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Biochemical Pharmacology 66 (2003) 213–223
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Role of tissue transglutaminase in GTP depletion-induced apoptosis of insulin-secreting (HIT-T15) cells

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Received 25 June 2002; accepted 5 December 2002

Abstract

The role of tissue transglutaminase (tTG), a calcium-dependent and GTP-modulated enzyme, in apoptotic death induced by GTP depletion in islet β-cells was investigated. GTP depletion and apoptosis were induced by mycophenolic acid (MPA) in insulin-secreting HIT-T15 cells. MPA treatment increased *in situ* tTG activity (but not protein levels) in a dose- and time-dependent manner in parallel with the induction of apoptosis. MPA-induced increases of both tTG activity and apoptosis were entirely blocked by co-provision of guanosine but not adenosine. MPA-enhanced tTG activity could be substantially reduced by co-exposure to monodansylcadaverine or putrescine (tTG inhibitors), and largely blocked by lowering free Ca^{2+} concentrations in the culture medium. However, MPA-induced cell death was either not changed or was only slightly reduced under these conditions. By contrast, a pan-caspase inhibitor (Z-VAD-FMK) entirely prevented apoptosis induced by MPA, but did not block the enhanced tTG activity, indicating that GTP depletion can induce apoptosis and activate tTG either independently or as part of a cascade of events involving caspases. Importantly, the morphological changes accompanying apoptosis could be markedly prevented by tTG inhibitors. These findings suggest that the effect of the marked increase in tTG activity in GTP depletion-induced apoptosis of insulin-secreting cells may be restricted to some terminal morphological changes.
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Keywords: Tissue transglutaminase; Apoptosis; Mycophenolic acid; Guanine nucleotides; Caspase; Islet β-cells

1. Introduction

Apoptosis plays an important physiologic role in embryonic development and in adult tissue homeostasis by eliminating excess and aged cells. Apoptotic cell death is also involved in the pathogenesis of many diseases such as cancer, neuronal injury, and diabetes. There are some typical morphological changes occurring during apoptosis, such as cytoplasmic shrinkage, membrane protuberances, chromatin condensation, and DNA fragmentation [1,2]. Several signaling pathways implicated in the initiation and execution of apoptosis have been identified, including the involvement of a family of asparate-specific cysteine proteases called caspases [3,4]. Recent studies suggested that other enzymes such as tissue transglutaminase (tTG) might participate in the induction of apoptosis [5–8]. It has been reported that tTG selectively accumulates and its activity is markedly increased in cells undergoing apoptosis [5–8]. However, its exact role in apoptosis, albeit extensively studied in recent years [5,7–9], is still not clear.

tTG (EC 2.3.2.13), or type II transglutaminase, belongs to a group of enzymes which include at least nine members that may be biochemically and immunologically distinct, such as blood coagulation factor XIIIa (plasma type) and epidermal (keratinocyte) transglutaminase (type 1) [5,10,11]. These enzymes are involved in a broad range of biological processes such as apoptosis, blood coagulation, wound healing, and keratinocyte terminal differentiation. They catalyze Ca^{2+}-dependent cross-linking reactions by establishing irreversible ε-(γ-glutamyl)lysine and *N*,*N*-bis(γ-glutamyl)polyamine bonds between proteins [5–7,12]. Transglutaminase activity has been found in pancreatic islets and may play a role in the process of glucose-induced

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Abbreviations: GNs, guanine nucleotides; IMPDH, Inosine 5′-monophosphate dehydrogenase; MPA, mycophenolic acid; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; Z-VAD-FMK, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; MDC, monodansylcadaverine.

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doi:10.1016/S0006-2952(03)00262-4

insulin release [13,14], but it is unclear whether this activity is attributable to tTG.

tTG is a cytosolic protein with molecular mass of ~80 kDa (slightly different among species). This enzyme is a bifunctional protein since it also possesses properties of a GTP-binding protein/GTPase (and therefore it is also called as G_h); it couples certain receptors to phospholipase C_δ in some cells [15]. Its activity is regulated by intracellular levels of both Ca^{2+} and GTP; an increase of the former or a reduction of the latter stimulates its transglutaminase activity [7,16,17]. Therefore, tTG activity may be suppressed in the presence of normal ambient GTP concentrations to protect cell and tissue integrity. Other factors such as redox potentials (GSSG and *S*-nitrosylation), polyamines, and sphingosylphosphocholine may also modulate tTG activity [7,18,19].

Inosine 5′-monophosphate dehydrogenase (IMPDH, EC 1.1.1.205) is a rate-limiting enzyme for the synthesis of GTP, dGTP, and other guanine nucleotides (GNs) [20,21]. Alterations in the expression or activity of IMPDH may be decisive to cell proliferation, differentiation, and apoptosis [2,20,22–24]. These effects are probably mediated directly by GTP or indirectly via GTP-binding proteins [21]. Mycophenolic acid (MPA) is a potent and specific inhibitor of IMPDH [20,22,25]. It is widely used as an antitumor and immunosuppressive drug, like other IMPDH inhibitors such as mizoribine, ribavirin, and tiazofurin [22,25,26]. We previously demonstrated that depletion specifically of GTP by using MPA- and mizoribine-induced apoptosis of insulin-secreting cells (HIT-T15, INS-1, and isolated rat islets) [2]. In this study, the possible role of tTG in GTP depletion-induced cell death was investigated in HIT-T15 cells. We found that *in situ* activity of tTG was increased in MPA-treated cells in parallel with the induction of apoptosis in terms of both time-course and dose-dependency. However, significant suppression of tTG activity by using its inhibitors, such as monodansylcadaverine and putrescine, or by lowering free Ca^{2+} concentrations in culture medium failed to block most aspects of apoptosis. Furthermore, tTG activity remained high in GTP-depleted cells even when apoptosis was blocked by a pan-caspase inhibitor, indicating that in MPA-treated cells tTG is stimulated by lowered GTP levels rather than being a secondary outcome of caspase activation. However, GTP depletion also caused rounding-up of cells which was markedly inhibited by tTG inhibitors. Our data together indicate that any role of tTG in the apoptosis induced by GTP depletion might be largely restricted to the accompanying morphological changes.

