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Thapsigargin Enhances Cell Death in the Gastrointestinal Stromal Tumor Cell Line, GIST-T1, by Treatment with Imatinib (Glivec)

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Imatinib is a specific inhibitor of c-KIT that has recently been approved for the treatment of chronic myeloid leukemia and gastrointestinal stromal tumor (GIST). Thapsigargin is an inhibitor of calcium transport to the endoplasmic reticulum (ER) and can inhibit protein maturation. In this study, we evaluated the synergistic cytotoxic effect of thapsigargin and imatinib on the GIST cell line, GIST-T1. Cell viability and cell death were determined by 3,-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry, respectively. In addition, the amount and activation of c-KIT on the cell surface were measured by flow cytometry and western blot analysis, respectively. Thapsigargin alone (for 5 hr incubation) was shown to decrease the amount of c-KIT on the GIST-T1 cell surface and to slightly inhibit the associated tyrosine kinase activity. Interestingly, thapsigargin significantly enhanced the cell death induced by imatinib compared with the effect of imatinib alone. The results further suggested that thapsigargin induced the increased cell death in GIST-T1 cells co-treated with imatinib, at least in part, *via* disrupting the translocation of c-KIT to the cell surface, and decreasing the activated c-KIT molecules.

Key words —— gastrointestinal stromal tumor, thapsigargin, imatinib, c-KIT, GIST-T1

INTRODUCTION

Gastrointestinal stromal tumors (GIST) are thought to derive from mesenchymal cells of the digestive tract. These tumors are characterized by the expression of both c-KIT and CD34 on the cell membrane. Recent studies linked a mutation in c-kit with oncogenic activation giving rise to GIST.[1,2] The c-kit proto-oncogene encodes for a tyrosine kinase receptor. Binding of c-KIT by the ligand, stem cell factor (SCF) activates the tyrosine kinase function to transduce downstream signaling to the nucleus by phosphorylation of tyrosine residues in signaling proteins. It is thought that deregulation of this kinase activation is one of the principal mechanisms underlying abnormal cell growth and survival of malignant cells.[2]

Protein kinase inhibitors that disrupt the autonomous signaling loops have recently been developed for clinical use.[3–9] Imatinib was designated as an inhibitor of BCR/ABL, platelet-derived growth factor receptor (PDGFR), and c-KIT.[10] Imatinib has been used effectively for the treatment of unresectable or metastatic GIST with constitutive activation of c-KIT.[7,8]

Thapsigargin is an agent that induces cell death *via* the endoplasmic reticulum (ER) stress response as it inhibits calcium uptake into the ER.[11] Newly synthesized proteins undergo folding in the ER, and high luminal calcium is necessary for the proper folding of several proteins. A protein will only be exported from the ER if it is correctly folded.[12–14] So thapsigargin inhibits protein synthesis and process-

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ing.[15)]

It is very important to investigate the reagents which enhance the cytotoxic effect to the GIST with STI571. We report here that thapsigargin enhances the cell death induced by imatinib in GIST-T1 cells, which are a cell line that was established from a patient with metastatic GIST.[16)] To our knowledge, GIST-T1 is one of only two GIST cell lines that exist. GIST-T1 has a heterogenic 57-bp deletion in exon 11 of the *c-kit*, which results in the constitutive activation of c-KIT. We also address the mechanism by which thapsigargin has its effect on cell death in GIST-T1 cells treated with imatinib by investigating the maturation process and activation of c-KIT.

MATERIALS AND METHODS

Reagents —— Dulbecco's Modified Eagle's Medium (DMEM) and antibiotics (penicillin, streptomycin) were purchased from Invitrogen Corporation (New York, U.S.A.). Thapsigargin was purchased from Sigma (St. Louis, U.S.A.). Anti-phosphotyrosine antibody (PY20) was purchased from Zymed Laboratories (San Francisco, U.S.A.). Anti-c-KIT (K963) was purchased from Immuno-Biological Laboratories (Gunma, Japan). Imatinib, Glivec capsules (Novartis, Switzerland) were diluted in water (5 μg/μl) and stored at –20°C.

