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Tamoxifen Induces Mitochondrial-dependent Apoptosis via Intracellular Ca^{2+} Modulation

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In the present work, we show that tamoxifen(Tam)-induced cytotoxicity is due to the mitochondrial-dependent pathway triggered by the intracellular Ca^{2+} increase in MCF-7 human breast cancer cells. Tam induced the intracellular Ca^{2+} increase. According to the experimental results with Ca^{2+} channel blockers, Tam-induced Ca^{2+} uptake seemed to depend on the voltage-sensitive Ca^{2+} channel at the early stage, but at later stages the intracellular Ca^{2+} increases are more likely due partly to the release of stored Ca^{2+} and partly to the capacitative Ca^{2+} or other entry pathways. Tam-induced Ca^{2+} increase led to the release of cytochrome c from mitochondria into the cytosol and the change of mitochondrial membrane potential. In MCF-7 cells, caspase-7 plays a key role in the downstream of apoptosis because caspase-3 is absent. In the cells treated with Tam, caspase-7 cleavage was increased almost two-fold. There was no marked alteration in the level of anti-apoptotic Bcl-2 protein; however, the cells showed increased expression of pro-apoptotic Bax protein more than two-fold in response to Tam. These results imply that the apoptotic signaling pathway activated by Tam is likely to be mediated via the mitochondrial-dependent pathway.

Key words – Apoptosis, calcium, cytochrome c, MCF-7, tamoxifen

Introduction

Tamoxifen (Tam) has been used in the treatment of breast cancer for more than three decades; however, the precise molecular mechanisms underlying Tam-induced cytotoxicity are yet unclear. Recent reports have shown that Tam inhibits the proliferation and induces apoptosis not only in estrogen receptor (ER)-positive breast cancer cells but also in ER-negative breast cancer cells and other cancer cells [10,14,25]. Non-genomic effects of Tam were suggested in both ER-positive and ER-negative cell lines; including the inhibition of protein kinase C [3], interferences with the function of ion channels [8], and the activation of extracellular signal-regulated kinase (ERK1/2) [26]. Recently, it has also been reported that Tam induces apoptosis by the initiation of a mitochondrial death program [10,15].

Apoptosis is physiological cell death regulated by genetic mechanisms and is principally characterized by morphological and biochemical changes in their nuclei, including chromatin condensation and internucleosomal DNA fragmentation [24]. Proteolytic cascades involving a family of proteases, called caspases, play a role in the signaling and execution stage of apoptosis. In particular, caspase-3 is the most active effector caspase in the execution stage of apoptosis by cleaving specific cellular substrates in both extrinsic (death receptor-dependent) and intrinsic (mitochondrial-dependent) pathways [2,9]. The activation of caspase-3 during apoptosis is regulated by multiple distinct pathways. Death receptors such as Fas/CD95 activate caspase-8, an initiator caspase, and the activated caspase-8 in turn activates caspase-3. Another pathway to activate caspase-3 is mediated by the alteration of mitochondria. Apoptosis-inducing stimuli lead to the release of cytochrome c from mitochondria and other apoptosis-inducing factors, which induce activation of pro-caspases and caspases. The release of cytochrome c can be related to the full opening of the mitochondrial permeability transition pore. The activation of caspase-3 during Tam-induced apoptosis has already been reported in ER-negative breast cancer cell lines [14]; however, caspase-3 is known to be absent in ER-positive MCF-7 breast cancer cell due to a 47-base pair deletion in exon 3 of caspase-3 gene. Instead of caspase-3, caspase-7 is known to act as a major downstream caspase in both intrinsic and extrinsic pathways in MCF-7 cells [22].

Tam-induced rapid cell death related to mitochondrial death programs has already been reported [10,15]. Tam-in-

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duced cytotoxicity was associated with release of mitochondrial cytochrome c, a decrease of mitochondrial membrane potential, an increase in production of reactive oxygen species, and stimulation of mitochondrial NO synthase [10,15].

During the course of mitochondrial function disruption, Ca^{2+} was also found to increase in Tam-treated cells. Ca^{2+} was the first identified endogenous substance to function as both a first and second messenger via the stimulation of an extracellular Ca^{2+} sensing receptor [23]. The concentration of intracellular free Ca^{2+} is maintained at about 100 nM, a very low level relative to the extracellular fluid (1.2 mM). The concentration of Ca^{2+} in the nuclear matrix or in the mitochondria matrix is similar to that of the intracellular Ca^{2+}; however, other intracellular organelles maintain a large Ca^{2+} concentration gradient versus the cytoplasm. The most important Ca^{2+} storage compartment is the endoplasmic reticulum (ER) in most cells.