2. Materials and methods

2.1. Materials

MPA, phenazine methosulfate (PMS), guanosine, adenosine, EGTA, EDTA, phenylmethylsulfonylfluride (PMSF), pepstatin A, aprotinin, and RPMI 1640 powder were purchased from Sigma. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) was from Promega. Fetal calf serum was purchased from Gibco-BRL. Bovine serum albumin (BSA) and leupeptin were from Boehringer Mannheim. *N*-Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK), nitrocellulose membranes (0.45 μm), protein assay dye reagent, and Kaleidoscope prestained molecular weight standards were purchased from Bio-Rad. 5-(Biotinamido) pentylamine, horseradish peroxidase (HRP)-conjugated streptavidin were from Pierce. Ammonium persulphate, bromophenol blue, and TEMED were purchased from Pharmacia Biotech. Anti-transglutaminase type II (polyclonal) antibody was from Upstate Biotech. and HRP-conjugated anti-goat IgG from Santa Cruz Biotech.

2.2. Cell culture

Insulin-secreting HIT-T15 (passage 76–83) cells were maintained in Falcon flasks in RPMI 1640 supplemented with 10% decomplemented fetal calf serum (v/v), 100 IU penicillin/mL, and 100 μg streptomycin/mL and the medium was changed every 48 hr. For experiments, cells were seeded in dishes or multi-well plates at a density of 1.0×10^6/mL with or without test agents for various time periods. MPA was added to the culture when cells had already approached more than 80% confluence.

2.3. Determination of cell death by MTS test and flow cytometry

MPA stock solution (2 mg/mL) was prepared in ethanol and added into cell culture medium directly to reach final concentrations of 0–10 μg/mL. The cells were treated by MPA up to 48 hr as per our previous protocols to induce apoptosis [2,27].

Cell viability was monitored by MTS test [2,27], which detects the reduction of a tetrazolium salt into a colored, soluble formazan in intact living cells by metabolic dehydrogenases [28]. A mixture of MTS (inner salt) and an electron-coupling reagent (phenazine methosulfate; PMS) was added to the cells in 96-well culture plates and incubated for 30 min at 37°. Absorbance of the formed formazan at 490 nm was measured by using a plate reader directly without additional processing. We confirmed that the intensity of absorbance from the formazan product is directly proportional to the number of living cells added into the wells.

Apoptosis reflecting DNA fragmentation was determined by propidium iodide staining and flow cytometry as described in details in our previous study [27]. This assay measures fragmented nuclei and thus detects the subdiploidy apoptosis (sub-G1 phase). Briefly, HIT cells were cultured in 6-well plates with test agents for indicated periods. All cells in the wells were collected and fixed in 70% ethanol. After centrifuging at 200 *g* for 5 min at room temperature to remove cell debris, cells were subjected to

propidium iodide staining. Flow cytometric analysis was carried out using a fluorescence-activated-cells-sorter (EPICS Elite ESP, Beckman Coulter). Ten thousand cells were evaluated for each sample. The data were processed and analyzed using WinMDI software (Scripps Institute).

2.4. Preparation of cell homogenates

Cells cultured in dishes were washed with cold phosphate-buffered saline (PBS), scraped in a homogenizing buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL each of aprotinin, leupeptin, and pepstatin), and sonicated on ice. The samples were stored at −70° if not immediately used for tTG assay or Western blotting.

Protein concentrations in the homogenates were determined by the Bradford (Bio-Rad) assay using BSA as standard. The color change of Coomassie brilliant blue G-250 dye (shift from 465 to 595 nm when binding to protein) was measured in 96-well plates with a plate reader, and compared to a standard curve of BSA concentrations between 200 and 1400 μg/mL.

2.5. Assay of in situ tTG activity

In situ tTG activity was measured in cell extracts following the procedures by Zhang *et al.* [17], in which a biotinated tTG substrate was loaded into living cells and the generated product was measured by a streptavidin-linked enzyme assay.

HIT-T15 cells were cultured in 24-well plates and treated with different concentrations of MPA alone or combined with other test agents for 24 or 48 hr. On the day of experiment, cells were preincubated in 250 μL Krebs-Ringer-bicarbonate-Hepes buffer containing 2 mM of the tTG substrate 5-(biotinamido) pentylamine (supplemented with 0.1% BSA and 10 mM glucose) for 1 hr. After one wash with cold PBS, 0.2 mL homogenizing buffer was added to each well and cells were subjected to sonication (2 × 10 s) on ice. Protein contents were determined by Bio-Rad assay. Ten micrograms of homogenate protein was diluted to 50 μL with coating buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EGTA, and 5 mM EDTA) and added to the 96-well microtiter plates. After incubation overnight at 4°, 200 μL of blocking buffer (100 mM boric acid, 20 mM sodium borate, 80 mM NaCl (BBS) containing 5% BSA, 0.01% Tween-20, and 0.01% SDS) was added to each well. The plates were incubated for 2 hr at 37°. After one wash with a rinse buffer (BBS supplemented with 1% BSA and 0.01% Tween-20), 100 μL of HRP-conjugated streptavidin (1:2000) in BBS with 1% BSA and 0.01% Tween-20 was added and the plates were left at room temperature for 1 hr. After four rinses, 200 μL of substrate solution (0.04% *O*-phenylenediamine dihydrochloride in 50 mM sodium citrate phosphate buffer, pH 5.0) was added to each well. The plates were incubated at room temperature for 15 min and the reaction was terminated by adding 50 μL of 3 M HCl. Optical density of HRP-generated product was measured with a plate reader at 492 nm. tTG activity was expressed as the percentage of basal activity in control samples.

2.6. Detection of tTG by Western blotting

Aliquots of cell homogenates (20 μg protein each) were boiled in a loading buffer (100 mM Tris–HCl, pH 6.8, 200 mM dithiothreitol, 20% glycerol, 4% SDS, and 0.2% bromophenol blue) for 5 min. The samples were loaded on 10% SDS–polyacrylamide gels (SDS–PAGE) and subjected to electrophoresis. Afterwards, the separated proteins were transferred to nitrocellulose membranes. The membranes were first blocked in TBST buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.2% Tween-20) containing 5% dried nonfat milk for 2 hr at room temperature, and then blotted with a polyclonal antibody against tTG (1:1000) for 2 hr. After three washes in TBST (5 min each), the membranes were incubated with HRP-conjugated secondary antibodies (anti-goat IgG-HRP) for 60 min. Following three washes in TBST (15 min each), light emission was detected by using the ECL system (Pierce). The exposed films were analyzed by densitometry.

