labeled with anti-rabbit-FITC conjugated IgG (Jackson ImmunoResearch Laboratories, Inc.PA, USA) diluted to 1:400 for 20 min at room temperature in the dark. After two more washes, flow cytometric

analysis was performed on a FACSCalibur system (Becton Dickinson, CA, USA) using CellQuest™ software with 10,000 events recorded for each sample. Data was acquired in single parameter histogram with appropriate particle size and light scatter gating.

4. Immunoblot analysis

Cells were washed once in PBS and lysed in a lysis buffer (Cell signaling, Beverly, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin and 1 mM PMSF. Protein concentrations were determined using the Bradford protein assay kit (BioRad, Hercules, CA, USA). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co, Bedford, MA, USA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% non-fat dried milk for 1 hr at room temperature, membranes were washed twice with TBS-T and incubated with primary antibodies for 1 hr at room temperature or for overnight at 4°C. The membrane were washed three times with TBS-T for 10 min, and then incubated for 1 hr at room temperature with horseradish

peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities were quantified using a Photo-Image System (Molecular Dynamics, Uppsala, Sweden).

5. Confocal microscopy and fluorescence measurements

The measurement of cytosolic free Ca^{2+} was performed by confocal microscopy analysis. Neonatal rat cardiomyocytes were plated on glass coverslips coated with laminin (5 mg/cm^2) for 1 day in cell culture medium (α-MEM containing 10% fetal bovine serum, Gibco BRL) and 0.1 μM BrdU. After incubation, the cells were washed with modified Tyrode's solution containing 0.265 g/l $CaCl_2$, 0.214 g/l $MgCl_2$, 0.2 g/l KCl, 8.0 g/l NaCl, 1 g/l glucose, 0.05 g/l NaH2PO4 and 1.0 g/l NaHCO3. Cells were then loaded with 5 mM of the acetoxymethyl ester of Fluo-4 (Fluo-4 AM, Molecular Probes, Eugene, OR) for 20 min, in the dark and at room temperature, by incubation in modified Tyrode's solution. Fluorescence images were obtained using an argon laser confocal microscope (Carl Zeiss Inc., Thornwood, NY). This fluorochrome is excited by the 488 nm line of an

argon laser and emitted light is collected through a 510-560 nm bandpass filter. Relative changes of free intracellular Ca^{2+} were determined by measuring fluorescent intensity.

6. Caspase-3 assay

Relative caspase-3 activity was determined using the ApopTarget™ Capase-3 Colorimetric Protease Assay, according to manufacturer's instructions (Biosource). This assay is based on the generation of free DEVD-*p*NA chromophore when the provided substrate is cleaved by caspase-3. Upon cleavage of the substrate by caspase-3, free *p*NA light absorbance can be quantified using a microplate reader at 405nm. Briefly, the cultured neonatal cardiomyocytes (3×10^6) after different treatments were harvested in lysis buffer (1M DTT), and cell extracts were centrifuged to eliminate cellular debris. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Aliquots (50 µl) of the cell extracts were incubated at 37°C for 2 h in the presence of the chromophore substrate. Free DEVD- *p*NA was determined colormetrically. The comparison of absorbance of *p*NA from apoptotic samples with uninduced controls allowed determination of the fold increase in caspase-3 activity.

7. Statistical analysis

Data are presented as mean ± S.E.M. of more than three separate experiments performed in triplicate. Where results of blots are shown, a representative experiment is depicted. Comparisons between multiple groups were performed with one-way ANOVA (Analysis of Variance) with Bonferroni's test. Statistical significance was defined as $p < 0.05$ and $p < 0.01$.

III. RESULTS

1. Change of tTG expression in apoptotic cardiomyocyte

To determine whether the treatment of cardiomyocytes with H_2O_2 led to its enhanced expression of tTG, we measured tTG levels by immunocytochemistry and flow cytometry. Confocal microscopy demonstrates low levels of immunoreactive tTG in the cytosol of control rat neonatal cardiomyocytes but stimulation of 500 μM H_2O_2 for 2 hours induced the increased level of immunoreactive tTG (Figure 1A). At the same time, flow cytometry results established the fact that stimulation of 500 μM H_2O_2 for 2 hours in cardiomyocyte increased the level of surface tTG protein

expression (Figure 1B).

