대 한 체 질 인 류 학 회 지
Korean J Phys Anthropol
22(3): 245～254, 2009

Cell Surface Tissue Transglutaminase-induced Activation of Phosphoinositol 3-Kinase/Akt Pathway

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Abstract : Multifunctional tissue transglutaminase (tTGase) is found in the cytoplasm and cell surface, as well as in the extracellular matrix. However, it is difficult to determine the exact function of tTGase in each cell compartment. This study focused on the potential role of cell surface tTGase in the process of "outside-in" signal transduction.

Immunofluorescence study and western blotting was performed to localize the overexpression of tTGase. tTGase-overexpressed H9c2/tTGase cells were treated with anti-tTGase antibody to evaluate the potential functions of tTGase on the outside-in signal process.

The tTGase level markedly increased in each cell compartment and the culture media of H9c2/tTGase cells that show overexpression of tTGases. Anti-tTGase monoclonal antibody reduced tTGase levels in the whole lysate of H9c2/tTGase cells, and concomitantly increased the activity of the Akt. The results suggest that the cell surface expression of tTGase may be associated with an intracellular signaling pathway *via* the phosphoinositol-3 kinase/Akt. Phosphorylation of mitogen activated protein kinase family, ERK1/2, and Jun N-terminal Kinase (JNK), was also inhibited in the anti-tTGase antibody-treated H9c2/tTGase cells.

These results suggest that cell surface tTGase may regulate intracellular signaling pathways in an autocrine or paracrine manner.

(Received 1 July 2009, revised 28 August 2009, accepted 9 September 2009)

Key words : Tissue transglutaminase, PI-3K/Akt, Paracrine

Introduction

Tissue transglutaminase (tTGase or TGase 2) is a calcium-dependent cross-linking enzyme that catalyzes a covalent iso-peptide bond between two proteins. tTGase has been implicated in the regulation of numerous physiological processes such as apoptosis (Fesus et al. 1987, Piacentini et al. 1991), wound healing (Griffin et al. 2002), cell adhesion and morphology change, cell differentiation (Maccioni and Seeds 1986, Tucholski et al. 2001), and cell survival (Antonyak et al. 2001, Boehm et al. 2002). Some reports suggest that tTGase regulate the tumor growth and metastasis (Johnson et al. 1994, van Groningen et al. 1995).

Although tTGase primarily localizes in the cytoplasm,

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considerable tTGase localization has also been reported in the nucleus (Lesort et al. 1998). In addition, tTGase is present on the cell surface and in the extracellular matrix (Upchurch et al. 1991, Aeschlimann et al. 1995, Verderio et al., 1998). tTGase is thought to exert a distinct physiological role in different cellular compartments (Kostakis and Griffin 2007). A large number of intracellular and extracellular proteins were identified as targets of tTGase-mediated cross-linking of proteins (Lorand and Graham 2003). Extracellular matrix (ECM) macromolecules, including fibronectin, vitronectin, laminin, and collagen (Sane et al. 1988, Aeschlimann and Paulsson 1991, Barsigian et al. 1991, Kleman et al. 1995) have been reported to serve as substrates for tTGase. tTGase in the ECM is able to bind and interconnect ECM proteins including fibronectin (Fellin et al. 1988, Turner and Lorand 1989, Akimov and Belkin 2001). Cell surface tTGase has also been reported to bind to integrins of the adhesion process via a direct noncovalent association with the β_1 and β_3 integrin subunits (Akimov et al. 2000). Recently, an interaction between the β_5 integrin and tTGase was also reported; up to 40% of β_5 integrins on the cell surface of various cells were found to be associated with tTGase. In endothelial cells, the interaction of cell surface tTGase with fibronectin stabilizes the ECM and anchors cells to the basement membranes (Martinez et al. 1994). Cell surface tTGase mediates the interaction of integrins with fibronectin, which subsequently promotes cell adhesion (Akimov et al. 2000). Furthermore, tTGase on the extra- and intracellular surface of the plasma membrane regulates cell migration, adhesion, differentiation, and wound healing (Akimov and Belkin 2001, Kang et al. 2004).

Moreover, we have previously observed a marked increase in tTGase concentration in the culture medium of H9c2 cells which overexpressed human tTGase protein. Based on the findings of the previous reports from ours as well as others, we hypothesize that tTGase released from the cells can transmit signals to neighboring cells by interacting with cell surface integrins. The aim of this study was to elucidate whether cell surface tTGase regulates the intracellular signal pathways from the outside of the cell.