2.7. Determination of lactate dehydrogenase (LDH) release

Cells (0.15×10^6) were seeded in 96-well plates and cultured in normal medium for 2 days and then treated in serum-free culture medium with test agents for 24 or 48 hr. FCS was excluded during the treatment periods since the medium containing FCS exhibited unknown factors which increased the blank signal dramatically (equivalent to three times of LDH activity in HIT cell homogenates) and made it impossible to meaningfully measure the activity of released LDH in our samples. At the end of treatment, equal amounts of supernatant from each well were mixed with the LDH assay solution (kits from Sigma) and incubated for 30 min at 37°. The absorbance at 340 nm was measured and the LDH activity was calculated according to the instructions provided the assay kit supplier.

2.8. Statistics

All data are expressed as mean ± SEM and statistically analyzed using Student's *t*-test or ANOVA test.

3. Results

3.1. MPA effects on in situ tTG activity and apoptosis in HIT cells

As demonstrated previously [2,27], sustained depletion of GTP with MPA for 24 or 48 hr induced apoptotic death

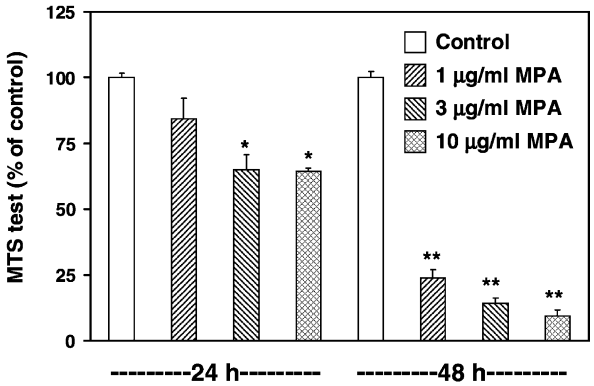

Fig. 1. Induction of apoptosis of HIT cells by MPA treatment in a dose-dependent manner. Cell seeded on 96-well plates were treated with various concentrations of MPA for 24 or 48 hr. Cell viability was evaluated by MTS test (formazan production). Data are mean ± SEM of four independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control.

of HIT cells as assessed by MTS test (Fig. 1). This convenient assay is widely used for determining the number of viable cells [29,30]. Our previous studies indicate that this test is a good way to monitor apoptotic cell death once the presence of the latter has been formally established by more specific means such as EM and DNA-laddering [2] and more recently by flow cytometry [27]. The *in situ* tTG activity was significantly increased in parallel under the same conditions (Fig. 2). This effect was time-dependent. At 10 μg/mL MPA, the enzyme activity was increased by 33% after treatment of 24 hr and increased further (+133%) after 48 hr. The two effects of MPA (promotion of cell death and enhancement of tTG activity) were closely co-related in a dose-dependent manner (Fig. 3). Furthermore, data in Fig. 3 demonstrated that both actions were prevented by the provision of guanosine (which restores GTP content and GTP/GDP ratio) but not by adenosine (which restores any possible reduction in

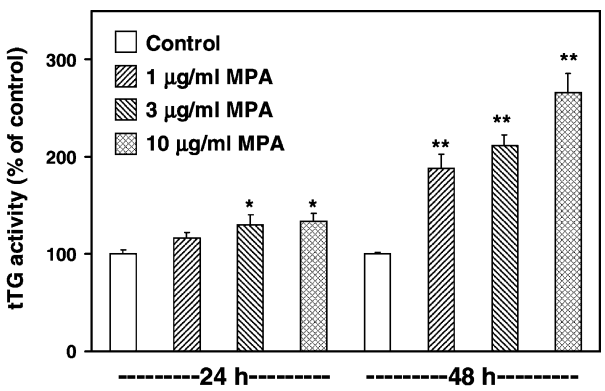

Fig. 2. Increase of *in situ* tTG activity by GTP depletion after MPA treatment in a dose-dependent manner. Cell seeded on 96-well plates were treated with various concentrations of MPA for 24 or 48 hr. *In situ* tTG activity was measured by a streptavidin-linked enzyme assay detecting the formation of a tTG-dependent product from a loaded substrate. The tTG activity in control cells is 0.158 ± 0.008 (arbitrary O.D. unit at 492 nm/10 μg protein during 2 hr). Values are mean ± SEM of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control.

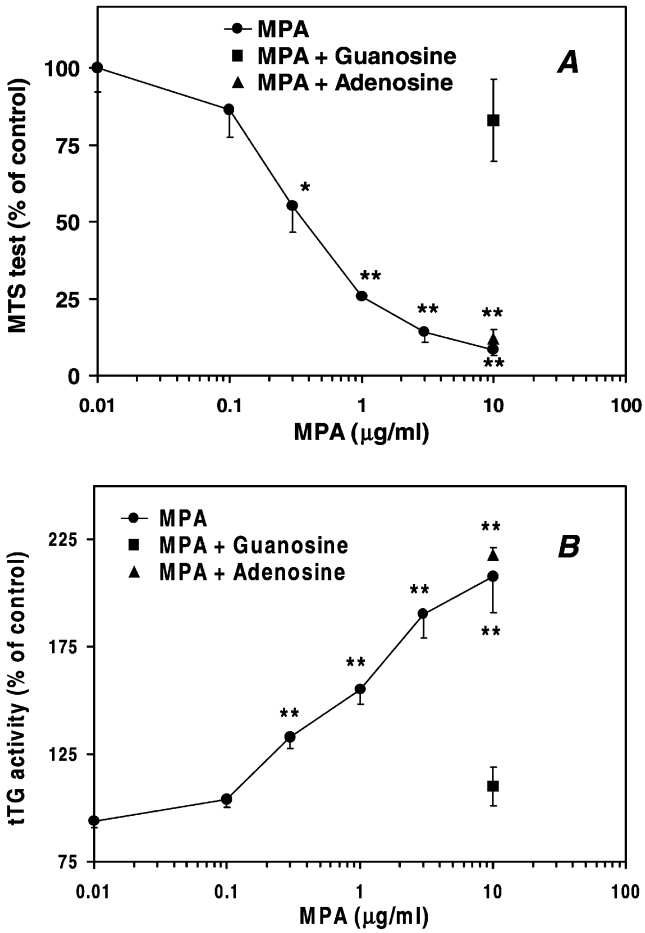

Fig. 3. Prevention of MPA-promoted induction of cell death (A) and the increase of *in situ* tTG activity (B) by the provision of guanosine but not adenosine. HIT cells in 96-well plates were cultured with a series of concentrations of MPA for 48 hr. Guanosine (500 μM) or adenosine (500 μM) was also included where appropriate. Cell viability was evaluated by MTS test (formazan production) and *in situ* tTG activity was measured by the formation of product from a loaded substrate for the enzyme. The tTG activity in control cells is 0.166 ± 0.009 (arbitrary O.D. unit at 492 nm/10 μg protein during 2 hr). Data are mean ± SEM of four independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control.