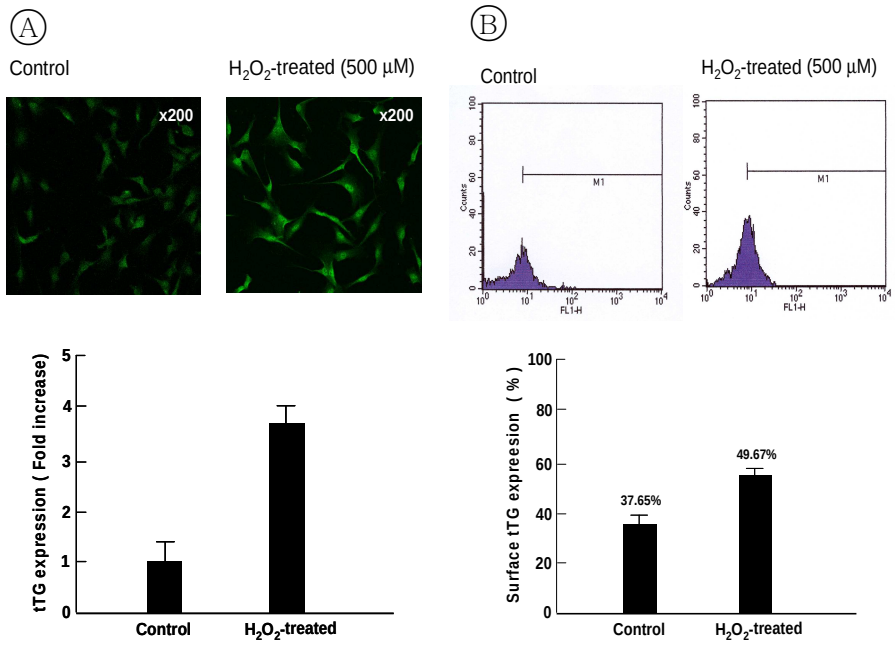

Figure 1. Change of tTG expression in apoptotic cardiomyocytes induced by H_2O_2. Cardiomyocytes were plated in triplicate wells of 4 well plates and treated with 500 μM H_2O_2 for 2 hours. Change of total tTG expression in cardiomyocytes was detected by the immunocytochemistry. Each result was quantified by scanning densitometry (A). Flow cytometry of surface tTG in cardiomyocytes exposed to 500 μM H_2O_2 for 2 hours (B). These experiments were repeated three times.

tTG is induced in a variety of cell types on exposure to retinoic acid (RA). Figure 2 shows similar activation of tTG when cardiomyocytes are stimulated

with RA. The induction of tTG with atRA is dose-dependent. Increasing steady-state expression levels of tTG emerge as low as 20 μM RA, with peak expression at 100 μM RA.

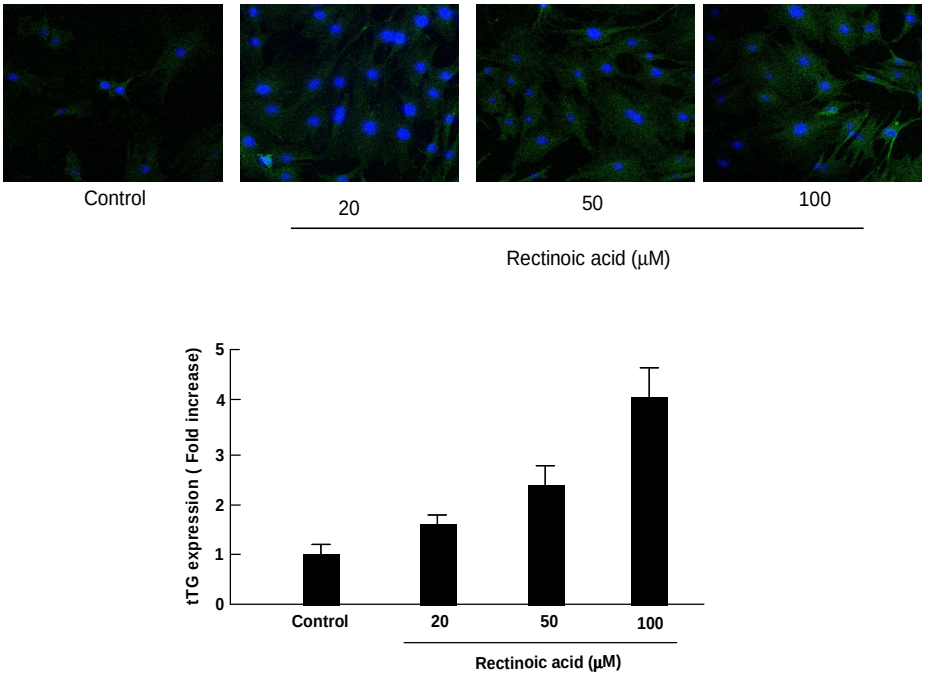

Figure 2. Change of tTG expression in retinoic acid-treated cardiomyocytes. Cardiomyocytes were plated in triplicate wells of 4 well plates and treated with various concentration of retinoic acid for 24 hours. Change of total tTG expression in cardiomyocytes was detected by the immunocytochemistry. Each result was quantified by scanning densitometry and repeated three times.

