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Study of the mechanism underlying the inhibitory effects of transglutaminase II on apoptosis in the osteosarcoma MG-63 cell line under hypoxic conditions

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Abstract. The aim of the present study was to investigate the association between the apoptosis phenomenon in the MG-63 osteosarcoma cell line, and transglutaminase II (TG2) expression. The relationship between the anti-apoptotic mechanism of TG2 and the expression of cytochrome *c* as well as caspase-3 under hypoxic conditions was also verified. A hypoxic culture of MG-63 cells was prepared. The hypoxia and TG2 siRNA hypoxia groups were established, and the cultures were incubated for 12 h under hypoxic conditions. TG2 activity, TG2 protein expression and its mRNA level were investigated. Cytochrome *c* and caspase-3 protein levels in the TG2 nucleus and cytoplasm were measured. The apoptotic rate was also monitored. The results showed that TG2 activity, TG2 protein expression and its mRNA level in the hypoxia group were significantly higher than those of the siRNA hypoxia group. The results showed statistically insignificant differences ($P<0.05$). By contrast, a comparison of the two groups in the cytoplasm yielded no statistically significant differences ($P>0.05$). Cytochrome *c* and caspase-3 protein levels in the hypoxia group were significantly higher than those of the TG2 siRNA hypoxia group. The results showed statistically significant differences ($P<0.05$). By contrast, the protein levels in the cytoplasm were significantly lower than those of the TG2 siRNA hypoxia group, with differences being statistically significant ($P<0.05$). The differences in apoptotic rates between the hypoxia and TG2 siRNA hypoxia groups were also statistically significant ($P<0.05$). Under hypoxic conditions, a high TG2 expression inhibited the apoptosis of the MG-63 osteosarcoma cell line. This effect was probably associated with its suppressive activity on the transportation of cytochrome *c* and caspase-3 from nucleus to cytoplasm.

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Introduction

Transglutaminase II (TG2) is a 32.5 kb gene that is located in the q12 of the 20th chromosome. The molecular weight of the product of this gene is 80 kDa. The TG2 protein is a unique member of the glutamine transaminase family and contains 687 amino acid residues forming four unique structural domains in each monomer. The presence of TG2 has been reported in many locations inside cells, including the cytoplasm, nucleus, mitochondria, cell membrane surface and extracellular matrix. TG2 has a Ca^{2+}-dependent activity and catalyzes protein crosslinking and the GTP-dependent function of G protein. TG2 also demonstrates disulfide bond isomerase activity as well as protein kinase function (1,2).

It has been previously demonstrated that TG2 may have dual properties, acting as a pro- and anti-apoptotic protein (3). The results of a previous study (4) showed that TG2 played an anti-apoptotic role in HEK293 cells through: i) Consumption of the Bax protein, ii) reduction of caspase-3 and caspase-9, iii) inhibition of cytochrome *c* release from the nuclei into the cytoplasm and iv) depolarization of the mitochondrial membrane in high concentrations of Ca^{2+}.

The present study established a hypoxia culture model for osteosarcoma cells to investigate the associatoin between TG2 expression and the apoptotic process in the MG-63 osteosarcoma cell line. The connection between the anti-apoptosis mechanism of TG2 and the expression of cytochrome *c* as well as caspase-3 under hypoxic conditions was also investigated.

Materials and methods

Cell line, reagents and instruments. The materials used in the present study included the MG-63 osteosarcoma cell line, serum and RPMI-1640 medium (Gibco, Grand Island, NY, USA). Mouse-anti-human TG2 monoclonal antibody (Cat. no. sc-73612), cytochrome *c* (Cat. no. sc-13560) and caspase-3 (Cat. no. sc-7272) monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). BCIP/NBT, Ponceau staining solution, as well as AP-labeled anti-mouse IgG and HRP-labeled goat anti-mouse IgG were purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). A FACSort Flow

Cytometer (BD Biosciences, San Diego, CA, USA) was also used in the present study.

Experimental process. RPMI-1640 medium containing 10% bovine serum was used to prepare the cell cultures. The cultures were incubated at 37˚C with 5% CO_2, and 0.25% trypsin was used for digestion and passaging. For experiments of the present study, the cells were collected in the logarithmic phase, in their second or third generation. The cell cultures were then placed in a CO_2 anoxic incubator (5% CO_2, 95 %N_2) at 37˚C to establish an anoxic model.

To determine experimental grouping, the cells were collected in the logarithmic phase and seeded in orifice plates containing 2 ml of media ($5x10^5$/well). When the cell density reached >90%, the cells were divided into the hypoxia and TG2 hypoxia groups and co-cultured for 12 h.

