Nuclear Translocation of Tissue Type Transglutaminase during Sphingosine-Induced Cell Death:
A Novel Aspect of the Enzyme with DNA Hydrolytic Activity

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Introduction

Transglutaminase (TGase; R-glutaminyl-peptide:γ-glutamyltransferase, EC 2.3.2.13) is an enzyme that catalyzes, in a Ca²⁺-dependent manner, an acyl-transfer reaction between peptidyl glutamine residues and primary amines including proteinaceous ε-amino group of lysine residues (Folk and Finlayson, 1977; Lorand and Conrad, 1984; Chung, 1975; Greenberg et al., 1991; Folk, 1980). Distinct isoforms of this enzyme family are known to participate in various biological processes such as growth and differentiation of cells as well as plants and bacteria (Folk and Finlayson, 1977; Lorand and Conrad, 1984; Chung, 1975; Greenberg et al., 1991; Folk, 1980; Aeschlimann et al., 1994; Margosiak et al., 1990; Ando et al., 1989).

Tissue type (type 2) TGase comprises a major subfamily of the enzyme. It is distributed in the cytoplasm of cells and has long been proposed to participate in various biological processes such as growth and differentiation of cells (Birckbichler et al., 1981; Murtaugh et al., 1983; Knight et al., 1991), and more recently in cell death (Piacentini et al., 1991; Piacentini and Autuori, 1994; Amendola et al., 1996; Zhang et al., 1995; Szondy et al., 1997). Recent studies revealed further interesting aspects of physiological significance of tissue TGase. Firstly, the enzyme activity was pivotal in the ability of lesioned fish optic nerve to cross-link interleukin-2 molecules into a dimerized form, reducing the neurotoxicity of ambient oligodendrocytes and underlying regeneration of injured optic neurons (Eitan and Schwartz, 1993; Eitan et al., 1994). Secondly, in addition to its conventional cross-linking activity, tissue TGase is a nucleotide binding protein with GTPase and ATPase activity (Achyuthan and Greenberg, 1987; Lee et al., 1989; Takeuchi et al., 1992; Lee et al., 1993; Monsonego et al., 1997; Lai et al., 1996; Singh and Cerione, 1996; Takeuchi et al., 1994). The demonstration of a novel function as a G protein in α₁-adrenoceptor-coupled cell signaling system (Nakaoka et al., 1994; Chen et al., 1996; Feng et al., 1996) has evoked wider interest in the physiological relevance of the tissue TGase.

In this report, we expand the multifunctional activities of tissue TGase to include a possible role...
in cell death. During sphingosine-triggered death of human melanocytic A375-S2 cells, we demonstrate a loss of tissue TGase from the cytoplasm and a concomitant translocation of the enzyme to the cell nuclei. We also propose a biological relevance to this translocation of the enzyme by showing that it possesses a DNA hydrolytic activity that is Mg\(^{2+}\)-dependent, but not Ca\(^{2+}\)-dependent, and that is inhibited by Zn\(^{2+}\).

**Materials and Methods**

**Cell culture and chemicals**

A375-S2 cells (human melanoma cell line, ATCC CRL-1872, Nakai et al., 1988) were maintained in fetal calf serum (10%-)-containing RPMI 1640 medium, which was originally prepared for lymphocyte culture (Moore et al., 1967). D-sphingosine and D,L-erythro-dihydrosphingosine (DL-sphinganine) were the products of Sigma. These lipids were first dissolved in methanol, diluted with Hepes buffer containing bovine serum albumin (fatty acid-free grade, Sigma) and then with H\(_2\)O, resulting in a 2 mM solution in 25 mM Hepes (pH 7.4) containing 2 mg/ml bovine albumin and 10% methanol (a similar protocol was reported by Hauser et al., 1994). Guinea pig liver TGase was purchased from Sigma and Takara (Kusatsu, Japan). Although these commercial preparations were electrophoretically confirmed for purity, we further applied them to GTP-agarose (Sigma) column chromatography prior to use (Lee et al., 1989; Takeuchi et al., 1994).

