# Mechanism on the Cell Death of T-lymphocytes Induced by Organotin *in Vitro*

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## Summary

Organotin compounds are toxic to the immune system. Alkyltin compounds such as dibutyltin (DBT) and tributyltin (TBT) cause severe thymus atrophy. At histopathological examination, there is marked depletion of lymphocytes and the depletion is due to the cell death. In this study, the mechanism on the cell death induced by DBT or TBT was investigated from the angle of apoptosis in T-lymphocytes.

The results revealed that DBT induced caspase-independent cell death (necrosis), whereas TBT induced caspase-dependent cell death (apoptosis). Furthermore, we demonstrated the mechanism on TBT-induced apoptosis. Bid cleavage by activation of caspase-8 produced tBid and released cytochrome *c* from mitochondria. Release of cytochrome *c* induced the activation of caspase-3 through caspase-9. CAD activated by caspase-3 induced DNA fragmentation. That is to say, it was elucidated that TBT induced caspase-dependent and mitochondria-mediated cell death.

Organotin compounds exert powerful toxic action on the immune system, brain nervous system and endocrine system. Organotin compounds such as dibutyltin (DBT), dioctyltin (DOT) and tributyltin (TBT) compounds caused immunodeficiency of cell-mediated immunity and T cell-dependent humoral immunity due to severe thymus atrophy[1-8]. This atrophy depends on marked depletion of lymphocytes and the depletion is due to the cell death. Moreover, under the long-term exposure from 5 weeks to 8 weeks, dibutyltin-induced atrophy is reversible, whereas tributyltin-induced atrophy is irreversible. These results suggest that there might be different mechanism between the cell death induced by dibutyltin and tributyltin[1-8].

On the other hand, there are morphologically and biochemically two distinct forms in the mode of cell death, referred to as apoptosis and necrosis. Apoptotic cell death is morphologically characterized by cell shrinkage, the blebbing of plasma membranes, nuclear breakdown and DNA fragmentation. The biochemical hallmark of apoptosis is the cleavage of chromosomal DNA into nucleosomal units, which appears to be the final blow in the cell death process. Apoptosis is mediated by the activation of a family of cysteine proteases with specificity for aspartic acid residues, referred to as caspases.

Caspases are synthesized as precursor forms, and an apoptotic signal converts the precursors to mature enzymes, which subsequently cleave other caspases that are downstream in the cascade. Caspases activated by apoptotic signals cleave various cellular substrates which may be responsible for the morphological changes that occur in the cells.

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Fas ligand is expressed in activated T cells, and induces apoptosis in target cells by binding to Fas, its receptor. The Fas engagement activates a cascade of caspase proteases, as found in most apoptotic processes. Activation of cell surface receptor Fas leads to rapid inactivation of the electron transfer activity of cytochrome $c$ and subsequent release of cytochrome $c$ from mitochondria. In addition, the activation of cell surface death receptor leads to rapid activation of caspase-8, the apical caspase in the Fas-induced apoptotic pathway. The activation of caspase-8 initiates two pathway leading to the activation of downstream caspases. One is the activation of downstream caspases like caspase-3, caspase-6 and caspase-7 by directly cleaving them, the other is the activation of these downstream caspases indirectly by causing cytochrome $c$ release from mitochondria that triggers caspase activation through Apaf-1.

The release of cytochrome $c$ triggers the interaction of Apaf-1 and caspase-9, which in turn results in the activation of caspase-9. Activated caspase-9 then cleaves and activates procaspase-3, an event that leads to the cleavage of other death substrates, cellular and nuclear morphological changes, and ultimately, cell death[9].

Bid, a BH3 domain-containing proapoptotic Bcl-2 family member, is a specific proximal substrate of caspase-8 during Fas-induced apoptosis that functions upstream of $Bcl_{XL}$[10]. That is to say, Bid acts downstream of caspase-8 and upstream of $Bcl_{XL}$ in Fas-induced apoptosis. The cleavage of Bid by caspase-8 mediates the mitochondrial damage in the Fas pathway of apoptosis[11]. While full-length Bid is localized in cytosol, truncated Bid (tBid) translocates to mitochondria and thus transduces apoptotic signals from cytoplasmic membrane to mitochondria. tBid induces first the clustering of mitochondria around the nuclei and release of cytochrome $c$ independent of caspase activity, and then the loss of mitochondrial membrane potential, cell shrinkage, and nuclear condensation in a caspase-dependent fashion[9]. Full-length Bid can not induce apoptosis, while tBid induces apoptosis very rapidly and efficiently. tBid-induced cell death is inhibited completely by $Bcl_{XL}$.