In recent years, great attention has been placed on the possible correlation between the effects of members of the Bcl-2 family of proteins on Ca^{2+} homeostasis and their role in the control of apoptosis [4,15]. Bcl-2 was the first family member proposed to have the ability to alter intracellular Ca^{2+} homeostasis [1]. Anti-apoptotic members in the Bcl-2 family, such as Bcl-2 and Bcl-XL, are preferentially localized in the mitochondrial membrane and protect mitochondria from perturbation, thereby preventing cell death [11]. The specific localization of Bcl-2 in the membranes of mitochondria and ER, and the demonstration that Bcl-2 acts as an ion channel when inserted into lipid bilayers suggest that Ca^{2+} signaling could be a target of the action of this anti-apoptotic oncoprotein [20]. Under normal conditions, mitochondrial Ca^{2+} uptake appears to serve as an activating signal to increase metabolism. However, it has been shown that mitochondrial Ca^{2+} uptake promotes cytochrome c release in cell exposed to the proapoptotic agent, staurosporine [17].

In this study, we investigated the signaling mechanism of apoptosis induced by Tam in the following aspects: i) involvement of intracellular calcium modulation, ii) caspase activation, iii) cytochrome c release from mitochondria, and iv) disruption of mitochondrial membrane potential.

Materials and methods

Chemicals

([Z]-4-[1-(p-[Dimethylaminoethoxy] phenyl)-2-phenyl-1-butenyl] phenol) (TAM), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium (MTT), ionomycin calcium salt, 1,4-dihydro=2,6=dimethyl-4=(2=nitrophenyl l)-dimethyl ester (Nifedipine), 2-{[3-(trifluoro methyl)phenyl]amino} benzoic acid (Fluofenamic acid), 1,2-Bis(2-aminophenoxy)ethane N,N,N′,N′-tetraacetic acid tetrapotassium salt) (BAPTA), Fluo-3/AM, Pluronic F-127, propidium iodide (PI), Hoechst 33342, ribonuclease A, dimethylsulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM, with L-glutamine and 1,000 mg/L Glucose) were purchased from Sigma Chemical Co (St. Loues, Mo, USA). Fetal bovine serum (FBS) was purchased from GIBCO (New York, USA). All other chemicals were purchased from standard commercial sources.

Cell culture

The MCF-7 breast carcinoma cell line was obtained from the Korean Cell Line Bank, and maintained in DMEM containing phenol red with 10,000 units/ml of penicillin G, 10 mg/ml streptomycin, and 10% heat-inactivation FBS in a humidified atmosphere of 95% air; 5% CO_2 at 37℃. The culture media were changed every 2 or 3 days. Cells were harvested using trypsin-EDTA and then subcultured at weekly intervals.

Measurement of cell viability

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. MCF-7 cells were plated at 5 x 10^4 cells/ml in 48 multi-wells in DMEM with 10% fetal bovine serum and were allowed to adhere overnight. Cells were then treated with each different drug or with the same volume of vehicle (0.1% ethanol). For each assay, medium was aspirated from each well and replaced with 400 μl of 0.5 mg/ml MTT in phenol red-free DMEM medium. The treated cells were incubated in a 5% CO_2 incubator at 37°C for 4 hr. After incubation, the medium was removed and the converted dye was solublized in a 1:1 mixture of DMSO and EtOH. The color intensity was measured by the ELISA Reader (Tecan, Germany) with 570 nm filter.

Ca^{2+} mobilization

Intracellular Ca^{2+} was measured using Fluo-3/AM. The cells grown in DMEM were washed twice and resuspended in HBSS; 120 mM NaCl, 6 mM KCl, 1.5 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES and 12 mM glucose. For all Ca^{2+}-free experiments, $CaCl_2$ - free HBSS with 1mM

EGTA was used. For fluo-3/AM loading, 1 x 10^6 cells were resuspended in HBSS (or Ca^{2+} -free HBSS for Ca^{2+} starvation) containing 3 μM fluo-3/AM, 0.02 μM pluronic F-127. Incubation was performed in the dark at 37℃ for 25 min. Cells were then washed with HBSS (or Ca^{2+}-free HBSS) and split into 48 well plates at 2 X 10^5 cells/wells for each treatment. Fluorescent activity was measured (485 nm excitation/535 nm emission) every 1 or 5 min for 30min.

Assessment of morphology

Nuclear condensation and/or chromatin fragmentation found during early apoptosis was determined morphologically by the confocal laser scanning microscopy (CLSM) (LSM510, Carl Zeiss, Oberkohen, Germany) after labeling PI and Hoechst dye. Cells with each treatment were harvested onto a clean, fat-free glass slide with cellspin (Hanil Sci. Industrial, Korea). The cells were then fixed for 30 min in 4% paraformaldehyde and then stained in 4 μg/ml Hoechst 33342 or 10 μg/ml PI with 50 μg/ml ribonuclease A for 1 hr at 37°C. The cells were observed and photographed by CLSM (LSM510).