Materials and Methods

1. Reagents and antibodies

Anti-tTGase antibody was obtained from Neomarkers (USA); and the anti-MMP-9 antibody was from Chemicon. Anti-phospho-Akt antibody, anti-NF-κB antibody, anti-FAK antibody and anti-phospho-GSK-3α/β (Ser21/9) antibody were purchased form Cell signaling (Beverly, MA, USA). Anti-β1 integrin antibody was from BD biosciences (San Diego, CA, USA).

2. Cell culture

The rat cardiomyoblast cell line (H9c2) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). H9c2 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

3. Confocal microscopy

H9c2/pcDNA cells and H9c2/tTGase cells were cultured in cover slips for 24 h. After fixation with 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS), cells were incubated with anti-tTGase antibody (1 : 100 dilution) for 2 h, and washed 3 times with PBS. The cells were then incubated with anti-β1 integrin (1 : 100 dilution) for 2 h. After reaction, cells were washed 3 times with PBS before treatment with secondary antibodies (FITC conjugated goat anti-mouse IgG, Texas red conjugated goat anti-rabbit) for 2 h under darkness. The slides were coverslipped with aqueous mounting

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medium and observed using an Olympus Fluoview FV1000 confocal microscope (Japan).

4. Stable transfection of tTGase

Human tTGase cDNA in the vector pcDNA3 (Invitrogen, Carlsbad, CA, USA), was used to transfect into rat cardiomyoblasts (H9c2) using CytoPure-huv (Qbiogene, Inc., Irvine, CA, USA), according to the manufacturer's instructions. Briefly, the cDNA was mixed with the CytoPure-huv reagent and incubated for 15 min at room temperature (RT). The DNA-CytoPure mix was added to the cells. After 6 h of incubation, the supplemented culture medium was added to the cell culture. One day after the transfection, G418 (Sigma, St. Louis, MO, USA) was added to the culture medium at 300 μg/mL as a selection reagent. The cell cultures were maintained in this medium for 4 weeks. Clones of cells derived from these transfections were subcultured and expanded as single clones. After expansion and subculture, the derived cell lines were assayed for the presence of TGase 2 using western blotting.

5. Western blotting

Cultured cells were washed twice with 1 × PBS, lysed by adding 600 μL (per 100 mm plate) of RIPA buffer containing 1% NP40, 0.5% Na deoxycholate, 0.1% SDS, PMSF (100 μg/mL), aprotinin (30 mg/mL, Sigma) and Na orthovanadate (1 mM). Protein concentrations were measured using the BCA protein assay kit. Cell lysates were subjected to 7.5% SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated in blocking solution (5% skim milk in tris-buffered saline (TBS) containing 0.05% Tween 20) for 30 min at RT and incubated overnight with an anti-mouse tTGase antibody (1 : 1,000 dilution in TBS containing 0.05% Tween 20) at 4°C. The membranes were washed twice with TBS buffer, treated with a goat anti-mouse IgG-HRP antibody (1 : 5,000 dilution) for 1 h, and washed. Immunodetection was performed using the enhanced chemiluminescence plus kit (PerkinElmer, Waltham, Massachusetts, USA) according to the manufacturer's instruction.

6. Zymography

The gelatinase activity of conditioned medium was detedcted through gelatin zymography. Serum-free media (40 μL) of conditioned for 24 h (40 μL) were subjected to SDS-PAGE using 7.5% acrylamide gels containing 0.1% gelatin. The gels were then incubated for 30 min at RT in 2.5% Triton X-100 in PBS. This step was repeated twice, before transferring the gels to the reaction buffer (50-mM Tris-Cl, pH 8.0; 10-mM $CaCl_2$) for overnight incubation at 37°C. The gels were then stained with Coomassie Brilliant Blue R-250 and briefly destained in 10% acetic acid and 40% methanol. Gelatinolytic activity was detected as transparent bands on a blue background of the stained gel.