Apoptosis is also accompanied by the internucleosomal degradation of chromosomal DNA. Recently, a caspase-activated DNase (CAD) and its inhibitor (ICAD) have been identified in the cytoplasmic fraction[12]. Activation of CAD downstream of the caspase cascade is responsible for internucleosomal DNA degradation during apoptosis and ICAD works as an inhibitor of this process. In addition, of caspases, particularly caspase-3 is activated during apoptosis and cleaves inhibitor (ICAD) of CAD and inactivates its CAD-inhibitory effect[13], suggesting that ICAD could be inactivated by an apoptotic signal.

In this study, the mechanism on organotin-induced cell death in T-lymphocytes was investigated from the angle of apoptosis as compared with dibutyltin and tributyltin.

## Materials and methods

### 1. Culture of T-lymphocytes and treatment

Thymus of wistar-derived male rats (3 week ages) was gently pressed through a stainless mesh. T-lymphocytes were washed PBS and suspended RPMI 1640 medium (GIBCO) at $8.0 \times 10^6$ cells/mL. Cells were incubated with 1 μM DBT or TBT in a 5.0 % $CO_2$ humidified incubator at 37℃. T-lymphocytes were pretreated with caspase-9 inhibitor (Z-LEHD-FMK) (Trevigen, Inc.) for 1 h.

### 2. Preparation of cytosolic fractions

T-lymphocytes were collected, washed PBS. Cells were resuspended in cell extraction buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 % Triton X-100, 10 % glycerol) and 2 % Protease Inhibitor Cocktail (NACALAI TESQUE, INC.). Cell suspensions were homogenized by using a glass dounce homogenizer and subjected to centrifugation at 10,000 rpm for 5 min. The resulting supernatants are cytosolic frantions. Cytosolic fractions without mitochondria were extracted using Mitochondria/Cytosol Fraction Kit (BioVision, Inc.). Protein

content was determined with BCA$^{TM}$ Protein Assay Kit (PIERCE).

### 3. Detection of caspase-8, -9, and -3 activity

Activity of caspase-8, -9, and -3 were evaluated by using Caspase-8 Apoptosis Detection Kit , Caspase-3 Apoptosis Detection Kit (Santa Cruz Biotechnology, Inc.), and Caspase-9 Fluorometric Assay Kit (BioVision, Inc.). Cytosolic fractions (5.0 μg) were placed in a 96-well plate with 50 μL reaction buffer and caspase substrate using fluorometric caspase-8 substrate (IETD-AFC), caspase-9 substrate (LEHD-AFC), caspase-3 substrate (DEVD-AFC) was added each well. Plates were incubated at 37℃ for 2 h and caspase activity was measured level of free AFC using a plate reader (Flex Station96, Molecular Devices) with a 400 nm excitation filter and a 505 nm emission filter.

### 4. Western Blot analysis

Cytosolic fractions (5.0 μg) were separated by various appropriate concentrations of SDS-PAGE; 15 % for cytochrome $c$, Bid; 12 % for CAD, ICAD, β-actin, and blotted to PVDF membrane. Antibodies used were cytochrome $c$, Bid (Santa Cruz Biotechnology, Inc.), CAD (MERCK), ICAD (stressgen), β-actin (SIGMA).

### 5. WST-8 assay

WST-8 Assay was perfomed by Cell Counting Kit-8 (DOJINDO). Cell suspensions were cultured with DBT or TBT in a 5.0 % $CO_2$ humidified incubator at 37℃ for 10, 60, 120 and 180 min. WST-8 solution was added and incubated for 2 h at 37℃. Cell suspensions were seeded at a density of $5.0 \times 10^5$ cells/well in 96-well plate. The absorbance at wavelength of 450 nm was measured by plate reader (SPECTRA MAX 190, Molecular Devices).

### 6. Detection of DNA fragmentation

DNA was prepared for gel electrophoresis as previously described[14]. Electrophoresis was performed in a 2.0 % agarose gel. DNA fragmentation was visualized by ethidium bromide staining.