Pure siRNA was designed and synthesized by Thermo Fisher Scientific (Waltham, MA, USA). The siRNA sequences for the TG2 transfection and control groups were: sense, 5'-GGGCGAACCACCUGAACAATT-3' and antisense, 3'-TTCCCGCUUGGUGGACUUGUU-5', and sense, 5'-UAGCGACUAAACACAUCAAUU-3' and antisense, 5'-UUGAUGUGUUUAGUCGCUAUU-3', respectively. Transfection was performed according to the protocol provided in the Dharmafect 3 kit (Lafayette, CO, USA). Subsequent experiments were carried out.

Observation indices and test methods. The microtiter plate method was employed to detect TG activity. Cells cultured under hypoxic conditions for 12 h were collected, re-cultured, and then marked with 5-biotin acyl amino amylamine (Beyotime, Shanghai, China). The cells were ultra-centrifuged at 2,000 x g for 10 min at 4˚C. Cell extracts were used to envelope the 96-well microtitration plate for 16 h at 4˚C. A total of 5% bovine serum albumin and PBS was used for incubation for 1 h at room temperature. The plates were incubated with HRP streptavidin for 45 min and washed using PBS. O-phenylendiamine dihydrochloride (Amresco, Solon, OH, USA) was added to the microtitration plate for 5-15 min at room temperature, and 1 mol/l sulfuric acid was used to stop the reaction. After staining, absorbance at 490 nm was measured using a microplate spectrophotometer (Biocompare, San Francisco, CA, USA).

The semi-quantitative RT-PCR method was employed to detect TG2 content. TRIzol reagent was used for total RNA extraction, and a spectrophotometer was employed for quantification as well as the purity test. The TG2 product sequence was: (5'-3') sense: GGGGTGAGAGAGGAAAGACC, and antisense: TGCAGTCTAGGGAGCTGGAT. The product size was 167 bp, and the annealing temperature was 58˚C. The PCR reaction was carried out after the cDNA synthesis, and was completed using reverse transcription. Agarose electrophoresis was used for product identification. Grey values were analyzed after images were captured, and the ratio of the target and reference genes was used as the mRNA relative amount.

Western blot analysis was used for TG protein detection. Cytochrome *c* and caspase-3 kits were used to extract the proteins from the nucleus and cytoplasm, respectively, and Coomassie brilliant blue protein (Amresco) was used for quantification purposes. Proteins were isolated in SDS-PAGE and then transferred to a nitrocellulose membrane, blocked, washed, and incubated with TG2, Bax and CytC primary antibodies (1:1000 dilution) for 2 h at 37˚C. Secondary antibodies (1:5000) were added and incubated for 1 h at 37˚C and then Ponceau staining solution was used for detection.

The sub-G1 method was employed to detect apoptotic cells. Following digestion with trypsin, the cells ($2x10^5$) were collected and fixed with 70% ethanol, and kept at 4˚C overnight. Subsequently, the cells were rinsed with PBS and stained with propyl bromide (Amresco). FACSort FCM was used for the detection.

Statistical analysis. SPSS 19.0 (IBM, Armonk, NY, USA) was used for the statistical analyses. Measurement data were presented as mean ± standard deviation, and the independent t-test was used for comparisons between two groups. Count data were expressed as a case number or percentage, and the χ^2 test was used for comparisons between two groups. P<0.05 was considered statistically significant.

Results

Comparison of TG activity, protein level and mRNA level between the two groups. TG2 activity, TG2 protein expression and its mRNA levels in the nucleus for the hypoxia group were significantly higher than those of the TG2 siRNA hypoxia group, and the differences were statistically significant (P<0.05). We compared the two groups in the cytoplasm, and the differences were not statistically significant (P>0.05) (Table I).

Comparison of cytochrome c protein and caspase-3 protein levels. The cytochrome *c* protein and caspase-3 protein levels in the nucleus for the hypoxia group were significantly higher than those of the TG2 siRNA hypoxia group. By contrast, in the cytoplasm, the proteins levels were significantly lower than those of the TG2 siRNA group, and the differences were statistically significant (P<0.05) (Table II).

Comparison of the cell apoptotic rate. The apoptotic rate of the hypoxia group was significantly lower than that of the TG2 siRNA hypoxia group, and the differences were statistically significant [(21.3±4.4)/(42.6±8.7)%, t=5.758, P=0.031].

Discussion

Few studies have focused on the role of TG2 during the process of osteosarcoma cell apoptosis and the mechanism controlling this regulation. In the present study, this process was elucidated and the regulation mechanism of the apoptotic process was determined. Previous findings (5) have shown that TG2 may be induced under hypoxic conditions and promote apoptosis in tumor cells. Various reports have confirmed that TG2 is involved in the regulation of the apoptosis phenomenon (6).