Results

1. Localization of cell surface tTGase

To determine the level of cell surface tTGase in H9c2/tTGase cells, immunoblotting and confocal microscopy were performed using an anti-tTGase antibody. The cell surface expression of tTGase was higher in H9c2/tTGase cells than in MOCK vector-transfected cells (Fig. 1A). Imperforated cells were stained with an anti-tTGase antibody and viewed under the confocal microscope to detect the cell surface tTGase (Fig. 1B). The expression of tTGase proteins on the cell surface of H9c2/tTGase cells markedly increased as compared with MOCK vector-transfected cells. Furthermore, the tTGase levels in the culture media also markedly increased (Fig. 1C).

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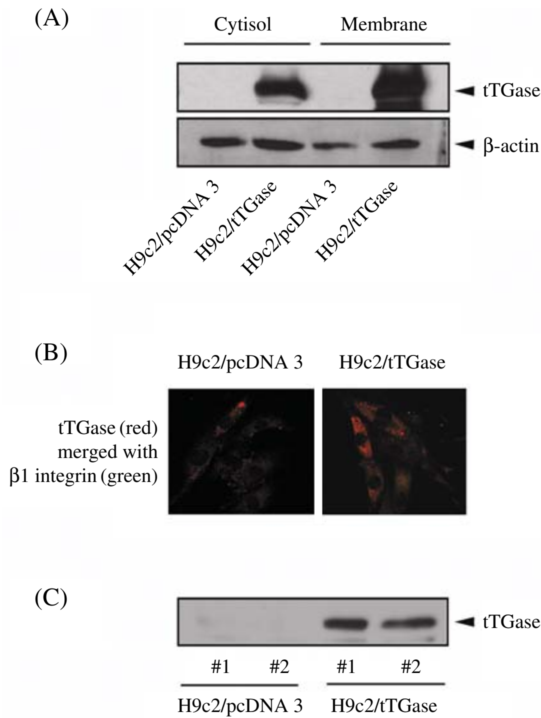

Fig. 1. Expression of tTGase in the H9c2/tTGase cells. Cytosolic and membrane fraction (A). Increased cell surface expression of tTGase protein as analyzed by immunofluorescence (B). Imperforated cells were stained with anti-tTGase antibody (red) and then anti-β1 integrin antibody (green) and the immunoreactivity was measured by confocal microscopy. tTGase levels were increased in the culture media of the H9c2/tTGase cells (C).

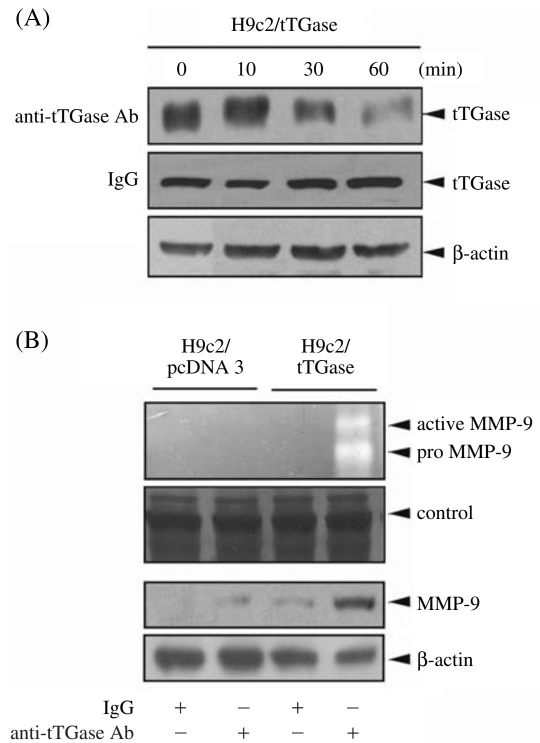

Fig. 2. Reduction in the tTGase level (A). Cytoplasmic tTGase level was reduced in a time-dependent manner by anti-tTGase antibody treatment (6 μg/mL, 2 h). H9c2/tTGase cell lysates were used for analyzing cytoplasmic tTGase levels. Recovered gelatinase activity in anti-tTGase antibody-treated H9c2/tTGase cells (B). Conditioned media from cells were analyzed for gelatinase expression by gelatin substrate zymography. The same cell lysate was used to determine MMP-9 levels by western blotting.