ATP content) [31–33], indicating that the enhanced tTG activity, similar to the induction of apoptosis, in MPA-treated cells is due specifically to the depletion of GNs.

The increased tTG activity during MPA treatment could result from elevated tTG mass and/or stimulation of enzyme activity. To address this question, we evaluated tTG protein levels by Western blotting. Surprisingly, MPA treatment did not enhance tTG mass but rather moderately decreased its levels at higher concentrations (Fig. 4), suggesting that GTP depletion may have a negative effect on tTG protein synthesis and/or degradation. Neither tTG degradation products nor its polymers could be detected in our studies (data not shown). The decrease of tTG mass was not observed at an early time point (24 hr) but was visible after 48-hr MPA treatment. This MPA-induced alteration in tTG mass was also blocked by addition of guanosine but not adenosine (Fig. 4). Thus, the increase of tTG activity in GTP-depleted

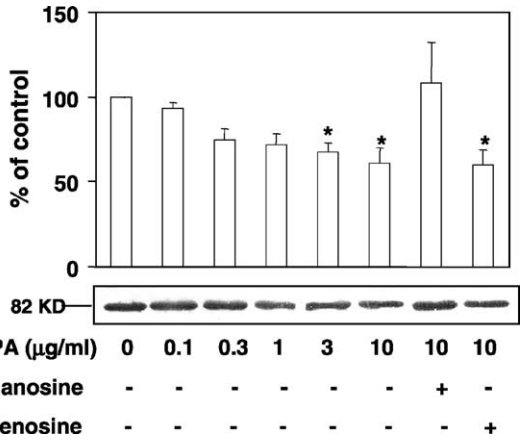

Fig. 4. Decrease of tTG expression at protein level after 48-hr MPA treatment. tTG was determined by Western blotting followed by densitometry analysis. The effect of MPA on the decrement of tTG mass was blocked by inclusion of guanosine (500 μM) but not adenosine (500 μM). The blots shown are representative of four independent experiments under the same conditions. Bar values are mean ± SEM from densitometric analysis of these experiments. $^{*}P < 0.05$ vs. control.

β-cells was due to the activation of the enzyme *per se* rather than an increase of its mass.

3.2. Effects of tTG inhibitors on tTG activity and apoptosis during MPA treatment

The relationship between increased tTG activity and apoptotic cell death during GTP depletion by MPA was studied under condition of tTG inhibition. In one series of experiments, two tTG inhibitors, monodansylcadaverine (MDC) or putrescine [34], were co-cultured together with 3 μg/mL MPA for 48 hr. The inhibitors had no apparent effect on cell viability and proliferation under basal conditions by themselves at the doses used in our study. In contrast, either 100 μM MDC or 6.25 mM putrescine was able to block near by half (42–43%) of the MPA-induced increase of *in situ* tTG activity (Fig. 5A). However, they did not rescue the cells from apoptosis under the same conditions (Fig. 5B). In addition, the tTG inhibitors did not alter tTG mass during MPA treatment as assessed by Western blotting (data not shown). In another series of experiments, free Ca^{2+} levels in culture medium were lowered to 80–120 nM by using the Ca^{2+} chelator EGTA, since tTG activity is not only regulated by GTP (suppression) but is also stimulated by Ca^{2+} [7,16,17]. The results revealed that lowering of extracellular free Ca^{2+} levels almost abolished (by 91%) the enhanced tTG activity due to MPA (Fig. 6A), but failed to prevent cell death triggered by GTP depletion (Fig. 6B). Lowering free Ca^{2+} levels to 80–120 nM itself did not damage cell viability.

In addition, MPA-induced apoptosis was also examined by flow cytometry which determines the subdiploidy apoptosis (sub-G1 phase) due to DNA fragmentation [27]. As shown in Table 1, 53% cells were detected undergoing apoptotic death after MPA treatment (3 μg/mL for 48 hr).

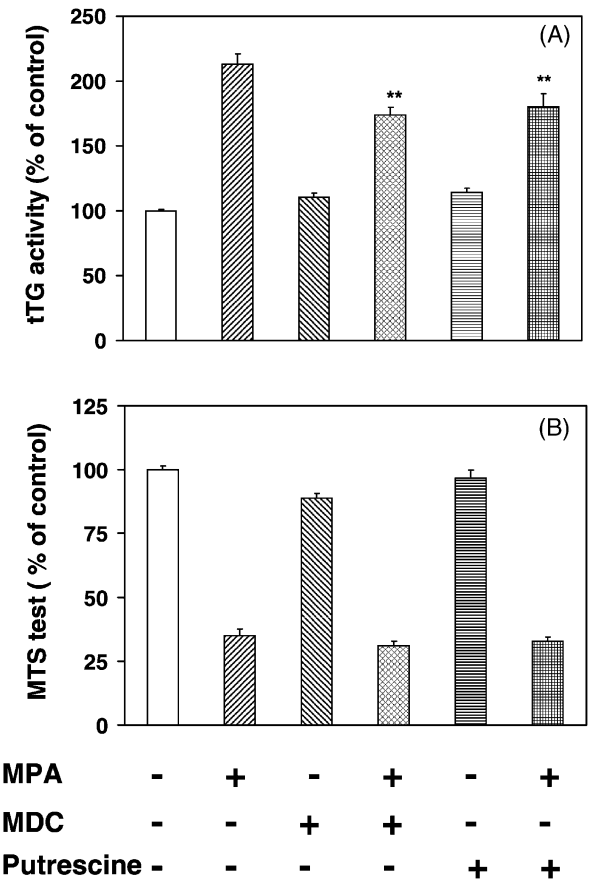

Fig. 5. Partial suppression of MPA-enhanced tTG activity (A) but failure of prevention of MPA-induced apoptosis (B) by two tTG inhibitors. HIT cells were treated with 3 μg/mL MPA alone or combined with 100 μM MDC or 6.25 mM putrescine in culture media for 48 hr. Cell viability was evaluated by MTS test (formazan production) and *in situ* tTG activity was measured by a streptavidin-linked enzyme assay. The tTG activity in control cells is 0.155 ± 0.005 (arbitrary O.D. unit at 492 nm/10 μg protein during 2 hr). Data are mean ± SEM of six independent experiments. $^{**}P < 0.01$ vs. MPA treatment alone.