2. Relation of tTG expression with apoptotic caspase-3 activity

Our present study has shown that H_2O_2 and RA, known as an inducer of apoptosis in cells, stimulate the expression of tTG. To determine whether the effect of increased tTG expression on the apoptosis of cardiomyocytes, cardiomyocytes were treated with or without 500 μM H_2O_2 and 100 μM RA, which induced increased expression level of tTG and changes of caspase-3 activity was measured. After stimulation of H_2O_2 or RA, the activity of caspase-3 was more increased than normal control (without H_2O_2 or RA) (Figure 3A and B).

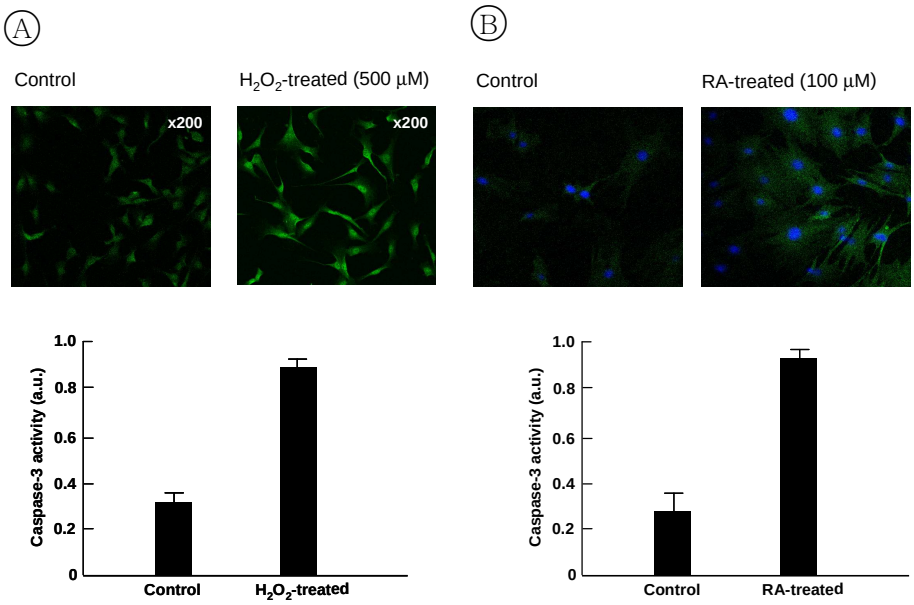

Figure 3. Relation of tTG expression with apoptotic caspase-3 activity.

Cardiomyocytes were plated in triplicate wells of 4 well plates and treated with 500 μM H_2O_2 (A) or 100 μM Retinoic acid (B). Change of total tTG expression in cardiomyocytes was detected by the immunocytochemistry.

Figure 4 demonstrated the relation between expression level of tTG and program cell death. The expression level of tTG was clearly increased within 24 hours after H_2O_2 stimulation. tTG protein remained elevated up to 24hours and shorter time-course studies reveal increases in tTG protein as early as 2 hours after H_2O_2 administration. Furthermore, the activity of caspase-3 was also increased according to increased expression of tTG.

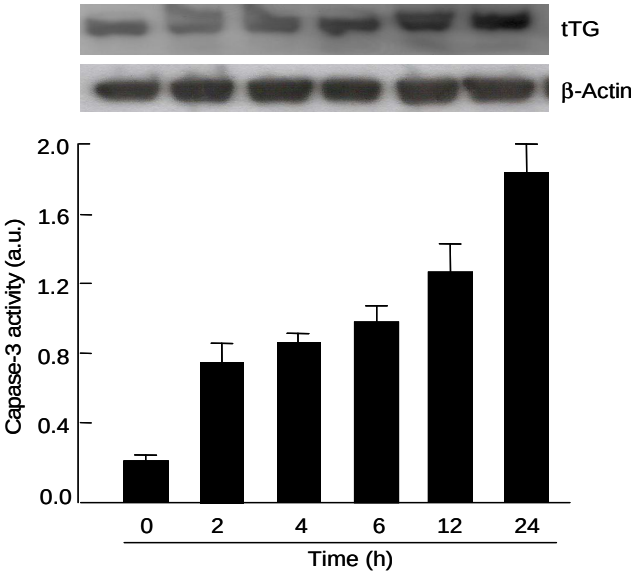

Figure 4. Relation of tTG expression with caspase-3 activity in cardiomyocytes induced by H_2O_2. Western blot analysis of tTG in cardiomyocytes exposed to 500 μM H_2O_2 within 24 hours after H_2O_2 stimulation. Each signal was quantified by scanning densitometry. Western blot was repeated three times. Relative caspase-3 activity was determined using the ApopTarget™ Capase-3 Colorimetric Protease Assay in cardiomyocytes stimulated with 500 μM H_2O_2 within 24 hours after H_2O_2 stimulation. Data denote the means ± S.E.M. of 2~3 replicate measurements in three different cell cultures.