The role of TG2 in the cell apoptosis is controversial (7,8). Previous results have shown that, under hypoxic oxidation conditions, TG2 promotes apoptosis. Reactive oxygen species (ROS) with a high level of induced Ca^{2+} enter the cell leading to the enhancement of TG2 enzyme activity and subsequently triggering apoptosis (9). Nevertheless, recent findings have shown that the expression of TG2 was not completely consistent with apoptosis (10). It has been previously reported that

Table I. Comparison of TG activity, protein level and mRNA level.

	Nucleus			Cytoplasm		
Group	TG2 activity	Protein level	mRNA level	TG2 activity	Protein level	mRNA level
Hypoxia group	26.4±20.3	31.5±3.4	26.9±20.1	11.6±1.8	12.9±2.7	14.7±1.9
TG2 siRNA hypoxia group	12.5±20.2	13.6±2.9	10.3±20.3	12.4±1.7	12.6±2.2	14.5±1.6
t-test	4.518	4.627	5.103	0.529	0.267	0.362
P-value	0.036	0.034	0.026	0.127	0.535	0.415

Table II. Comparison of cytochrome *c* protein and caspase-3 protein levels.

	Nucleus		Cytoplasm	
Group	Cytochrome *c*	Caspase-3	Cytochrome *c*	Caspase-3
Hypoxia group	31.6±2.5	26.7±1.8	5.6±0.4	4.9±0.6
TG2 siRNA hypoxia group	13.5±2.3	12.6±1.5	17.9±2.1	16.6±1.5
t-test	5.174	4.926	6.517	6.238
P-value	0.023	0.035	0.014	0.016

in several rapidly dividing tumor cells, despite a high level of TG2 expression, cell apoptosis was initiated (8). Additionally, in endothelial cells, when TG2 was consumed the cell cycle was arrested, and apoptosis was induced (11).The results from a recent study (12) revealed that TG2 upregulation was involved in increasing the survival rate in kidney cancer cells. The mutant TG2 (R580A) in the nucleus may offset its effect in promoting apoptosis in the cytoplasm. These results suggest that TG2 promotes apoptosis, and possesses anti-apoptotic properties. This contradictory role was dependent on the cell type, stimulation type, TG2 location and its configuration within cells.

The results presented in this study clearly demonstrate that TG2 levels were significantly amplified inside the nucleus under hypoxic conditions while no detectable changes were observed in the cytoplasm. Under hypoxic conditions, TG2 expression and activity were increased significantly. Additionally, TG2 expression and activity were gradually increased with the prolonged hypoxia time. The TG2 activity, TG2 protein levels and mRNA levels were all higher in the hypoxia group compared to those of the TG2 siRNA hypoxia group. The differences were statistically significant. Comparative analyses on the two groups in the cytoplasm did not produce any statistical significant differences. The results suggested that TG2 expression under hypoxic conditions may be involved in observed resistance to the apoptotic process, and the nuclear expression of TG2 may be the influential factor promoting the apoptosis.

Apoptotic events in osteosarcoma cells were triggered through the death receptor and ligands, and mitochondrial pathways. The mitochondrial pathway stimulated mitochondria to release cytochrome *c* through certain apoptosis-stimulating factors.

Cytochrome *c* released into the cytoplasm reacted with apoptotic protease activating factor-1 to form a protein complex which attached to caspase-9 and formed the apoptotic body. Activated caspase-9 stimulated caspase-3, which subsequently triggered the cell apoptosis (13).

Caspase-3 is an important effector in the caspase family and is normally present in the cytoplasm in the form of a 32 kDa zymogen. The activated form of caspase-3 contains two large subunits (17 kDa) as well as two small subunits (12 kDa) and can induce lytic events in the nucleus and cytoplasm that eventually lead to apoptotic events (14). Fesik (15) clearly showed that TG2 combined with nuclear, cytoplasmic and mitochondrial proteins via non-covalent bonds. Under hypoxic conditions, the cross-linked polymers formed by the activated form of TG2 in the cytoplasm combine with caspase-3 and produce insoluble complexes with inhibitory activities towards caspase-3, thus resisting the hypoxia-induced apoptosis. The anti-apoptotic effect was associated with TG2 positioning in the cytoplasm and its configuration, which is not dependent on the function of TG2 conversion enzymes.

The results have shown that there was no amplification in caspase-3 activity and expression in the hypoxia group. However, when TG2 siRNA transfected the hypoxia group, the inhibition of TG2 expression upregulated caspase-3 expression, thereby increasing its activity and the rate of cell apoptosis in the TG2 siRNA hypoxia group. Thus, the high expression of TG2 exerted anti-apoptotic effects by inhibiting caspase-3.