Measurement of cytochrome c release

Cells were attached on a slide glass by cytospin centrifugation for 2 min at 900 rpm using cellspin, and then the cells were fixed with 4% paraformaldehyde (Sigma, MO, USA) at room temperature for 20 min. Fixed cells were washed three times with PBS for 5 min and incubated with 0.2% Triton X-100 for 15 min. After three washes with PBS, the cells were incubated with a cytochrome c antibody in 1% bovine serum albumin (BSA) at room temperature for 2 hr. For a secondary reaction, the cells were incubated with a rabbit FITC-conjugated secondary antibody at room temperature for 2 hr. For the counter staining of nuclei, cells were incubated with PI (0.05 mg/ml) at room temperature for 30 min. The sample were washed several times with PBS and mounted and stored at 4°C in the dark until observation. The cells were observed by CLSM.

Measurement of mitochondrial membrane potential change ($\Delta\Psi m$)

To measure $\Delta\Psi m$, the fluorescent probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimida-zolocarbocyanine iodide) was used. Cells of 5 x 10^5 were incubated in darkness with 100 nM JC-1 at 37°C for 30 min. After washing with PBS, the cells were suspended in the PBS and then mounted with coverslip. The cells were observed by CLSM.

Western blot analysis

Cells were collected by centrifugation at 5,000 rpm for 10 min at 4°C. Proteins were extracted by washing cells twice with ice-cold PBS and incubating them for 60 min on ice in lysis buffer containing 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. For Western blotting, an aliquot of 60 μg of protein was separated by sodium dodecylsulfate-polyacrylamid gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose transfer membrane. Blotting was performed using the primary antibodies of anti-Bcl-2 (1:1000 dilution) and anti-Bax (1:1000 dilution), and then with the proper secondary antibody (Sigma, MO, USA). The immuncomplexes were detected by using the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech Korea, Inc., Seoul, Korea).

Statistical analysis

All experiments were carried out at least in triplicate. Results were expressed as mean ± SEM. Statistical significances between the control and the treatment group were determined by one-way analysis of variance and then followed by the Student T test. In all cases, a p value of less than 0.05 was considered statistically significant.

Results

Effect of Tam on cell viability

The effect of Tam on viability of MCF-7 cells was examined using the MTT staining method. When the cells were treated for 4 days with 1-10 μM of Tam, cell viability was decreased in a dose- and time-dependent manner as shown in Fig 1.

At the lower concentrations (under 4 μM), Tam did not affect cell viability. However, a significant cytotoxicity by Tam was noticed at the concentration of 10 μM. Cells treated with 10 μM Tam showed 64%, 58%, and 21% viability at 2, 3, and 4 days of incubation, compared to the control.

Tam has been known to induce apoptosis in MCF-7 cells. To examine that in the present experiment, we studied the morphological change of the nuclei in the cells

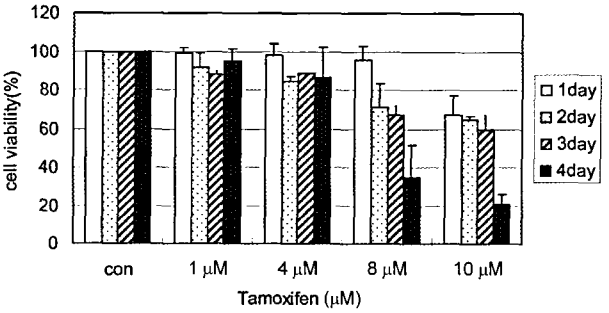

Fig. 1. Effect of Tam on cell viability in MCF-7 breast cancer cells. Cell viability assay was done by MTT staining method as described in "Materials and Methods". Data are expressed as percent change of the control (con). Data shown are means of three independent experiments, bars, SE.

treated with Tam by staining with Hoechst and PI dye. MCF-7 cells treated with 10 μM Tam showed severe chromatin condensation and nuclear fragmentation which was a typical early event of apoptotic cell death (Fig. 2).

Effect of Tam on intracellular Ca^{2+} level

Ca^{2+} homeostasis is very important for many cell function, including growth, differentiation, and death. Intracellular Ca^{2+} level change was examined to find out whether the cytotoxic effect of Tam was related to the intracellular Ca^{2+} modulation. Tam at the concentration of 10 μM caused a significant increase in the intracellular calcium level in the cells grown at medium containing 1.5 mM Ca^{2+} (Fig. 3A). The level of intracellular Ca^{2+} increased continuously until 10 min of incubation time and remained a sustained phase

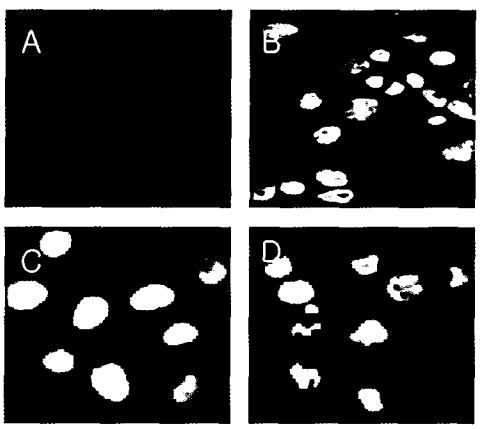

Fig. 2. Induction of apoptosis in breast cancer cells by Tam. MCF-7 cells were treated with 10 μM of Tam for 36 hr and stained with PI (A,B) or Hoeschst 33342 (C,D). A,C; untreated control. B,D; treated with 10 μM Tam. Data shown are from one of three independent experiments.