3. Effect of tTG expression level on proliferation and survival signal molecules

In the mechanisms of cellular survival and proliferation, the activation of ERKs plays an important role in gene regulation and PI3K/Akt signaling pathway is also pro-survival and anti-apoptotic signal. Akt is phosphorylated at two sites due to activation of enzyme activity: Thr308 in the catalytic domain and Ser473 in the cytoplasmic domain. ERK is one of dual specificity kinases in MAPKs. Phosphorylation of Akt at Ser473 and ERKs (42 and 44 kDa) was detected by immunoblot assay. The phosphorylation activities of both Akt and ERK were decreased in 500 μM H_2O_2 stimulated cardiomyocyte

compared with normal cells and cells transfected with tTG induced more decreased activities of both Akt and ERK. But the activities of Akt and ERK were increased in tTG down-expressed cells cells (Figure 5).

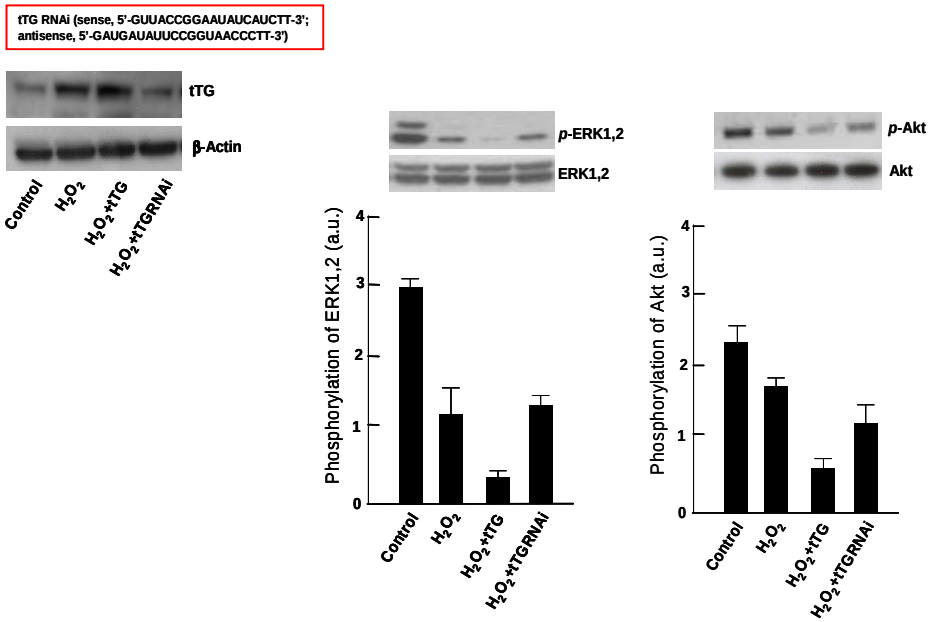

Figure 5. Effect of tTG on activity of proteins related to survival of H_2O_2 stimulated cardiomyocytes. Cardiomyocytes were treated with siRNA for 12hr to block each gene, and then incubated for 2 hours with 500 μM H_2O_2. Western blot analysis of phosphorylaton of ERK and Akt in cardiomyocytes exposed to 500 μM H_2O_2 for 2 hours. Each signal was quantified by scanning densitometry and the figure shows the level of each activity relative to the maximal level of p-ERK and p-Akt. Western blots were repeated three times.

4. Effect of tTG expression level on apoptotic signal molecules

It has been known that H_2O_2 is one of apoptotic stimulus in cardiomyocyte. Apoptotic cell death is triggered by extrinsic, receptor-mediated, or intrinsic, mitochondria-mediated, signaling pathways that induce death-associated proteolytic and/or nucleolytic activities. The net result of extrinsic apoptotic signaling is the activation of caspase-8 and intrinsic apoptosis through the mitochondrial pathway is partly regulated by the interaction of Bcl-2 family proteins. These proteins may be anti-apoptotic (Bcl-2) or pro-apoptotic (Bax) and pro-apoptotic protein induces conformational change which triggers the release of cytochrome c from the mitochondrial membrane space into the cytosol. H_2O_2 induces the increased expression of pro-apoptotic protein, caspase-8, Bax and cytochrome c and the decreased expression of anti-apoptotic protein, Bcl-2 than normal condition. Following change of tTG expression in 500 μM H_2O_2 stimulated cardiomyocyte, the expression level of caspase-8, Bax and cytochrome c was more increased in tTG over-expressed cells but inhibition of tTG expression in 500 μM H_2O_2 stimulated cardiomyocyte induced the decreased caspase-8, Bax and cytochrome c expression (Figure 6, 7A and B). The case of Bcl-2 expression was dramatically opposed to the pattern of Bax and cytochrome c expression

(Figure 7C).