**Analysis of cell death**

Following the overnight incubation (3.0x10\(^3\) cells per well on a 96-well plate, 5.0x10\(^5\) cells per 25 cm\(^2\) flask, and 4.6x10\(^6\) cells per 225 cm\(^2\) flask), cells were treated with vehicle buffer or either sphingosine or sphinganine (final concentration of 40 mM) that was preheated at 50 °C. Two minutes later (a significant portion of cell population already showed their morphological abnormality at this moment, see Fig. 1), cells were scraped from 225 cm\(^2\) flasks, pelleted by a low speed centrifugation and washed twice with 3 ml phosphate-buffered saline at 4°C. This step was performed in 4 min. Cell nuclei were separated from 1% NP-40 lysate and were pelleted as reported (Bates et al., 1994). The post-nuclear cell lysate was concentrated to 300-400 μl by Centricon 30 (Amicon, MA) with 40 mM Tris (hydroxymethyl) aminomethane-HCl (pH 7.5) containing 150 mM sodium chloride, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin and 1 mM dithiothreitol. The pelleted cell nuclei were briefly (4 sec x 3) sonicated in 300 μl of the same buffer (Takaku et al., 1995). Both lysate and nuclear fractions were analyzed on sodium dodecylsulfate-polyacrylamide gel electrophoresis (8% acrylamide) followed by immunoblotting with a monoclonal antibody CUB74 (Birckbichler et al., 1985). Tissue TGase was detected by ECL (enhanced chemiluminescence) method (Takaku et al., 1995; Ohashi et al., 1995), using a kit (Amersham). Cell death was estimated by assaying lactate dehydrogenase activity (Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988) retained in the surviving cells on a 96-well plate, using a LDH-Cytotoxicity testing kit (Wako Pure Chemicals, Osaka). Cells were also monitored for their morphology under a light microscope.

**DNA hydrolytic assay**

pGEM-3Z plasmid (Promega, 0.8 μg) and purified guinea pig TGase (0.14 μg) were incubated at 37 °C for 1 h in 10 μl of 25 mM Tris-acetate buffer (pH 7.6) containing 10 mM magnesium chloride, 0.1 M potassium acetate, 0.5 mM dithiothreitol, 50 μM EDTA and 10 μg/ml bovine serum albumin. The mixture was then analyzed on 1% agarose electrophoresis. Effect of divalent cations (2 mM CaCl\(_2\) and 2 mM ZnCl\(_2\)) was also tested.

**Other assays**

The TGase activity retained in the post-nuclear cell lysate was determined as incorporation of \([^{14}C]\) putrescine into N,N-dimethylcasein (Ohashi et al., 1995; Lorand et al., 1972) after a 2 h reaction to assure detectable activity. The activity released into the culture medium was also determined after concentrated through a GTP-agarose column (Takeuchi et al., 1994). Proteins were determined by the Bradford method (Bradford, 1976).
Results and Discussion

Severe toxicity of sphingolipids to A375-S2 cell line

Sphingosine and sphinganine function as lipidic mediators in the sphingomyelin signaling cycle, exerting multiple cellular effects on proliferation, survival and death processes (Kolesnick and Golde, 1994; Spiegel and Merrill, 1996; Hannun, 1996). A375-S2 cells started their death schedule by a sphingosine-treatment. After a 2 min exposure to sphingosine, morphological changes of the cells were already evident with the characteristic appearance of clusters of round-shaped cells (Fig. 1C). At 5 min or later, the damage to the cells was more prominent as featured by rough indentations and blebbing at the cell surface (Fig. 1D). Within 20 min, most cells became detached from the flask, aggregated, and were floating in the culture medium (Fig. 1E). Sphinganine exerted a similar, though slightly milder, effect on the cells (data not shown). To the contrary, untreated control cells and vehicle (bovine albumin/ethanol)-treated mock cells (see Materials and Methods), maintained their normal appearance throughout this time scale (Fig. 1A, B and F). Concurrently, a rapid reduction in the lactate dehydrogenase activity was observed in the sphingosine- and sphinganine-treated cells (Table I), confirming a severe toxicity of such sphingolipids to A375-S2 cells. Concentration of these lipids lower than 20 \( \mu M \) produced only a transient change in cell morphology (data not shown).

Intracellular dynamics of tissue type TGase

We next examined the intracellular redistribution of tissue TGase provoked by a sphingosine treatment of A375-S2 cells. In a preliminary experiment, bovine albumin, which was included in the culture medium, was found to contaminate the lysate fraction prepared from dying cells, giving rise to difficulty with accurate protein quantitation. We thus ran the following experiments not on the 'protein' basis but on the 'cell number' basis.