Table 1
Analysis of MPA-induced subdiploidy apoptosis by flow cytometry

Condition	Apoptotic cells (%)
Control	8.4 ± 1.3
MPA, 3 μg/mL	53.2 ± 2.7
Putrescine, 6.25 mM	11.4 ± 3.2
MDC, 100 μM	8.7 ± 2.6
Lowering free Ca^{2+}	10.7 ± 3.3
MPA + putrescine	54.3 ± 6.1
MPA + MDC	51.3 ± 1.9
MPA + lowering free Ca^{2+}	42.3 ± 1.8*
MPA + 100 μM Z-VAD-FMK	13.9 ± 2.0**
MPA + 200 μM Z-VAD-FMK	12.2 ± 1.7**

HIT cells in 6-well plates were cultured under the above conditions for 48 hr. All cells were collected and stained by propidium iodide and subjected to flow cytometric analysis as detailed in the Section 2. The cells in sub-G1 phase whose DNA were fragmented were counted as subdiploidy apoptosis. Values are mean ± SEM from three to seven independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. MPA alone.

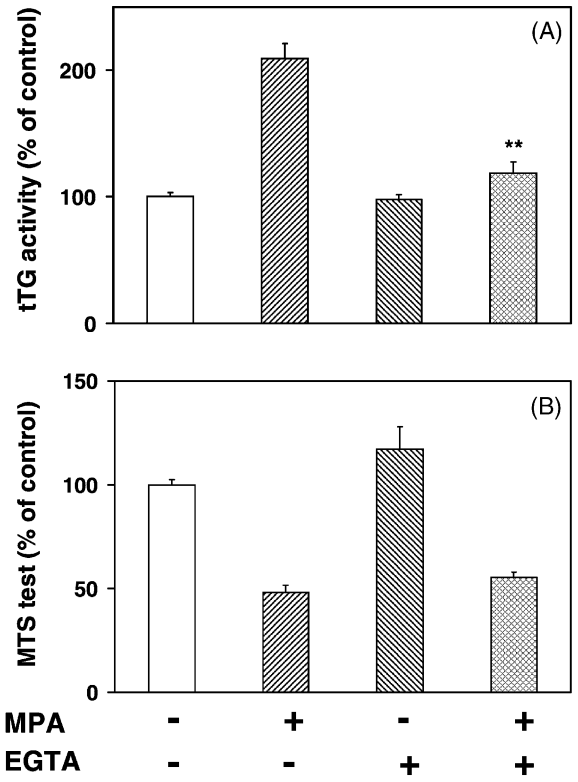

Fig. 6. Significant suppression of MPA-enhanced tTG activity by EGTA without prevention of MPA-induced apoptosis. HIT cells in 24-well plates were cultured with 3 μg/mL MPA for 48 hr. The Ca^{2+} chelator EGTA (0.83 mM) was also included where appropriate. tTG activity (A) was measured by the formation of a product from a loaded substrate for the enzyme and cell viability (B) was evaluated by MTS test. The tTG activity in control cells is 0.189 ± 0.008 (arbitrary O.D. unit at 492 nm/10 μg protein during 2 hr). Values are mean ± SEM of five independent experiments. $^{**}P < 0.01$ vs. MPA treatment alone.

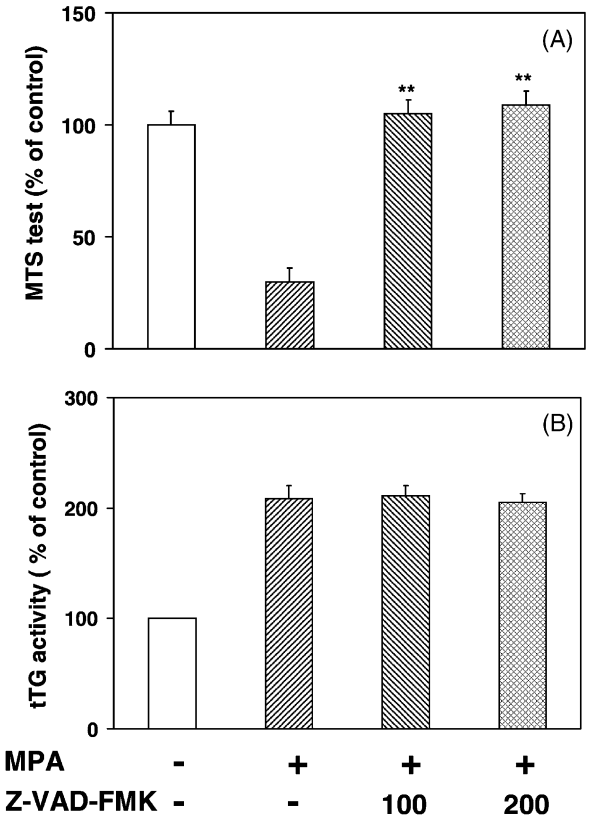

Fig. 7. Prevention by a pan-caspase inhibitor of apoptosis but not the increased tTG activity induced by MPA. HIT cells in multi-well plates were cultured with 3 μg/mL MPA for 48 hr and the pan-caspase inhibitor Z-VAD-FMK (100 or 200 μM) was also included upon appropriate. Cell viability was evaluated by MTS test (A) and *in situ* tTG activity was measured by a streptavidin-linked enzyme assay (B). The tTG activity in control cells is 0.158 ± 0.009 (arbitrary O.D. unit at 492 nm/10 μg protein during 2 hr). Data are mean ± SEM of three independent experiments. $^{**}P < 0.01$ vs. MPA treatment alone.

Putrescine, MDC, and lowering free Ca^{2+} did not affect cell viability by themselves. In agreement with the results from MTS test (Fig. 5B), neither putrescine nor MDC was able to rescue the cells from MPA-induced cell apoptosis (Table 1). However, lowering free Ca^{2+} was able to slightly (by 29%) but significantly relieve MPA-evoked cell death. These data suggested that while tTG might have some small involvement, it appeared not to play a major role in the induction of apoptosis due to GTP depletion.

3.3. Effects of caspase inhibitor on tTG activity in GTP depletion

It is well established that caspase activation is involved in both initiation and execution of apoptosis [3,4]. To study whether the increased tTG activity during GTP depletion by MPA is related to caspase activation, a cell-permeable pan-caspase inhibitor, Z-VAD-FMK [35,36], was employed. This peptide derivative is able to bind specifically to general caspases but the modification of its C-terminal aspartic acid with fluoromethyl ketone (FMK) renders it become an irreversible caspase inhibitor rather a substrate [37]. At 100 μM, Z-VAD-FMK completely prevented apoptosis induced by GTP depletion as assessed by MTS test (Fig. 7A) as well as the accompanied activation of several caspases [27]. Similarly, MPA-induced apoptosis was prevented by the caspase inhibitor as determined by flow cytometry (Table 1). However, the same concentration, or an even higher one (200 μM), of the pan-caspase inhibitor did not prevent the increase of *in situ* tTG activity caused by MPA treatment (Fig. 7B). This suggested that the enhanced tTG activity occurs either up-stream or independent of caspase activation during MPA-treatment.