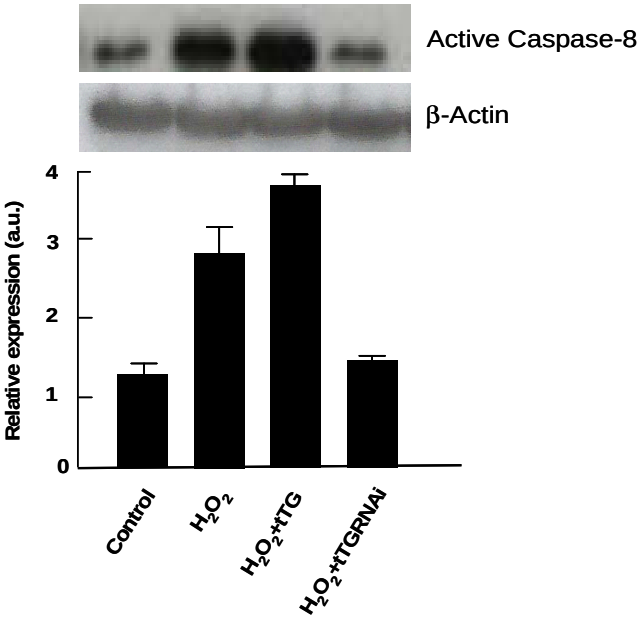

Figure 6. Effect of tTG on activity of proteins related to extrinsic apoptosis in cardiomyocytes. Cardiomyocytes were treated with siRNA for 12hr to block each gene, then incubated for 2 hours with 500 μM H_2O_2.Western blot analysis of caspase-8 in cardiomyocytes exposed to 500 μM H_2O_2 for 2 hours. Each signal was quantified by scanning densitometry and the figure showed the levels of each activity as relative value of the maximal level of active caspase-8. Western blot was repeated three times.

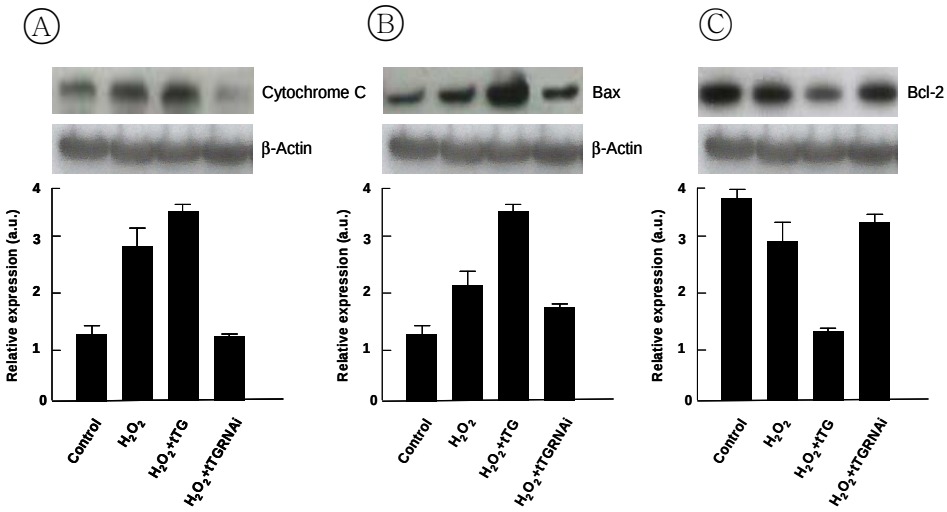

Figure 7. Effect of tTG on activity of proteins related to intrinsic apoptosis in cardiomyocytes. Cardiomyocytes were treated with siRNA for 12hr to block each gene, and then incubated for 2 hours with 500 μM H_2O_2.Western blot analysis of cytochrome c, Bax and Bcl-2 in cardiomyocytes exposed to 500 μM H_2O_2 for 2 hours. Each signal was quantified by scanning densitometry and the figure showed the levels of each activity as relative value of the maximal level of cytochrome c, Bax and Bcl-2. Western blot was repeated three times.

5. Effect of tTG expression level on calcium overload and related signal molecules

Phospholipase C (PLC) hydrolyzes the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2) to generate diacylglycerol

(DAG) and inositol 1,4,5-triphosphate (IP3). DAG and IP3 mediate the activation of protein kinase C (PKC) and intracellular Ca^{2+} mobilization, respectively. Reactive oxygen species can be generated by H_2O_2 and promote large entry of Ca^{2+} into rat neonatal cardiomyocyte. To examine intracellular Ca^{2+} overload in 500 μM H_2O_2 stimulated cardiomyocyte, the fluorescence intensity method was used in cardiomyocyte loaded with fluo-4 AM. The result shows that the intracellular calcium level of 500 μM H_2O_2 stimulated cells was much more increased than normal cells but these cells transfected with tTG or siRNA for tTG induced the increase or reduction of intracellular calcium level (Figure 8).