## Results and Discussion

### 1. Activation of caspases

Caspases are synthesized as precursor forms, and an apoptotic signal converts the precursors to mature enzymes, which subsequently cleave other caspases that are downstream in the cascade. Caspases activated by apoptotic signals cleave various cellular substrates which may be responsible for the morphological changes that occur in the cells.

To evaluate the activation of caspases leading to apoptosis, time-dependent activities of caspase-8, -9, and -3 were first determined by using fluorometric assay. The weak activities of caspase-8, -9, and -3 were observed in DBT-exposed T-lymphocytes. In contrast, the strong activities of caspase-8, -9, and -3 were observed in TBT-exposed T-lymphocytes (Fig. 1). These results revealed that TBT-induced cell death was largely dependent on caspase activation, whereas DBT-induced cell death was independent on caspase cascade.

### 2. Release of cytochrome $c$ into the cytosol

The release of cytochrome $c$ into the cytosol triggers the interaction of Apaf-1 and caspase-9, which in turn results in the activation of caspase-9. Activated caspase-9 then cleaves and activates procaspase-3, an event that leads to the cleavage of other death substrates, cellular and nuclear morphological changes, and ultimately, cell death[11].

Therefore, time-dependent release of cytochrome $c$ into the cytosol was examined using western blot analysis. As shown in Fig. 2, the strong expression was observed by TBT-treatment for 10 min. In other words, TBT-treated T-lymphocytes caused release of cytochorome $c$ from mitochondria into the cytosol.
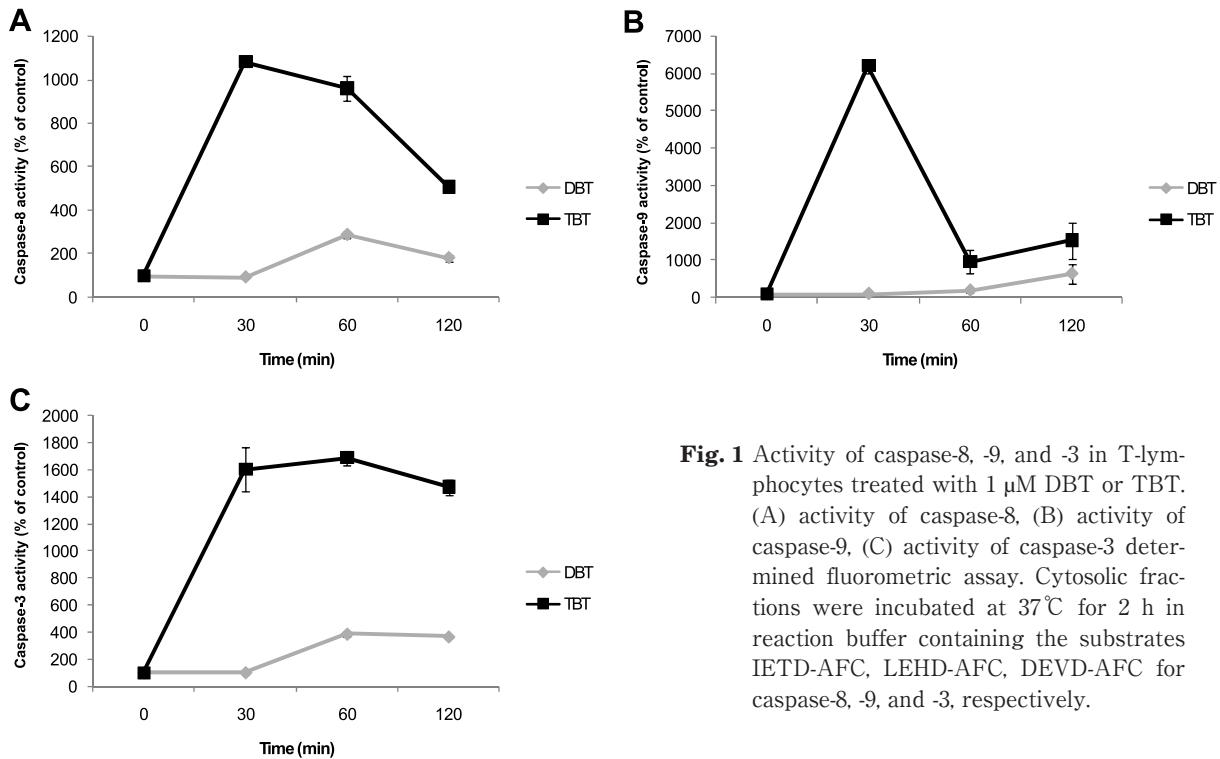
**Fig. 1** Activity of caspase-8, -9, and -3 in T-lymphocytes treated with 1 μM DBT or TBT. (A) activity of caspase-8, (B) activity of caspase-9, (C) activity of caspase-3 determined fluorometric assay. Cytosolic fractions were incubated at 37℃ for 2 h in reaction buffer containing the substrates IETD-AFC, LEHD-AFC, DEVD-AFC for caspase-8, -9, and -3, respectively.
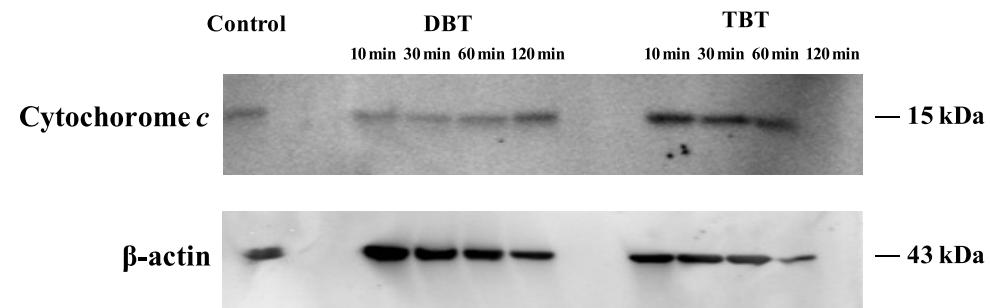