2. Surface tTGase-induced reduction of intracellular tTGase

To investigate the role of cell surface tTGase in the outside-in signal pathway, H9c2/tTGase cells were treated with a monoclonal antibody against tTGase (6 μg/mL, 2 h) as stimulatory ligand at various time intervals. As shown in Fig. 2A, the level of cytoplasmic tTGase reduced in a time-dependent manner. The cells in the control group were treated with an anti-mouse IgG, which was used as reference antigen, standard, and blocking agent. These cells showed no significant changes in the intracellular tTGase expression levels. To confirm the effect of tTGase antibody treatment on the intracellular tTGase level, the activity and expression of MMP-9 were examined by immunoblotting and zymography. According to our previous study, the activity of MMP-9 was reduced in H9c2 cells by overexpression of tTGase (Ahn et al. 2008). The MMP-9 activity in the culture media of H9c2/ tTGase cells was reversed to that of the control cells after anti-tTGase antibody treatment (Fig. 2B). The level of the MMP-9 protein expression also increased after treatment with anti-tTGase antibody (Fig. 2B). These results suggest

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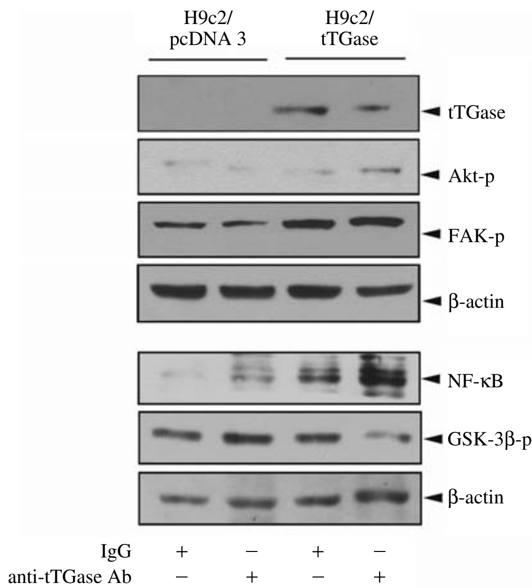

Fig. 3. Anti-tTGase antibody treatment-induced activation of PI-3K/Akt pathway. PI-3K activity in the same cell lysate used to analyze tTGase level was determined using Akt phosphorylation (Akt-p) and FAK phosphorylation (FAK-p) as readouts. GSK-3β inhibition activity and NF-κB activation supported the tTGase-induced activation of PI-3K/Akt pathway.

that cell surface signaling by tTGase decrease the expression of cytoplasmic tTGase.

3. Surface tTGase-induced activation of phosphoinositide 3-kinase /Akt pathway

Retinoic acid (RA) increased expression and activation of tTGase through the PI-3K/Akt pathway (Antonyak et al. 2002). PI-3K/Akt pathway is, in general, associated with integrin-induced signal transduction into the cell (Parsons and Parsons 1997, Schlaepfer and Hunter 1998, Cary et al. 1999). Since surface tTGase interacts with integrins through fibronectin (Akimov et al. 2000), we hypothesized that signals originating from cell surface tTGase protein may also be transmitted through the PI-3K/Akt pathway. PI-3k activation was examined by measuring Akt phosphorylation using an phospho-Akt-specific antibody. As shown in Fig. 3, Akt phosphorylation was augmented by treatment with anti-tTGase antibody. The treatment of H9c2/tTGase cells with an anti-mouse IgG antibody did not induce the phosphorylation of Akt. In order to confirm the activation, immunoblotting was conducted to detect phosphorylation levels of glucose synthase kinase-3β (GSK-3β). Glycogen synthase kinase 3β (GSK-3β) is a unique serine/threonine kinase that is inactivated by phosphorylation. In response to insulin binding, PKB/AKT phosphorylates GSK-3beta on serine 9, which prevents GSK-3β from phosphorylating glycogen synthase (Cross et al. 1995). As shown in Fig. 3, GSK-3β was downregulated in H9c2/tTGase cells treated with the anti-tTGase antibody. Moreover, phosphorylation of NF-κB, a functional target of Akt (Ozes et al. 1999, Romshkova et al. 1999), was increased, as shown in Fig. 3, thereby indicating that tTGase induce the activation of the PI-3K pathway.