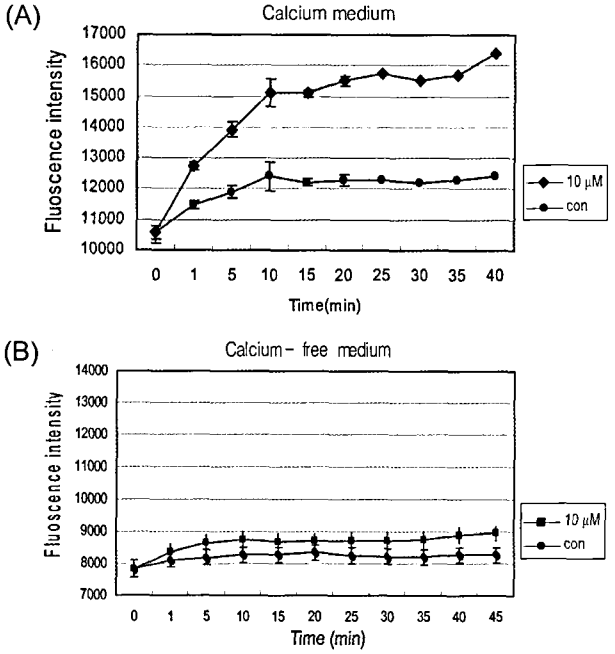

Fig. 3. Intracellular Ca^{2+} level change by Tam treatment in 1.5 mM Ca^{2+} medium (A) and Ca^{2+}-free medium (B). For each experiment, $1x10^6$ cells were seeded in 48 multi-well plate containg HBSS. $CaCl_2$ was omitted and EDTA was added into the reaction medium for Ca^{2+}-free experiment. Details are mentioned in "Materials and Methods". Data shown are means of three independent experiments, bar, SE.

after that up to 40 min. To check if the Ca^{2+} level increase was due to Ca^{2+} influx from the exterior, the same experiment was performed in the EDTA treated Ca^{2+} -free medium. As shown in Fig 3B, the Ca^{2+} level increase was not prominent, compared to the high level found in the calcium containing medium. The minor increase in the intracellular Ca^{2+} level in the Ca^{2+}-free medium might have resulted from the leak of the stored intracellular Ca^{2+} pool. Thus, the main source of intracellular Ca^{2+} increase induced by Tam treatment is most likely due to the Ca^{2+} influx from outside. To investigate which type of Ca^{2+}channels in the cell membrane mediate the Tam-induced Ca^{2+} influx, two types of Ca^{2+} channel blockers were used; nifedipine (Nif), a voltage-sensitive Ca^{2+} channel blocker; and flufenamic acid (FA), a nonselective cation channel (NSCC) blocker. When the cells were placed in the Ca^{2+} medium, Tam treatment induced the increase of intracellular Ca^{2+} level within 5 min. However, 50 μM of Nif blocked the Tam-induced intracellular Ca^{2+} increase at the early stage of incubations (Fig. 4). As incubation continued, cytosolic Ca^{2+} level increased slowly. FA at 50 μM reduced

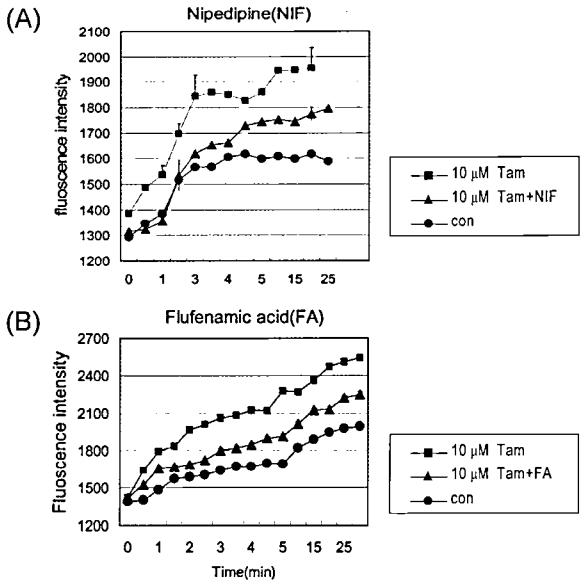

Fig. 4. Effect of Ca^{2+} channel blockers on the Ca^{2+} increase induced by Tam. Intracellular Ca^{2+} was measured by using Fluo-3/AM as described in "Materials and Methods". Cells of $2x10^5$/well were pretreated with 50 μM of each Nif (A) or FA (B) for 4 hr, then were exposed to Tam for 10 min in Ca^{2+} medium. Data shown are the means of three independent experiments, bars, SE.

the Tam-induced Ca^{2+} influx but didn't completely block the increase of intracellular Ca^{2+}. These results indicate that Tam-induced Ca^{2+} uptake seemed to depend on mainly the voltage-sensitive Ca^{2+} channel at the early stage, but at later stages the intracellular Ca^{2+} increases are more likely due partly to the release of stored Ca^{2+} (as shown in Fig 3B) and partly to the capacitative Ca^{2+} or other entry pathways.