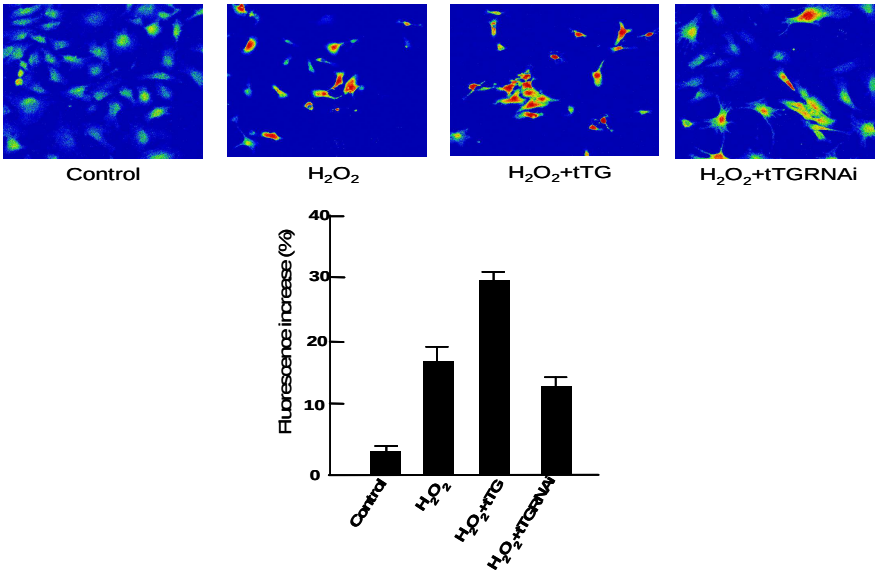

Figure 8. Effect of tTG on intracellular calcium concentration of apoptotic cardiomyocytes induced by H_2O_2. Neonatal cardiomyocytes were transfected with or not siRNA to a final concentration of 10nM or vehicle then incubated for 2 hours with 500 μM H_2O_2. Cytosolic free Ca^{2+} concentration was determined with relative fluorescence intensity. Data denote the means ± S.E. of 2~3 replicate measurements in three different cell cultures.

Additionally, the pattern of PLC-δ1 expression and PKC activation was the same as intracellular Ca^{2+} change of above data. PLC-δ1 expression and PKC activation in tTG over-expressed cells were more decreased than only 500 μM H_2O_2 stimulated cells but those of tTG down-expressed cells were enhanced (Figure 9).

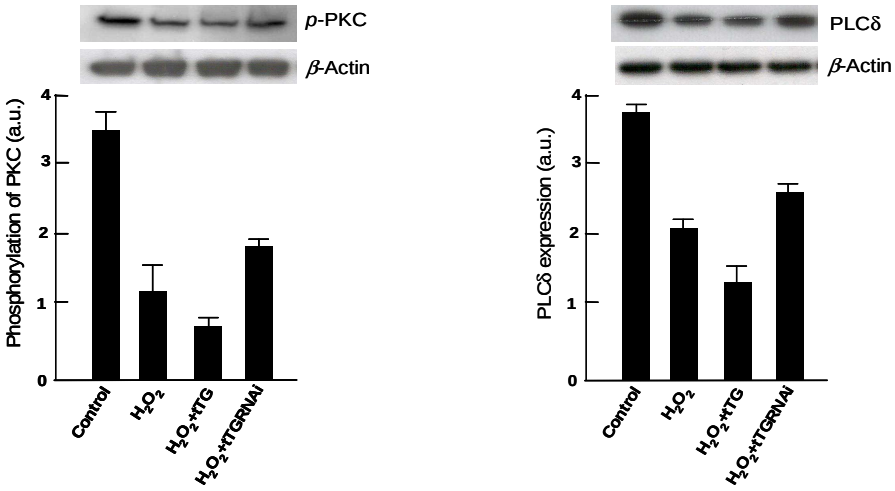

Figure 9. Effect of tTG on activity of proteins related to calcium overload in cardiomyocytes. Cardiomyocytes were treated with siRNA for 12hr to block each gene, and then incubated for 2 hours with 500 μM H_2O_2. Western blot analysis of PKC and PLCδ in cardiomyocytes exposed to 500 μM H_2O_2 for 2 hours. Each signal was quantified by scanning densitometry and the figure showed the levels of each activity as relative value of the maximal level of PKC and PLCδ. Western blot was repeated three times.

6. Effect of CRT expression level on tTG related signal molecules

To examine whether calreticulin (CRT) influences the change of tTG related signal molecules, the change of tTG expression and pro-/anti-survival signal molecules by CRT was examined. Our results demonstrated that CRT over-expressed cardiomyocytes enhanced tTG expression much more than cells treated with only 500 μM H_2O_2. On the other hand, the expression of tTG in cells transfected with siRNA for CRT was decreased (Figure 10B). Due to this change of tTG expression by CRT, survival and apoptotic signaling molecules were also controlled by CRT expression. That is, overexpression of CRT in 500 μM H_2O_2 stimulated cardiomyocytes leaded the increase of caspase-3 activity and the decrease of ERK phosphorylation compared with

H_2O_2 only treated cells (Figure 10A and C).