4. Surface tTGase-induced inhibition of JNK and ERK1/2 pathway

In order to determine the mechanism of intracellular tTGase inhibition by anti-tTGase antibody, the mitogen-activated protein kinase (MAPK) pathway was evaluated by immunoblotting. The ERK1/2 pathway has been reported to contribute to the upregulation of tTGase. In addition, decreased tTGase expression has been reported to be mediated by p38 MAPK, c-Jun NH_3-teminal kinase (JNK), and the PI-3K pathways in NIH 3T3 cells (Akimov and Belkin 2003). RA is a well known activator of tTGase and has been shown to induce the expression and activation of tTGase through the ERK1/2 and the PI-3K pathways. Incubation of H9c2/tTGase cells with an anti-tTGase antibody for 2 h did not significantly alter p38 MAPK phosphorylation (Fig. 4). The activity of JNK was examined by an anti-JNK antibody using the same cell lysates used to analyze the induction of p38 MAPK. An anti-tTGase

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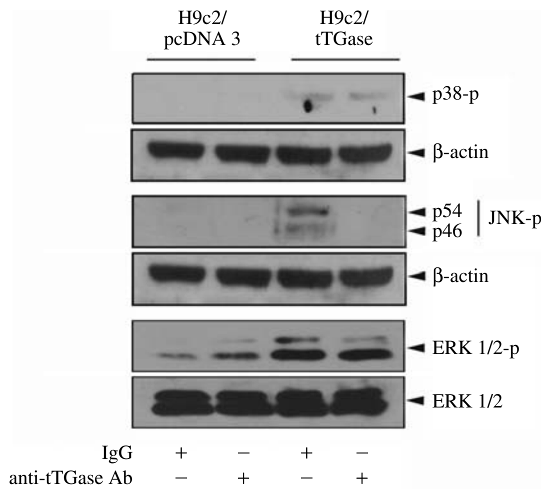

Fig. 4. Effect of anti-tTGase antibody treatment on the activity of MAP kinase in H9c2/tTGase cells. H9c2/tTGase cells with or without anti-IgG and anti-tTGase antibody treatment were used for determining MAP kinase activity.

antibody reduced the phosphorylation of JNK in H9c2/tTGase cells (Fig. 4). Additionally, an anti-tTGase antibody inhibited the phosphorylation of ERK1/2 in H9c2/tTGase cells (Fig. 4), suggesting that tTGase activates JNK and ERK1/2 *via* Akt activation.

Discussion

This study focused on investigating the potential role of cell surface tTGase in the outside-in signaling pathway of the cell. Here, we demonstrate that signals transmitted via cell-surface tTGase downregulate tTGase expression. The extracellular signals transmitted through this cell-surface protein activate the PI-3K/Akt pathway. In addition, it is observed that a reduction in tTGase protein expression is regulated by MAPK through the PI-3K pathway.

Our data show that intracellular tTGase levels were markedly decreased following anti-tTGase antibody treatment (Fig. 2). On the basis of this finding, we hypothesized that cell surface tTGase protein may serve a messenger function. We observed an increase in the levels of tTGase in the culture media due to the overexpression of tTGase-overexpressed cells (Fig. 1); this suggests that cell surface tTGase has another potential role in addition to its function as biological glue. On the basis of reports that have provided evidence for the multi-localization of tTGase, this protein is considered to serve distinct physiological functions in different cellular compartments (Singh et al. 1995, Losort et al. 1998, Kotsakis and Griffin 2007). However, it is still unclear whether the tTGase protein functions via some form of paracrine signaling.

In this study, anti-tTGase antibody treatment against cell surface tTGase was observed to result in the activation of the PI-3K/Akt pathway in H9c2/tTGase cells. Since activated Akt/PKB phosphorylates several downstream targets including GSK-3β (Doble and Woodgett 2003), endothelial nitric oxide synthase (Dimmeler et al. 1999, Fulton et al. 1999), and several apoptotic effectors (Datta et al. 1999, Brunet et al. 2001) within the cytoplasmic, mitochondrial, and nuclear compartments (Meier et al. 1997, Bijur and Jope, 2003), the reduction in the levels of activated GSK-3β (Fig. 3) supports the fact that cell-surface tTGase activate PI-3K/Akt pathway in some ways. Further, it was observed that activation of NF-κB also resulted in PI-3K/Akt pathway activation. These results suggest that the signals from cell-surface tTGase are transmitted into the cell via the PI-3K/Akt pathway. On the basis of the close relationship between the PI-3K/Akt pathway and integrins (Parsons and Parsons 1997, Schlaepfer and Hunter 1998, Cary et al. 1999) and the role played by tTGase with respect to integrin-fibronectin interaction (Akimov et al. 2000), we suggest that integrins may serve as a bridge between cell-surface tTGase and the PI-3K/Akt pathway.