Effect of BAPTA and ionomycin on Tam-induced apoptosis

In order to determine the co-relationship between Ca^{2+} increase and the apoptotic death, cells were pretreated with 10 μM of BAPTA, an intracellular Ca^{2+} chelator , for 2 hr before being treated with Tam. Cell viability and intracellular Ca^{2+} level change was then studied in cells treated with and without Tam. As shown in Fig. 5A, BAPTA pretreatment almost completely blocked Tam-induced intracellular Ca^{2+} increase. BAPTA also significantly abolished the cytotoxicity of Tam (Fig. 5B). When the effect of BAPTA on Tam-induced apoptosis was checked by CLSM, BAPTA prevented the nuclear shrinkage caused by the Tam treatment (Fig. 6). If the increase of intracellular Ca^{2+} level induced by Tam was one of the main causes of

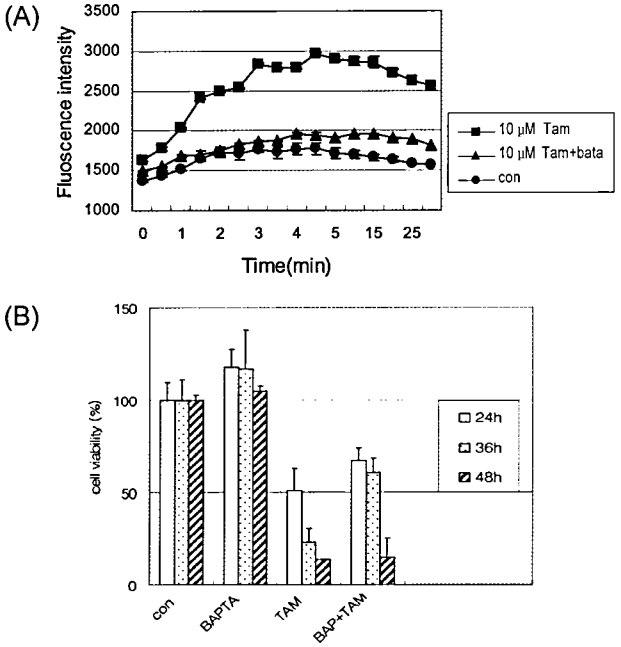

Fig. 5. Effect of BAPTA on intracellular Ca^{2+} change (A) and cell viability (B) in the cells treated Tam. For each experiment, $2x10^5$ cells/well were seeded into the 48 multi-well plate. Ca^{2+} level was measured in the cells exposed to BAPTA 2 hr before treatment with Tam. Data shown are the means of three independent experiments, bar, SE.

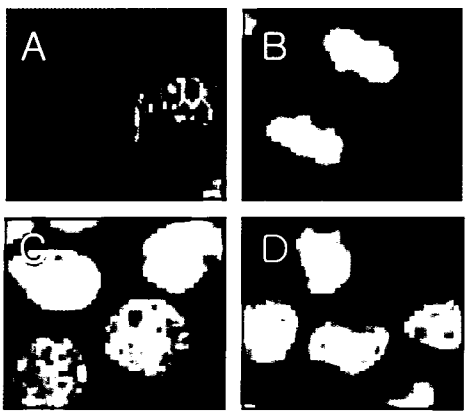

Fig. 6. Influence of BAPTA on Tam-induced apoptosis. A; control, B; 10μM Tam, C; 10 μM BAPTA, D; 10 μM Tam + 10 μM BAPTA. The experimental conditions are the same as in Fig. 5. The cells were stained with PI for 1 hr at 37°C, then observed under CLSM. Data shown are from one of three independent experiments.

cell death, an enforced Ca^{2+} increase by ionophore would give the same results. To check the above hypothesis, cells were treated with ionomycin. At the 10 μM of ionomycin, cells were found to undergo cell death in a time- and dose-dependent manner (Fig. 7). Cell viability dropped to

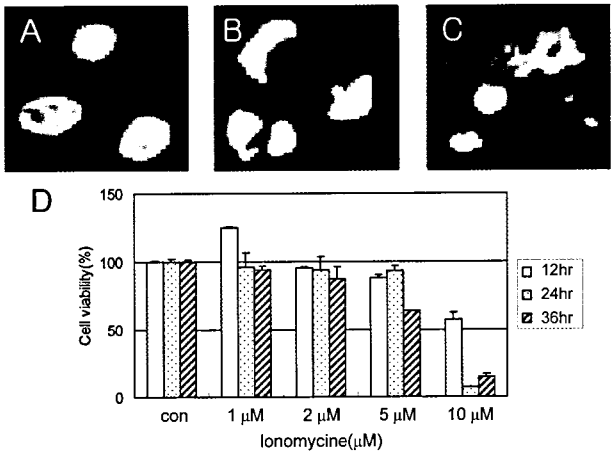

Fig. 7. Induction of apoptosis by ionomycin. The experimental conditions are as same as in Fig. 6. A; untreated control, B; 5μM ionomycin, C; 10 μM ionomycin. Data shown are from one of three independent experiments. D; cell viability change in ionomycin treated cells. Data shown are the means of three independent experiments, bars, SE.