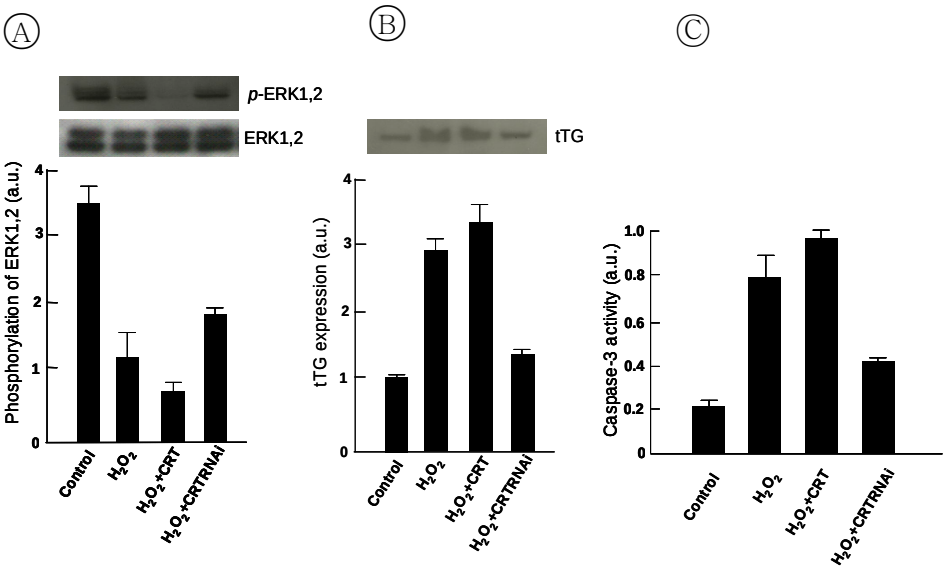

Figure 10. Effect of CRT expression on tTG-related signal molecules in cardiomyocytes. Cardiomyocytes were treated with siRNA for 12hr to block each gene, then incubated for 2 hours with 500 μM H_2O_2. Western blot analysis of ERK and tTG in cardiomyocytes exposed to 500 μM H_2O_2 for 2 hours. Each signal was quantified by scanning densitometry and the figure showed the levels of each activity as relative value of the maximal level of ERK and tTG. Western blot was repeated three times (A and B). Relative caspase-3 activity was determined using the ApopTarget™ Capase-3 Colorimetric Protease Assay in cardiomyocytes stimulated with 500 μM H_2O_2 within 24 hours after H_2O_2 stimulation. Data denote the means ± S.E.M. of 2~3 replicate measurements in three different cell cultures (C).

IV. DISCUSSION

The present study demonstrates that a novel GTPase, termed as $G\alpha_h$ and TG2, plays a pivotal role in neonatal rat ventricular cardiomyocyte under apoptotic condition. In addition, the expression level of tTG effect on myocardial survival under pathologic condition. The results indicate that H_2O_2 induced apoptosis in cardiomyocyte but cardiomyocyte transfected with SiRNA for tTG increased anti-apoptotic signals.

tTG is the most ubiquitous isoform belonging to TGases family. tTG is a versatile multifunctional protein involved in a variety of biochemical functions at various cellular locations.[16-18] Depending upon the nature of the group entering in the reaction, Ca^{2+}-dependent cross-linking activity of tTG is responsible for different related modifications such as incorporation of amine into proteins, protein-protein cross-linking as well as site-specific deamidation (if the entering group is a water molecule instead of an amine).[16] Mainly under pathological settings, tTG can also act extracellularly, it can be exposed on the external leaflet of the plasma membrane or released from cells, where it has been suggested to mediate the interaction between integrins and fibronectin with extracellular matrix (ECM).[19, 20]

In addition to the above mentioned activities, tTG can act as G-protein

binding and hydrolyzing GTP with an affinity and a catalytic rate similar to the α-subunit of large heterotrimeric G proteins and small Ras-type G proteins.[21] Under such circumstances, tTG couples α_{1b} and α_{1d} adrenoreceptors, thromboxane and oxytocin receptors to phospholipase C (PLC-δ1).[22] When the enzyme is in a GTP/GDP-bound form, it cannot act as transglutaminase.[23] The inhibition is suspended by Ca^{2+} which plays a role as molecular switch between these two functions.[24] Finally, based on in vitro observations, a Ca^{2+}-independent protein disulfide isomerase (PDI) function has been proposed for this enzyme. The PDI activity seems to rely on an independent active domain from that used for TGase activity, being the cysteine of this latter active site not responsible for PDI activity itself .[25]