3.4. tTG activity and cell morphology

Control HIT cells grew in clusters on culture plates and the cell boundary was ambiguous (Fig. 8A). MPA treatment caused the cells to round up and individual cells became recognizable (Fig. 8B). In addition, many of these cells lost their normal brightness. Guanosine, which abolished MPA-induced tTG activation (Fig. 3), restored normal morphology (Fig. 8C) while adenosine had no such effect (Fig. 8D). However, the morphological changes

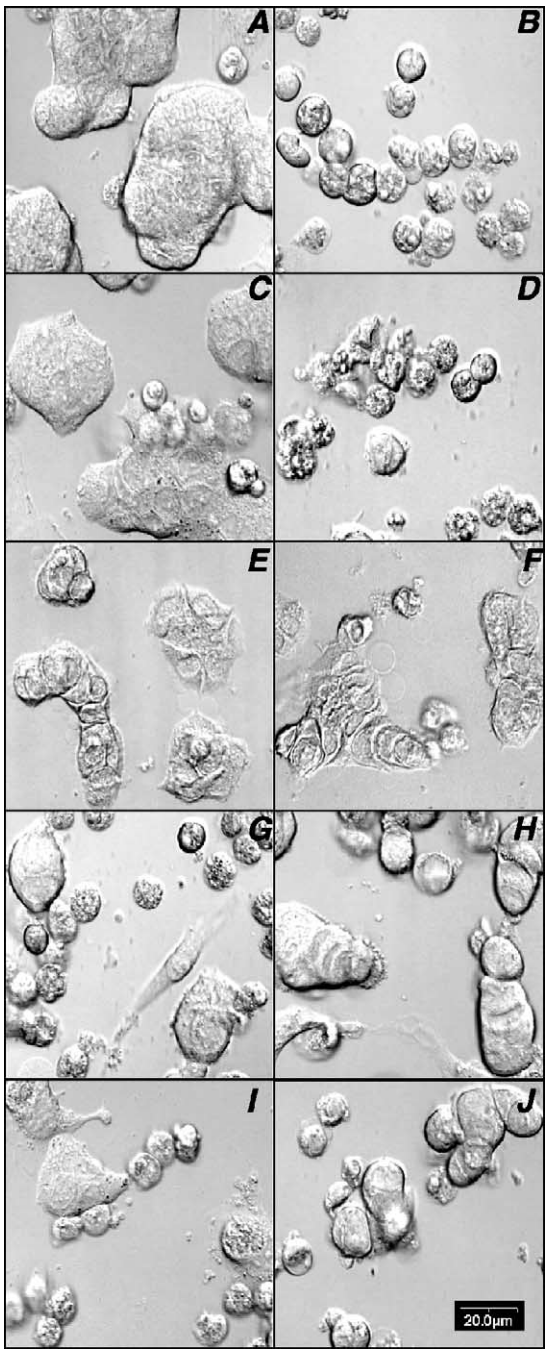

Fig. 8. Partial prevention of MPA-induced morphological alterations by tTG inhibitors. HIT cells in coverslip chambers were cultured for 48 hr in the following conditions: control (A); 3 μg/mL MPA (B); MPA plus 500 μM guanosine (C); MPA plus 500 μM adenosine (D); MPA plus 100 μM (E) or 200 μM (F) Z-VAD-FMK; MPA plus 6.25 mM putrescine (G) or 100 μM MDC (I); MPA plus putrescine and 100 μM Z-VAD-FMK (H); and MPA plus putrescine and 100 μM Z-VAD-FMK (J). Cell morphology was examined under differential interference contrast (DIC) by microscopy. Photos are representative of four independent experiments.

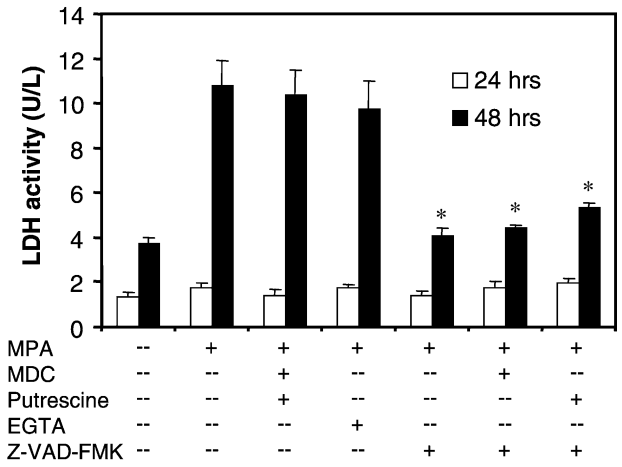

Fig. 9. LDH release from HIT cells after MPA treatment under various conditions. After HIT cells seeded in 96-well plates were treated with test agents in culture medium for 24 or 48 hr, the LDH activity in the supernatants was measured as described in the Section 2. Values are mean ± SEM of five independent experiments. $^{*}P < 0.01$ vs. MPA treatment alone. The final concentrations during treatment were: MPA, 3 μg/mL; MDC, 100 μM; putrescine, 6.25 mM; EGTA, 0.83 mM; and Z-VAD-FMK, 100 μM.

induced by MPA treatment could only be marginally reversed by the pan-caspase inhibitor (Fig. 8E and F), allowing for a possible role of tTG in this process compatible with its proposed role in protein cross-linking [5–7,12]. Indeed, either putrescine or MDC was able to markedly prevent the morphological changes in MPA-treated cells (Fig. 8G and I) and this effect was more apparent in the presence of a caspase inhibitor (Fig. 8H and J); these observations matched the observation that they could only produce incomplete inhibition of MPA-induced tTG activation (cf. Fig. 5A).