We also established that the cytochrome *c* and caspase-3 levels in the hypoxia group were all higher than those of the TG2 siRNA hypoxia group, while in the cytoplasm, the levels were significantly lower than those of the TG2 siRNA group. Differences were statistically significant. Differences in the apoptotic rate between the hypoxia and TG2 siRNA hypoxia

groups were also statistically significant. In the MG-63 osteosarcoma cells, TG2 exerted an anti-apoptotic effect by suppressing the cytochrome *c* and caspase-3 released from mitochondria to cytoplasm under hypoxic conditions.

In conclusion, the results of the present study show that TG2 activity in the MG-63 osteosarcoma cytoplasm was obviously enhanced under hypoxic conditions. Furthermore, the TG2 mRNA and protein levels were gradually amplified with a longer delay in hypoxic time. TG2 prevented the release of cytochrome *c* into the cytoplasm. TG2 formed cross-linked polymers in the cytoplasm and produced insoluble complexes with caspase-3, which downregulated caspase-3 expression, and exerted an anti-apoptotic effect. This may constitute a protective escape mechanism for the tumor cells to arrest the programmed cell death process. However, when TG2 siRNA transfected the hypoxia group, the inhibition of TG2 expression upregulated caspase-3 expression, and increased the apoptotic rate. The findings of this study provide a new starting point for future investigations into osteosarcoma treatment.

References

1. Jang GY, Jeon JH, Cho SY, Shin DM, Kim CW, Jeong EM, Bae HC, Kim TW, Lee SH, Choi Y, *et al*: Transglutaminase 2 suppresses apoptosis by modulating caspase 3 and NF-kappaB activity in hypoxic tumor cells. Oncogene 29: 356-367, 2010.
2. Shin DM, Jeon JH, Kim CW, Cho SY, Kwon JC, Lee HJ, Choi KH, Park SC and Kim IG: Cell type-specific activation of intracellular transglutaminase 2 by oxidative stress or ultraviolet irradiation: Implications of transglutaminase 2 in age-related cataractogenesis. J Biol Chem 279: 15032-15039, 2004.
3. Zhang YQ, Xiao CX, Lin BY, Shi Y, Liu YP, Liu JJ, Guleng B and Ren JL: Silencing of Pokemon enhances caspase-dependent apoptosis via fas- and mitochondria-mediated pathways in hepatocellular carcinoma cells. PLoS One 8: e68981, 2013.
4. Cho SY, Lee JH, Bae HD, Jeong EM, Jang GY, Kim CW, Shin DM, Jeon JH and Kim IG: Transglutaminase 2 inhibits apoptosis induced by calcium- overload through down-regulation of Bax. Exp Mol Med 42: 639-650, 2010.
5. Nurminskaya MV and Belkin AM: Cellular functions of tissue transglutaminase. Int Rev Cell Mol Biol 294: 1-97, 2012.
6. Waterhouse NJ, Goldstein JC, Kluck RM, Newmeyer DD and Green DR: The (Holey) study of mitochondria in apoptosis. Methods Cell Biol 66: 365-391, 2001.
7. Jeong SY and Seol DW: The role of mitochondria in apoptosis. BMB Rep 41: 11-22, 2008.
8. Facchiano A and Facchiano F: Transglutaminases and their substrates in biology and human diseases: 50 years of growing. Amino Acids 36: 599-614, 2009.
9. Fésüs L and Szondy Z: Transglutaminase 2 in the balance of cell death and survival. FEBS Lett 579: 3297-3302, 2005.
10. Caccamo D, Currò M, Ferlazzo N, Condello S and Ientile R: Monitoring of transglutaminase 2 under different oxidative stress conditions. Amino Acids 42: 1037-1043, 2012.
11. Nadalutti C, Viiri KM, Kaukinen K, Mäki M and Lindfors K: Extracellular transglutaminase 2 has a role in cell adhesion, whereas intracellular transglutaminase 2 is involved in regulation of endothelial cell proliferation and apoptosis. Cell Prolif 44: 49-58, 2011.
12. Ku BM, Kim DS, Kim KH, Yoo BC, Kim SH, Gong YD and Kim SY: Transglutaminase 2 inhibition found to induce p53 mediated apoptosis in renal cell carcinoma. FASEB J 27: 3487-3495, 2013.
13. Colak G, Keillor JW and Johnson GV: Cytosolic guanine nucleotide binding deficient form of transglutaminase 2 (R580a) potentiates cell death in oxygen glucose deprivation. PLoS One 6: e16665, 2011.
14. Gundemir S and Johnson GV: Intracellular localization and conformational state of transglutaminase 2: Implications for cell death. PLoS One 4: e6123, 2009.
15. Fesik SW: Promoting apoptosis as a strategy for cancer drug discovery. Nat Rev Cancer 5: 876-885, 2005.