56% of the control after 12 hr treatment. When PI staining was performed, ionomycin- induced cells underwent apoptosis. These findings further demonstrated that raising intracellular Ca^{2+} induced apoptosis in MCF-7 cells.

Cytochrome c release from mitochondria and mitochondrial membrane potential change (ΔΨm)

Cytochrome c released from mitochondria is known to have pivotal role in the progression of apoptosis. Therefore, to check if the apoptotic cell death induced by Tam was preceded by release of cytochrome c, immunocytochemistry was performed using specific antibody for cytochrome c. In the immunofluorescent study, cytochrome c in the control cell was found in a punctuate pattern, in keeping with its normal mitochondrial location (Fig. 8). However, a diffuse distribution of cytochrome c was found in the Tam-induced cells, which supported the hypothesis that Tam led to the release of cytochrome c from mitochondria into the cytosol. The change of ΔΨm in response to the Tam was investigated by using JC1 dye under CLSM. Tam treatment induced a shift from the JC1 aggregate (red) to the JC1 monomeric (green) form of the dye, indicating mitochondrial depolarization because of the loss of membrane potential (Fig. 9).

Effect of Tam on the caspase activation and Bcl expression

Caspase activation is regulated by the release of

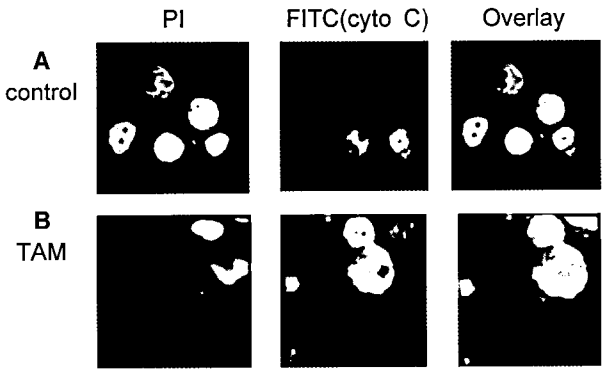

Fig. 8. Immunofluorescence analysis of Tam- induced cytochrome c release in MCF-7 cell. Cytochrome c was localized on mitochondria in the untreated cells (punctuate staining) but was found to be distributed throughout the cytosol (diffuse fluorescence in the cells) when treated with the Tam for 36 hr. The experimental conditions are mentioned in "Materials and Methods". A; control, B; 10 μM Tam.

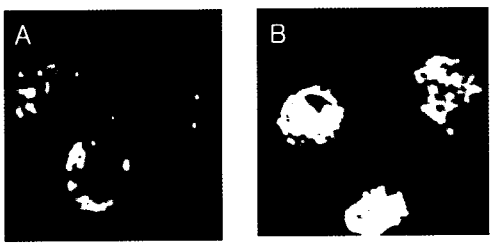

Fig. 9. Analysis of mitochondrial membrane potential change in Tam-induced apoptosis. JC1 dye - stained MCF-7 cells were analyzed with CLSM in the absence (A) and presence of Tam (B).The untreated cells showed a significant population of cells with the J aggregate (red) form of the JC1 dye indicating a normal mitochondrial membrane potential. A significant shift of J aggregate (red) to J monomer (green) was observed in the population of cells treated with 10 μM Tam for 36 hr. The experimental details are mentioned in "Materials and Methods". Data shown are from one of three independent experiments.

mitochondrial polypeptide activators including cytochrome c during apoptosis. MCF-7 breast cancer cells do not express caspase-3. Caspase-7, however, plays a key role in the downstream of apoptosis. To prove the involvement of caspase-7 in Tam-induced apoptosis, cells were treated with 10 μM Tam for 36 hr, and then the level of cleaved/activated caspase-7 protein was analyzed using Western blotting. As shown in Fig. 10, caspase-7 cleavage was increased almost two-fold by Tam treatment. Caspase-12, which is specifically localized to the ER, has been reported to cleave during ER stress-induced apoptosis. To investigate whether Tam-

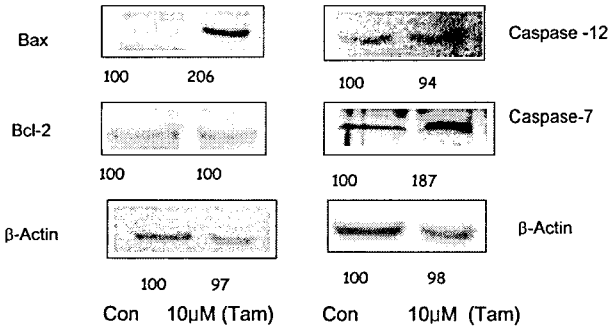

Fig. 10. Western blot analysis showing the effects of Tam on Bcl family proteins and caspase proteins levels. The experimental details are mentioned in "Materials and Methods". Data shown are from one of three independent experiments. The numbers are from densitometric scanning.

induced apoptosis is related to ER stress, the level of caspase-12 expression was also checked, but there was no alteration of its level. These results indicated that caspase-7 is related in Tam-induced apoptosis. The involvement of ER-associated caspase-12 is not clear and requires further study.