It has been proposed that an effect of tTG is to stabilization of dying cells by polymerization of substrate proteins [6,7]. Cross-linking of proteins by tTG might reduce the release of cytoplasmic materials from cells undergoing apoptosis and inhibition tTG therefore might increase the release in apoptotic cells. In order to address this issue, we assessed LDH release from MPA-treated cells under various conditions (Fig. 9). There was no apparent release of LDH after 24-hr treatment with MPA, whereas 48-hr exposure to MPA caused an increase of LDH release by 191%. Neither a combination of two tTG inhibitors (MDC and putrescine) nor lowering Ca^{2+} by EGTA affected the MPA-induced LDH release. However, the pan-caspase inhibitor (Z-VAD-FMK) prevented the MPA-induced LDH release, similar to its action in the protection of cells from death as assessed by MTS test and flow cytometry (cf. Fig. 7A and Table 1). As expected, inclusion of tTG inhibitors did not significantly alter the reversing effect by Z-VAD-FMK on LDH leakage. These data support our conclusion that tTG does not play a major role in the phenomena associated with apoptosis, including leaking of cytoplasmic contents.

4. Discussion

Normal intracellular levels of GTP (in the mM range) and other GNs are necessary for cell survival. Cell growth is affected by both accumulation [38] and depletion

[21–23,39] of GNs. Under the latter condition, cell growth is inhibited, with drastic reduction in DNA synthesis and alternations in RNA and protein synthesis. IMPDH is a key enzyme for the biosynthesis of GNs [21,22,39] and its specific inhibitors such as MPA interfere with DNA synthesis and induce death of various cells due to GTP depletion (down to μM ranges) [20,23,24]. IMPDH inhibition causes specific block of cells in the G1/S-phase of the cell cycle [40].

We have previously demonstrated that MPA and mizoribine, two structurally dissimilar IMPDH inhibitors, induce apoptosis of insulin-secreting cells [2]. The current studies were directed at two possible mechanisms underlying the apoptosis triggered by GN depletion. We focused on the possible role of tTG in apoptosis using our unique system in which the death of HIT cells is induced by MPA. We hypothesized that since tTG is GTP dependent [7,16,17] and has been implicated in at least the morphological changes of apoptosis [7,41,42], it would be a logical candidate to mediate at least a part of the cell death induced by GTP-deficiency. Although seven transglutaminases have been identified [11], the increase in transamidating activity in MPA-treated β-cells observed in our study appeared due to the activation of GTP-sensitive tTG for two reasons. First, only tTG and epidermal transglutaminase (TG3) are sensitive to GTP; the increased transamidation could be completely prevented by the provision of guanosine (cf. Fig. 3B) which restores the cellular GTP levels. Second, all other transglutaminases except tTG are distributed in specific tissues [11]. Our results for the first time revealed the presence of tTG in the β-cells by Western blotting. Our findings indicate that although an increase of tTG activity may accompany apoptosis, the activation of this enzyme may be not essentially required for the induction or execution of at least a significant number of the changes seen during apoptosis.

The relationship between tTG and apoptosis has been broadly investigated in recent years, and it has been proposed that the enzyme may be a complex player of the crucial balance between survival and death [5–8]. Several lines of evidence point to a role of tTG in apoptosis. First, a wide range of signaling pathways can lead to the induction of apoptosis associated with increased tTG activity, such as in response to interleukins [43], TGF-β [44], nitric oxide [19], thapsigargin (increasing intracellular Ca^{2+} levels) [45], and retinoids [9,44]. An augmentation of tTG expression (mRNA and/or protein), accompanied by cross-linking of polypeptides, occurred during apoptosis [5,9,44,45]. An increase of tTG activity was also observed in isolated human islets undergoing loss of β-cells by apoptosis [46]. Thus, tTG activation is often considered as a marker for apoptosis. Second, substantial knowledge of tTG in apoptosis has been accrued from studies using retinoic acid, a potent tTG inducer [9]. This agent and its derivatives induced a marked increase (as high as 10-fold) of tTG expression and inhibited cell growth in G1 phase [47–50], often followed by apoptosis [9,47–49] which could be blocked by inhibition of tTG activity with tTG inhibitors [47]. It appeared that retinoids exerted the effects via their nuclear receptors acting on the tTG promoter [51] and might be mediated by the repression of the expression of bcl-2, c-fos, and c-jun [48,49], but not of p53 and p21WAF1/CIP1 [49]; these proteins are important players in the apoptotic process. Third, experiments over-expressing tTG in fibroblasts and neuroblastoma cells provided further evidence for a role of tTG in the induction of apoptosis [42,52]. These cells displayed changes in morphology and adhesiveness characteristic of cells undergoing apoptosis as wells as enhanced susceptibility to apoptotic stimuli [42,52]. Consistent with these findings, transfection of cells with tTG antisense markedly decreased both spontaneous and retinoic acid-induced apoptosis [8,48,52]. However, it is important to note that most of these studies involved massive over-expression of tTG levels and/or use of stimuli such as retinoids which do not work through changes in GTP, as was the subject of our study.

In the present study investigating the possible role of tTG in apoptosis of HIT cells induced by GN depletion with MPA treatment, we did observed increased *in situ* activity of the enzyme after inhibition of GTP synthesis. Importantly, the time-course of the increase of *in situ* tTG activity was paralleled with the induction of programmed cell death. In contrast to the observations of increased tTG expression in other studies [9,44,45], however, this increment of tTG activity by MPA was not associated with an augmented expression of tTG protein (rather even with reduced mass at late treatment time periods) in our study. This phenomenon indicated that MPA altered tTG activity by lowering intracellular GTP level and not by increasing its synthesis, since tTG activity is negatively regulated by intracellular GTP levels [7,16,17]. This notion was further supported by our experiments using guanosine which, by restoring GNs to normal levels [31–33], blocked the increase of tTG activity and prevented HIT cells from apoptosis due to MPA. An increase of tTG activity during apoptosis but unaccompanied by amplified expression of tTG has also been observed in other studies [53,54].

Whether tTG is an upstream effector in the induction of GN depletion-induced apoptosis was examined by using its inhibitors. It has been reported that tTG inhibitors block apoptosis induced both by retinoic acid [47] and by expressing truncated DRPLA (dentatorubral-pallidoluysian atrophy) protein [55]. In our study, two specific tTG inhibitors (MDC or putrescine) [34] reduced MPA-evoked tTG activity by ∼43%. However, the inhibitors were barely able to relieve cell death caused by MPA treatment. In addition, tTG activity is also regulated by intracellular Ca^{2+} levels [7,16,17]. Although the enzyme is not active at the Ca^{2+} levels normally detected in viable cells (<1 μM) [10], the apoptosis-associated rise in cellular Ca^{2+} concentrations is sufficient to activate it [56]. We have previously observed that even 6-hr MPA treatment raised resting intracellular Ca^{2+} levels by 20% in HIT cells