Moreover, following anti-tTGase antibody treatment, we observed that the activities of both JNK and

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ERK decreased. These results are in agreement with the data published by Akimov and Belkin (2003), wherein it was shown that p38 MAPK, PI-3K, and JNK signaling pathways play a role in the downregulation of cell-surface tTGase in H-Ras- and Raf-1-transformed cells. However, in our study, we did not observe a change in p38 MAPK activity. This difference between the observations made in these two studies may be due to the different characteristics of the cell lines employed in both. Further, the study of Akimov et al. reported that ERK activation via constitutive pathways stimulates the expression of tTGase mRNA. However, the fact that ERK upregulates tTGase is in agreement with the findings of this study, because activated Akt has been shown to downregulate the ERK pathway through Raf-1 (Cantley 2002, Vivanco and Sawyers 2002). On the basis of these results, we consider the possibility that anti-tTGase antibody treatment downregulates tTGase expression via the PI-3K/Akt-MAPK pathway.

In summary, the role of tTGase has been implicated in the pathophysiology of various disease states including inflammation, tumor angiogenesis, and neurodegenerative conditions. However, it is difficult to define the exact function of tTGase in each of the disease states. The results presented in this paper indicate that tTGase released from cells may function as a messenger of signals between cells. These findings contribute to a better understanding of the diverse role of tTGase in the pathophysiology of various disease states including tumor angiogenesis, metastasis, and tumor growth.

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Korean J Phys Anthropol
22(3): 245 ~ 254, 2009

세포 표면 Tissue Transglutaminase에 의해 유도된 Phosphoinositol 3-Kinase/AKT Pathway 활성에 관한 연구

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간추림 : Tissue transglutaminase (tTGase)는 lysine과 glutamine residue를 서로 연결하여 Ca^{2+}의 조절 하에 Nε-(γ-L-glutamyl)-L-lysine (GGEL) isopeptide 결합을 형성한다. tTGase는 세포 안과 밖의 단백질들을 서로 결합하는 작용을 하므로 이와 같은 기능을 통해 상피조직의 기능성 방벽 또는 apoptosis와 같은 여러 가지 생명 현상에 관여하는 것으로 알려져 있다. tTGase는 주로 세포질 내에 존재하지만 세포에 따라서는 상당한 양의 tTGase가 핵에서 발견되기도 하며, 세포표면과 세포외기질 (extracellular matrix)에서 관찰된다. 본 연구는 세포표면부착 tTGase가 세포의 밖에서 세포막을 통과하여 세포질 내로 전달되는 과정에서 어떤 역할을 하는지 관찰하고자 하였다.

사람의 tTGase 단백질을 과발현 시킨 H9c2 세포주 (H9c2/tTGase)를 이용하여 세포면역형광염색, western blot 분석 방법을 이용하여 세포표면의 tTGase 단백질의 증가를 확인한 후 항 tTGase 항체를 처리하여 세포 외부로부터 내부로 전달되는 신호를 증폭하는 방법을 이용하여 세포표면의 tTGase가 관여하는 신호전달체계를 조사하였다.

과발현된 tTGase 단백질은 세포내부뿐만 아니라 세포표면에서도 증가하였으며, 세포배양액으로의 분비량 역시 증가하였다. H9c2/tTGase 세포의 세포표면에 부착된 tTGase 단백질을 통해 증폭된 신호를 세포내부로 전달한 결과 세포질 내부의 tTGase 발현이 감소하였다. 이와 같은 tTGase 단백질의 감소는 phosphoinositol 3-kinase/Akt (PI-3K/Akt) 신호전달계를 통해 mitogen-activated protein kinase (MAP kinase)에 전달된 신호에 의해 일어나는 것으로 사료된다.

본 연구의 결과들은 활성화된 세포로부터 유리된 tTGase가 세포표면에 부착됨으로써 세포내부의 신호전달체계에 영향을 미치는 것으로 사료되며, 이와 같은 결과는 암 발생과 전이 등의 세포부착과 관련된 질병현상의 이해를 돕기 위한 기초자료가 될 것이다.

찾아보기 낱말 : tissue transglutaminase, PI-3K, 신호전달

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