Sphingosine treatment evoked a dramatic loss of the classical enzymatic cross-linking activity of TGase in the cell lysate. As shown in Fig. 2A, we observed \( \approx 60\% \) loss of the activity as early as
2 min after the addition of sphingosine. This activity loss was partly explained by the concomitant emergence of the activity in the culture medium (Fig. 2B), suggesting a leak of the TGase activity from the damaged cells. Such a loss of TGase from the cells was further confirmed by immunoblotting analysis using CUB 74, a monoclonal antibody widely used to detect tissue type TGase (Takaku et al., 1995; Birkbichler et al., 1985; Ohashi et al., 1995) (compare the 77 kDa band between lanes 1 and 2, Fig. 2C). To our surprise, however, we found that a small, but significant portion of tissue TGase was redistributed to the nucleus by the sphingosine treatment (note the 77 kDa band appeared in lane 4, Fig. 2C, but not in lane 3 for the mock sample). In some experiments, a second 60 kDa band also emerged in the nuclear fraction as well, presumably suggesting a nuclear distribution of a proteolytic degradate of the enzyme during sphingosine-induced cell death (data not shown).

**DNA hydrolytic activity of tissue TGase**

Knowing i) the sphingosine-induced nuclear redistribution of tissue TGase (Fig. 2C), ii) the implication of the enzyme in cell death (Piacentini et al., 1991; Piacentini and Autuori, 1994; Amendola et al., 1996; Zhang et al., 1995; Szondy et al., 1997), iii) that the enzyme is also associated with a hy-
Table I. Lactate dehydrogenase assay of sphingolipid-treated A375–S2 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OD&lt;sub&gt;570&lt;/sub&gt;</th>
<th>Net absorbance (% Cell survival)</th>
</tr>
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<tbody>
<tr>
<td>Control (no cells)</td>
<td>0.177 ± 0.012</td>
<td>–</td>
</tr>
<tr>
<td>No treatment</td>
<td>0.577 ± 0.091</td>
<td>0.400 ± 0.079 (100.0 ± 19.8)</td>
</tr>
<tr>
<td>Mock (vehicle)</td>
<td>0.553 ± 0.047</td>
<td>0.376 ± 0.035 (93.9 ± 8.8)</td>
</tr>
<tr>
<td>Δ-Sphingosine</td>
<td>0.354 ± 0.028</td>
<td>0.177 ± 0.016 (44.3 ± 2.5)</td>
</tr>
<tr>
<td>ΔL-Sphinganine</td>
<td>0.433 ± 0.024</td>
<td>0.256 ± 0.012 (64.0 ± 3.0)</td>
</tr>
</tbody>
</table>

Note. A375–S2 cells were seeded on a 96-well plate (3x10<sup>3</sup> per well). After overnight culture in RPMI 1640 medium containing 10% fetal calf serum, cells received no treatment, vehicle for sphingolipid, 40 μM sphingosine and 40 μM sphinganine. Seven minutes later, medium was aspirated, and cells were washed with phosphate-buffered saline (100 μl x 2) and were then lysed with 0.1% Tween 20. Lactate dehydrogenase activity retained in the cells was assayed according to the manufacturer’s protocol. Values are expressed as mean ± SEM of triplicate determinations.

Although many reports have implicated that tissue type TGase plays a role in cell death, particularly in apoptotic cell death, the implication is mostly considered in terms of the classical cross-linking activity of the enzyme located in the cytoplasm (Greenberg et al., 1991). However, the nuclear localization of tissue TGase observed in A375-S2 cells that underwent sphingolipid-induced cell death (Fig. 2C) strongly suggests a novel aspect of the enzyme, when combined with its DNA hydrolytic activity (Fig. 3). It may be mentioned that the preexisting TGase, but not newly synthesized enzyme, was presumably relocated into the cell nuclei, since this relocation occurred in cells at an early phase (in 2 min after sphingosine treatment, Fig. 2C) of death process.

Moreover, the DNA hydrolytic activity shown in Fig. 3 expands the functional repertoire of tissue TGase, which has been hitherto reported to act as a classical cross-linking enzyme (Folk and Finlayson, 1977; Lorand and Conrad, 1984; Chung, 1975; Greenberg et al., 1991; Folk, 1980; Aeschlimann et al., 1994; Margosiaik et al., 1990; Ando et al., 1989). Ca<sup>2+</sup> or Zn<sup>2+</sup>, when tested alone in the absence of the enzyme, did not exhibit any observable effect (lanes 2 and 4).


Feng J.-F., Rhee S. G. and Im M.-J. (1996), Evidence that phospholipase deltal is the effector in the Gh (transglutaminase II)-mediated signaling. J. Biol. Chem. 271, 16451–16454.