**Fig. 2** Expression of cytochrome *c* into the cytosol in T-lymphocytes treated with 1 μM DBT or TBT. Cytosolic fractions without mitochondria were analyzed by 15 % SDS-PAGE and transferred to PVDF membrane and immunoblotted rabbit anti-cytochrome *c*, mouse anti- β-actin antibodies. β-actin was used as internal standard.

## 3. Cleavage of Bid

Bid, a BH3 domain-containing proapoptotic Bcl-2 family member, is a specific proximal substrate of caspase-8 during Fas-induced apoptosis that functions upstream of Bcl$_{XL}$[12]. The cleavage of Bid by caspase-8 mediates the mitochondrial damage in the Fas pathway of apoptosis[13]. Bid cleavage by activation of caspase-8 produces tBid which induces first the clustering of mitochondria around the nuclei and translocates from the cytosol to mitochondria and releases cytochrome *c*. Therefore, expression of Bid was investigated by using western blot analysis. As shown in Fig. 3, expression of Bid was strongly decreased by TBT-treatment for 30 min. Bid cleavage was observed significantly in TBT-treated T-lymphocytes. Bid cleavage in DBT-treated T-lymphocytes was weak. These results concluded that TBT-induced cell death involved activation of caspase-8, Bid cleavage, and release of cytochrome *c* from mitochondria.

**A**



Bid  Control  DBT  TBT  — 22 kDa

**B**
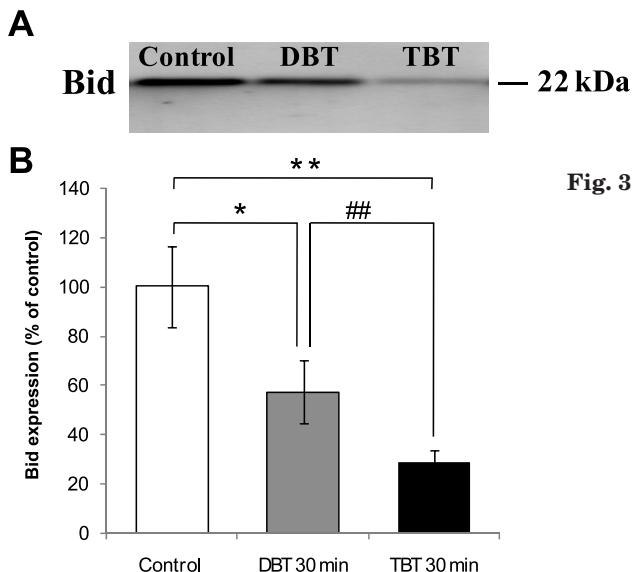


**Fig. 3** Detection of Bid cleavage. (A) Expression of Bid in T-lymphocytes treated with 1 μM DBT or TBT. Cytosolic fractions were analyzed by 15 % SDS-PAGE and transferred to PVDF membrane and immunoblotted rabbit anti-Bid, mouse anti-β-actin antibodies. (B) The relative percentage (% of control) of Bid proteins normalized for β-actin. Results are means ± SD (n = 4) and significance as *compared with Controls (*$p < 0.05$, **$p < 0.01$), significance as #compared with TBT-treated T-lymphocytes (##$p < 0.01$).