[33]. It is, thus, very likely that higher Ca^{2+} levels would be reached in these cells after longer MPA treatment, which may contribute to tTG activation. Therefore, a Ca^{2+} chelator was used to lower extracellular Ca^{2+} to 80–120 nM (much lower than 1 μM that activates tTG). This maneuver could almost abolish the increased tTG activity due to GN depletion by MPA treatment, but was still only capable of partially protecting the cells from death. Thus, these experiments inhibiting tTG did not clearly reveal a biologically significant role for this enzyme as an ''initiator'' of cell death induced by GTP depletion. In a study by Zhang *et al.* [57], MDC prevented the increase in the cross-linked envelopes caused by tTG activation, but failed to inhibit DNA fragmentation due to interferon-β. Therefore, it seems that the major role of tTG in apoptosis is to function as a downstream effector in the late phase of apoptosis [6,7]. By catalyzing the Ca^{2+}-dependent formation of an insoluble protein scaffold from stable cross-links between proteins during apoptosis [5–8], tTG could maintain the contents of the dying cells before their clearance by phagocytosis, and thus prevent the nonspecific leakage of harmful intracellular components (e.g. lysosomal enzymes, nucleic acids, etc.) and consequently inflammatory responses [7]. We found that 48-hr exposure to MPA caused a marked increase of LDH release. However, neither a combination of two tTG inhibitors (MDC and putrescine) nor lowering Ca^{2+} by EGTA affected the MPA-induced LDH release. These findings suggest that tTG is also unlikely to be involved in the terminal release of large molecular compounds from the apoptotic cell.

The possible role of tTG in maintaining cell integrity for apoptotic cells to avoid the release of cellular components leading to inflammation is not well defined. The liver cells undergoing apoptosis induced extensive cross-linking of cellular proteins resulting in the formation of SDS-insoluble shells (similar to cornified envelopes of epidermis) in the so-called ''apoptotic bodies'' that contain oligonucleosomal fragments [58]. The insolubility of these apoptotic bodies was evoked by protein cross-linking bonds formed by tTG [58]. A late study observed that an increase of tTG activity in hepatocytes was paralleled by levels of forming SDS-insoluble apoptotic bodies [59]. In addition, intense tTG immunostaining is also found in the apoptotic bodies present inside phagosomes within the cytoplasm of neighboring cells [59]. Thus, cross-linking proteins by tTG plays a role in the formation of apoptotic bodies ready to be cleaned by the engulfing cells. Many substrates of tTG are cytoskeletonal proteins and some are nuclear proteins such as Rb [7], but there is no evidence that LDH is a tTG substrate. Published studies did not reveal a relationship between LDH release and tTG activity in apoptotic cells in which the tTG was either knocked out or highly expressed [60,61]. In one study, however, the number of cross-linked apoptotic bodies *was* significantly reduced in the tTG knockout cells [60]. The postulated role of tTG in the maintenance of cell integrity may occur in a critical period during the apoptotic process, i.e. DNA fragmentation and formation of apoptotic bodies before engulfed by neighboring cells. In MPA-treated HIT cells, DNA fragmentation occurred after 24-hr treatment [2]. Taken together, the data suggest that while tTG is unlikely to mediate cytoplasmic leakage in terminally apoptotic or necrotic cells (at least of large molecular weight molecules such as LDH), cross-linking of proteins by tTG may well play an important role in the morphological changes during programmed cell death. For the latter effect, cytoskeletonal proteins (actin, microtubules, and intermediate filaments) may be implicated since they act not only as the anchorage sites but also the substrates for tTG in cells undergoing apoptosis [7,41,62]. We indeed observed that the alterations of HIT cell morphology which accompanied tTG activation during MPA treatment were blocked by guanosine and partially relieved by tTG inhibitors. We emphasize, however, that we cannot exclude the possibilities that other roles for tTG might have been unmasked if we had been able to inhibit it completely, or if we had studied different inducers of apoptotic cell death.

It is now known that caspases play an essential role in both initiation and execution of apoptosis [3,4]. Many of tTG-targeted proteins are also substrates of caspases [8] and thus there may exist a relationship between tTG and caspases. In our study, tTG activation by GTP depletion was not affected when a pan-caspase inhibitor blocked activity of all caspases [27]. More importantly, our data indicated that the rise in tTG activity was not merely secondary to the apoptosis induced by GTP depletion, since the pan-caspase inhibitor prevented the cell from death but did not suppress the augmented tTG activity. Alternatively, this observation cannot completely rule out the possibility that tTG activation is an upstream event of caspase activation in GTP-depleted cells; the unavailability of more potent tTG inhibitors impedes the assessment of this possibility. However, the morphological changes evoked by GTP depletion could not be completely reversed by the caspase inhibitor, allowing for the possibility of an additional important role of tTG in this aspect. This concept was further substantiated by the observation that tTG inhibitors were able to partially prevent MPA-induced changes in cell morphology, corresponding to the partial suppression of the enhanced tTG activity under such conditions. Interestingly, tTG is a caspase-3 substrate and is cleaved during apoptosis in lymphoid cells [63]. Cleavage of tTG causes loss of its cross-linking function, and this event was regarded as a valuable biochemical marker of caspase-3 activation during the late execution phase of apoptosis. This may explain our observation of reduction of tTG mass in HIT cells at late MPA treatment (48 hr) following activation of caspases. Alternatively, the reduction of tTG mass might be due to the activation of other proteases, since it has been reported that, in neuroblastoma SH-SY5Y cells, depletion of GTP increases tTG degradation by calpain after elevation of intracellular Ca^{2+}

levels [17], a phenomenon perhaps also occurring in our HIT cells [33].

In conclusion, tTG may play a role as a downstream regulator in GN depletion-induced apoptosis in HIT cells, but this role may be principally involved to the terminal morphological changes. Interestingly, it has been reported that an increase of tTG activity was not observed during apoptosis under some circumstances, such as by treatment with dexamethasone [64], peroxinitrate [65], and by Fas-receptor stimulation [66]. Moreover, apoptosis induced by various agents were not affected in tTG ''knock-out'' cells [60]. These studies, in conjunction with the current ones, suggest that the hypothesis that tTG is a universally crucial component of the main pathway of the early apoptotic programs remains to be substantiated.

Acknowledgments

We are grateful to Dr. R.P. Robertson for providing HIT-T15 cells. This work was supported by the National Medical Research Council of Singapore (R-364-000-004-213 and NMRC/0540/2001, to G.D.L.). J.H. is a recipient of Research Scholarship from the National University of Singapore. Portions of this work were presented at the 59th (in San Diego, CA, June 1999) Annual Scientific Sessions of American Diabetes Association.

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