The Bcl-2 family proteins are regulators that play a major role in the apoptosis. They exist as multigene subfamilies with many homologues that are individually expressed in various tissues. The Bcl-2 family includes death promoting (Bax) and death inhibiting (Bcl-2) members, most of which are resident proteins in the mitochondrial membrane. When we examined the expression level of them in the cells treated with Tam, there was no marked alternation in the level of Bcl-2 protein. However, the cells showed increased expression of Bax more than two-fold in response to Tam, as compared to the control (Fig.10). This result implies that the apoptotic signaling pathway activated by Tam is likely to be mediated via the mitochondrial-dependent pathway.

Discussion

Tam is already known to induce apoptosis in many cell systems including ER-positive cells and ER-negative cells. The action of Tam is partly through the activation of caspases in several cancer cell lines including human breast cancer and prostate cancer cells [5,14]; however, the precise mechanism of Tam-induced apoptosis is yet unclear. The present work demonstrates that Tam induces apoptotic cell death through the intracellular Ca^{2+} modulation in MCF-7 cells.

Ca^{2+} is an essential intracellular regulator of signal transduction [4,6]. Diseases are often associated with the perturbed regulation of intracellular free Ca^{2+} [7]. The pathological consequences of perturbed Ca^{2+} regulation are not surprising considering the role of Ca^{2+} in a variety of cellular processes as diverse as muscle contraction, apoptosis, gene expression, synaptic transmission, and proliferation. Because of the importance of Ca^{2+} in disease, transporters of Ca^{2+} such as voltage-gated plasmalemmal Ca^{2+} channels and Ca^{2+} - APTase can be potential therapeutic targets for which screening assays are useful. One area of research receiving attention in regard to alterations in Ca^{2+} homeostasis is cancer. Recently, Ca^{2+} influx via a pathway known as capacitative Ca^{2+} entry (CCE) has been observed in MCF-7 breast cancer cells [17], and inhibitors of Ca^{2+} influx can inhibit the proliferation of breast cancer cells [12]. Furthermore, an inhibitor of CCE blocks the growth of prostate cancer cells *in vitro* and a mouse *in vivo* model. Another Ca^{2+} pathway altered in cancer is the plasma membrane Ca^{2+}-ATPase, which is a pump responsible for maintaining low levels of resting intracellular Ca^{2+}. The expression of the plasma membrane Ca^{2+}-ATPase is altered in human breast cancer cell lines [13]. The above findings indicate that Ca^{2+} homeostasis is essential for the normal cell function.

In the present work, Tam increased intracellular Ca^{2+} levels via extracellular Ca^{2+} influx and partly by releasing stored intracellular Ca^{2+}. Nif, a voltage-sensitive Ca^{2+} channel blocker, could block the Tam-induced intracellular Ca^{2+} increase at only the early stage of incubations. FA could only partly reduce the Tam-induced Ca^{2+} influx. These results indicate that Tam-induced Ca^{2+} uptake seems to depend on the voltage-sensitive Ca^{2+} channel at the early stage, but at later stages intracellular Ca^{2+} increase are more likely due partly to the release of stored Ca^{2+} (even though we did not check the exact source of the intracellular Ca^{2+} leak).

In the present study, we showed that the increase in intracellular Ca^{2+} level was directly related to apoptosis. This finding was further checked by using BAPTA and ionomycin. The increase in intracellular Ca^{2+} by ionomycin was accompanied by apoptotic cell death. However, BAPTA, and intracellular Ca^{2+} chelator, abolished the cytotixic effect of Tam. In general, an increase in cytosolic Ca^{2+} stimulates the mitochondrial uptake of Ca^{2+}. Mitochondrial

Ca^{2+} accumulation, in turn, changes the mitochondrial microenvironment [20]. Mitochondrial Ca^{2+} overload is known to lead to the opening of the permeability transition pore. In the present work, intracellular Ca^{2+} level increases, induced by Tam, lead to mitochondrial permeability change, clearly demonstrated by JC1 dye staining. Tam treatment resulted in the membrane depolarization. The opening of a permeability transition pore by depolarization leads to the release of cytochrome c and other apoptosis inducing factors which can activate pro-caspases that eventually lead to cell death. The release of cytochrome c from the cells treated with Tam was clearly demonstrated by fluorescence microscopy in the present work.