## 4. Mitochondrial damage

Organotin compounds induce gross mitochondrial swelling. In addition, these compounds inhibit mitochondrial ATP production. Therefore, mitochondrial damage was investigated using WST-8 assay evaluating the activity of mitochondrial dehydrogenase. As shown in Fig. 4, mitochondrial dehydrogenase activity showed a down-regulation in T-lymphocytes treated with TBT. Mitochondrial function was lost in TBT-treated T-lymphocytes.

## 5. Link of caspase-9 and caspase-3

To confirm the link of caspase-9 and caspase-3, the activity of caspase-3 in T-lymphocytes preincubated with caspase-9 inhibitor (Z-LEHD-FMK) or treated with TBT were determined. As shown in Fig. 5, the activation of caspase-3 with TBT was suppressed by the pretreatment of caspase-9 inhibitor. This means that TBT induced the activation of caspase-3 through caspase-9.
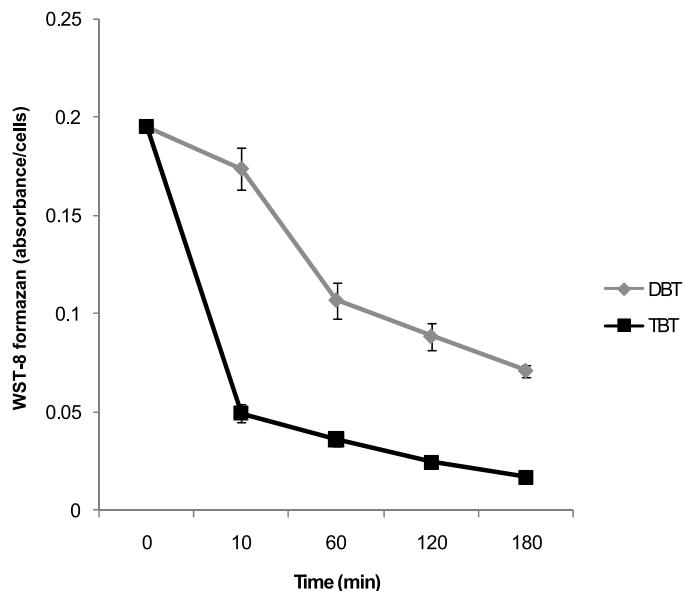


**Fig. 4** Detection of mitochondrial damage evaluated by mitochondrial dehydrogenase activity using WST-8 assay. Cell suspensions were cultured with 1 μM DBT or TBT in a 5.0 % $CO_2$ humidified incubator at 37 ℃.
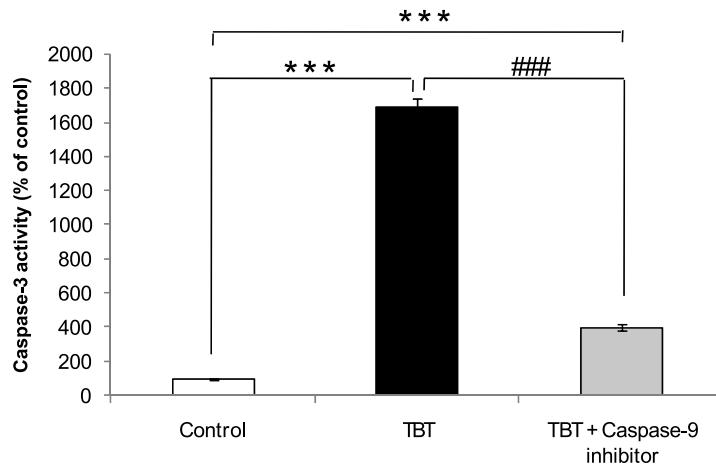
**Fig. 5** Activity of caspase-3 in T-lymphocytes treated with 1 μM TBT for 1h. Caspase-9 inhibitor (Z-LEHD-FMK) was preincubated for 1h at a concentration of 50 μM. Results are means ± SD (n = 5) and significance as *compared with controls (***$p$ < 0.001), significance as #compared with TBT-treated T-lymphocytes (###$p$ < 0.001).