The tTG gene is induced in cells programmed to die during embryonic development as well as in cells undergoing apoptosis in various physiological and pathological contexts.[26-30] Studies from different laboratories have suggested that tTG might have more than one function within the cascade of events leading to the establishment of the apoptotic phenotype. The data reported in previous paper confirmed that tTG indeed has a role in priming cells for apoptosis; in fact, overexpression of tTG in human neuroblastoma cells significantly increases both spontaneous and induced apoptosis. In the

last 15 years more than 300 papers have clearly shown that tTG acts as a proapoptotic enzyme. In keeping with this notion, previous studies indicated tTG KO mice show defects in the clearance and stability of apoptotic cells.[31] However, recently antiapoptotic activities for the enzyme have also been reported.[32–34]

To explore tTG function, we searched the effect of tTG protein expression level on pro- or anti-apototic signals in neonatal cardiomyocytes. The present study showed that cardiomyocytes transfected with tTG induced increased apoptotic signal molecules such as Bax, cytochrome c, caspase-8 and caspase-3. In relation to anti-apoptosis, cardiomyocytes transfected with siRNA for tTG resulted in a dramatic reduction in cardiomyocyte apoptosis. Changes in levels of intracellular calcium can activate signaling pathway to lead apoptosis and cell death caused by calcium overloading has been implicated in myocardium injury of hypoxia-reperfusion. The inhibition of Ca^{2+} overload and calcium related signal was observed in tTG down-expressed cells. Furthermore, our data showed that tTG expression and tTG-related apoptotic signal molecules were controlled by CRT expression level known as a major Ca^{2+} binding/buffering protein of the ER lumen and as such, is involved in several of the processes that comprise cellular Ca^{2+} homeostasis, including

Ca^{2+} storage in the ER, Ca^{2+} release from the ER, SERCA function, and activation of store-operated Ca^{2+} influx. That is to say, CRT overexpression enhanced the expression of tTG and pro-apoptotic signal molecules.

V. CONCLUSION

tTG might be essential for regulation of heart apoptosis. Our study demonstrated that tTG expression level modulates apoptotic signal molecule and undergo on intracellular calcium level and signal. Therefore, we have provided new insight into myocardial physiology of tTG so a better understanding of the role of tTG in the regulation of myocardial apoptosis may provide strategies to improve outcome.

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Abstract (in korean)

심근세포의 세포 사멸 기전에서의

tissue transglutaminase의 기능적 역할

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Tissue transglutaminase (tTG)는 포유동물의 조직에 산재되어 있으며 세포막에 결합되어 세포 외부나 세포막에 결합되어 세포 내부나 세포질에서 발견된다. 세포의 기능적 측면에서 tTG의 생리적 역할은 확실히 알려진 것이 없다. 대다수의 연구들은 tTG에 의한 아미드 교환반응이 세포사멸을 촉진 혹은 억제시킬 수 있는 반면, 이 효소의 구아노신 3인산과의 결합된 상태는 일반적으로 사멸로부터 세포를 보호한다는 점을 강조한다. 즉 이전의 연구들은 tTG가 세포사멸의 과정에서 이중성을 보여줌을 지적한다. 이번 연구에서 우리는 신생 백서의 심근세포의 세포사멸 과정에서 tTG가 상당한 역할을 할 것이라고 가정하였다. 우선, 신생 백서의 심근세포를

2시간 동안 500 μM H_2O_2 혹은 24시간 동안 100 μM retinoic acids로 처리 하였다. 심근세포에 세포사멸을 유발시키는 자극을 준 후 tTG의 단백질 발현의 증가를 확인하기 위해 면역세포염색분석과 유체 세포측정법을 사용하였다. Caspase-3 활성 측정법은 tTG의 발현과 세포 사멸 사이의 상당한 관련성을 보여주고 있다. 면역분석법은 tTG 단백질의 발현 증가가 p-ERK1,2, p-Akt 그리고 Bcl-2과 같은 세포 생존 혹은 항세포사멸 신호 분자를 상당히 억제하나 caspase-8, cytochrome c 그리고 Bax의 활성은 강화시킨다는 사실을 보여주고 있다. tTG의 단백질 발현은 또한 세포내 칼슘의 과부하를 조절하는데 이는 tTG의 유전자를 삽입하여 이를 과발현 시킨 심근세포를 500 μM H_2O_2로 처리한 결과 PLC-δ1의 단백질 발현과 PKC의 활성은 감소시키기 때문이다. 게다가 우리의 결과는 tTG의 단백질 발현과 tTG 관련 세포 사멸 분자들이 calreticulin (CRT)의 단백질 발현 수준에 의해 조절됨을 보여주고 있다. 그러므로 이 연구가 보여주고 있는 것은 tTG의 단백질 발현 수준이 세포사멸 신호를 조절하고 세포내의 칼슘의 수준과 신호를 매개한다는 것이다.

핵심되는 말: 심근세포, tissue transglutaminase (tTG), 세포사멸,
과산화 수소, Retinoic acids