Bax and Bid was also known to induce a change in mitochondrial membrane polarization. Bax is a pro-apoptotic member of Bcl-2 family. Bcl-2 and Bcl-X_L of this family can promote cell survival, but Bax promotes cell death. The overexpression of Bax accelerated cell death [18]. When we checked the expression of Bcl-2 family proteins in the cell treated with Tam, the level of Bcl-2 protein was not changed at all. However, the expression of Bax protein was increased more than two-fold, which might lead to the change of mitochondrial membrane permeability. Caspase activation is initiated by the release of mitochondrial proteins, including cytochorome c. Caspases lead to the drastic morphological changes of apoptosis by proteolysing and disabling a number of key substarates, including gelsolin, protein-tyrosine kinase (PTK), and focal adhesion kinase (FAK) [9]. In MCF-7 breast cancer cells which show the absence of caspase-3, caspase-7 is known to be in the downstream of the caspase cascade and plays a pivotal role in the terminal execution stage of apoptosis. Caspase-7 is able to cleave various substrates, which contributes to the typical biochemical feature of apoptosis. When we checked the level of cleaved caspase-7 by Western blotting, it was increased almost two-fold, indicating that Tam induced the apoptotic cell death through the activation of caspase-7 in MCF-7 cells.

Tam has been used to treat breast cancer for several decades; however, its molecular mechanisms are still unclear. Zhang *et al.* [24] suggested that Tam could be used as a potential modulator of tumor-associated Ca^{2+} signaling not only in human breast cancer cells but also in several other ER-free cells. They found that Tam increased the spatial expansion of Ca^{2+} wave and Tam pretreatment accelerated Ca^{2+} ionophore-induced cell death. In the present work, we proved that Tam induces apoptosis through the modulation of intracellular Ca^{2+} levels in association with the disruption of mitochondrial function.

Endoplasmic reticulum (ER) has been known as a major Ca^{2+} storage site. Recent study has shown that the ER Ca^{2+} store may play an important role in apoptotic induction [16]. Ca^{2+} released from ER induced mitochondria to release cytochrome c and Bax, a pro-apototic protein. To the contrary, a reduction of Ca^{2+} released from ER prevented mitochondrial damage. It has been reported recently that overexpression of Bax caused a loss of ER content [1]. Caspase-12 is specifically localized on the cytoplasmic side (outer membrane) of ER and is activated during ER stress-induced apoptosis [27]. In our present results, Tam treatment in MCF-7 cells induced two-fold increase in Bax expression. Thus, we checked if there were any changes in the caspase-12 protein level. As mentioned earlier, caspase-7 expression increased two-fold by Tam treatment, but the caspase-12 protein level was not changed at all. However, the above results cannot exclude the possibility of ER-mitochondrial cross-talk in Tam-induced apoptosis. Further studies should be performed to pin down the precise interaction of ER-mitochondrial Ca^{2+} modulation during Tam-induced apoptosis.

In summary, we have provided the evidence that Tam induces the increase in intracellular Ca^{2+} level in MCF-7 breast cancer cells. The Ca^{2+} level change leads to apoptotic cell death via the mitochondrial-dependent pathway including loss of mitochondrial membrane potential, cytochrome c release, and caspase activation.

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초록 : 탐옥시펜에 의해 유도된 세포 내 칼슘농도 변화와 미토콘드리아 의존적 세포사멸

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유방암 치료제로 사용되는 탐옥시펜 (Tam)은 MCF-7 세포주에서 세포증식을 억제하며 세포사멸을 유도한다. 본 연구에서는 Tam의 세포독성 효과가 세포 내 칼슘이온 농도 증가에 따른 미토콘드리아-의존 기작에 의하여 일어난다는 것을 보여준다. Tam에 의해 유도된 세포 내 칼슘이온 농도 증가는 주로 외부로부터의 칼슘 유입에 의한 것으로 생각된다. 칼슘 채널 억제제를 이용한 실험 결과에 의하며, 칼슘 증가 초기 단계는 주로 전압의존 칼슘채널에 의한 것이며 후기에는 세포 내 저장된 칼슘의 유출, 혹은 다른 방법에 의한 칼슘 유입으로 생각된다. Tam에 의한 세포 내 칼슘 증가는 미토콘드리아로부터의 cytochrome c 방출과 미토콘드리아막의 탈분극에 의한 membrane potential 변화를 초래하였다. 세포사멸에 주도적인 역할을 하는 caspase의 확인에 있어서는, MCF-7 세포는 caspase-3이 결핍되어서 caspase-7이 중심적인 역할을 하는 것으로 이미 알려져 있다. 본 연구에서 확인한 결과 Tam 처리시 caspase-7이 활성화되었으며, 또한 세포사멸 조절 단백질인 Bcl-2 종류 단백질들의 발현을 조사 한 결과 세포사멸 억제 단백질인 Bcl-2의 발현에는 변화가 없었으나 촉진단백질인 Bax는 Tam 처리시 단백질 양이 2배로 증가되었다. 이상의 결과에 의하면, Tam에 의해 유도되는 세포사멸과정은 세포질 내 칼슘이온 농도증가에 의한 미토콘드리아의 변화가 주도적인 역할을 하는 것으로 생각된다.