## 6. Cleavage of CAD inhibitor in CAD activation and DNA degradation

Activation of CAD downstream of the caspase cascade is responsible for internucleosomal DNA degradation during apoptosis and ICAD works as an inhibitor of this process[12]. In addition, caspase-3 cleaves inhibitor (ICAD) of CAD and inactivates its CAD-inhibitory effect[13], suggesting that ICAD could be inactivated by an apoptotic signal. Therefore, to confirm the cleavage of CAD inhibitor in CAD activation and DNA degradation, expression of CAD/ ICAD were analyzed by using western blot analysis. As shown in Fig. 6, expression of ICAD was not seen in TBT-treated T-lymphocytes for 1h. Reversely, strong expression of CAD was seen in TBT-treated T-lymphocytes., in other words, CAD was activated in TBT-treated T-lymphocytes. Therefore, DNA fragmentation was further confirmed in T-lymphocytes treated with TBT. As shown in Fig. 7, a ladder pattern of DNA fragmentation was observed in TBT-treated T-lymphocytes. These results concluded that CAD activated by caspase-3 induced DNA fragmentation in T-lymphocytes exposed with TBT.
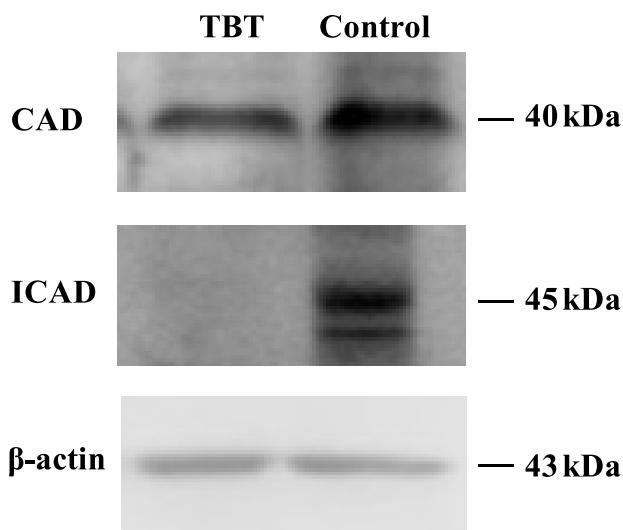


**Fig. 6** Expression of CAD/ICAD in T-lymphocytes treated with 1 μM TBT. Cytosolic fractions were analyzed by 12 % SDS-PAGE and transferred to PVDF membrane and immunoblotted rabbit anti-CAD, anti-ICAD, mouse anti- β-actin antibodies. β-actin was used as internal standard.
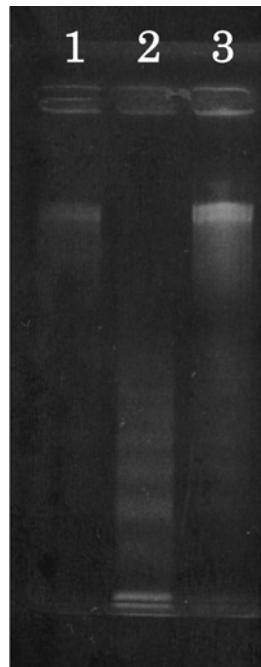
**Fig. 7** Detection of DNA fragmentation. DNA was analyzed by 2.0 % agarose gel electrophoresis. DNA extracted from (1) T-lymphocytes treated with 1 % ethanol, (2) T-lymphocytes treated with 1 μM TBT, (3) T-lymphocytes.

## Conclusion

We could discuss the mechanism on apoptosis of T-lymphocytes exposed with organotin by the established *in vitro* cell culture method. The results revealed that DBT induced caspase-independent cell death (necrosis), whereas TBT induced caspase-dependent cell death (apoptosis). Furthermore, we demonstrated the mechanism on TBT-induced apoptosis. Bid cleavage by activation of caspase-8 produced tBid and released cytochrome *c* from mitochondria. Release of cytochrome *c* induced the activation of caspase-3 through caspase-9. CAD activated by caspase-3 induced DNA fragmentation. That is to say, it was elucidated that TBT induced caspase-dependent and mitochondria-mediated cell death.

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