Cells and Cell Culture —— The human GIST cell line, GIST-T1 and human peripheral nerve sheath tumor cell line, HS-sch-2 were characterized in detail by Taguchi *et al.* and Sonobe *et al.*, respectively.[16,17)] Both cell lines were cultured in DMEM supplemented with penicillin, streptomycin, and 8% fetal bovine serum (FBS), and maintained in a 5% CO_2 atmosphere at 37°C in a humidified incubator.

3,-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide (MTT) Assay —— Each well of a 96-well plate was seeded with 1×10^4 cells/100 μl of cell suspension. After the initial 24 hr of culturing, imatinib, cisplatin, or thapsigargin were added to each well. After 24 hr incubation with/without reagents, 10 μl of MTT [Sigma-Aldrich, 2.5 mg/ml in phosphate-buffered saline (PBS)] was added in each well and incubated for 2 hr at 37°C. The resulting violet formazan precipitates were solubilized by the addition of 100 μl of 500 ml/l N,N,-Dimethyl formamide/100 g/l sodium dodecylsulfate (SDS) and the cells were then incubated for a further 4 hr at room temperature. Absorbances were then measured on a plate reader at 570 nm. Triplicate and three independent experiments were performed and similar results were obtained.

Flow Cytometry for the Determination of Cell Death —— 1×10^6 cells were seeded in a 6-cm plate and incubated for 24 hr. The cells were then treated with imatinib, cisplatin, or thapsigargin [final concentrations, imatinib (1 μg/ml), cisplatin (2 μg/ml), thapsigargin (100 nmol/l), imatinib (1 μg/ml) + cisplatin (2 μg/ml), or imatinib (1 μg/ml) + thapsigargin (10100 nmol/l)]. After 24 hr incubation, both adherent and non-adherent cells were trypsinized and washed twice with ice-cold PBS, and then centrifuged at 500 *g* for 5 min. Cells were treated with Annexin V-FITC Reagent (BioVision Palo Alto, CA, U.S.A.) according to the manufacturer's protocol. Apoptosis was then measured by flow cytometry (FACSCalibur, Becton Dickinson, NJ, U.S.A.) to determine the percentage of apoptotic cells with high FITC signal per 10000 gated events (using CellQuest soft ware, Becton Dickinson). Dead cells were indicated as Annexin V-positive cells. Cell death was represented as the early phase of apoptotic cells (right lower quadrant) and late phase of apoptotic cells or necrotic cells (right upper quadrant). Three independent experiments were performed and similar results were obtained.

Quantitive Analysis of c-KIT Expression on the Plasma Membrane —— 1×10^6 cells were seeded on 6-cm plates and incubated for 24 hr. The cells were then treated with thapsigargin (0, 300 nmol/l) for 5 hr. Cells were washed twice with PBS and treated with normal mouse IgG or an anti-c-KIT antibody and then FITC-conjugated secondary antibody in PBS. The amount of c-KIT on the cell surface was measured by flow cytometory. Three independent experiments were performed and similar results were obtained.

Western Blot Analysis —— 4×10^6 cells were seeded on 10-cm dish for 24 hr, and then treated with imatinib (0.1, 1 μg/ml) or thapsigargin (100, 200, 300 nmol/l) in medium for 5 hr. Cells were washed three times with ice-cold PBS and then lysed in RIPA buffer (Upstate Biotechnology, U.S.A.) containing 20 mmol/l sodium pyrophosphate, 20 mmol/l NaF, 1 mmol/l orthovandate, 2 mmol/l pyrophosphate, 1 mmol/l PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cell lysates containing comparable amounts of proteins, estimated by a Bradford assay (BioRad, Munchen, Germany) were subjected to direct western blot analysis.

Immunoelectromicroscopic Analysis —— Cells were treated with/without the indicated reagent for

6 hr. Adherent cells were washed twice with ice-cold PBS and scraped. Scraped cells were collected by centrifugation and the collected cells were fixed with 1% glutaraldehyde for 2 hr on ice. Fixed cells were dehydrated through graded ethanols, embedded in Lowicryl K4M (Electron Miroscopy Science, Washington, U.S.A.), and polymerized in an Ultraviolet Polymerizer Tur 200 (Dosaka EM Co., Kyoto, Japan) for 24 hr at –35°C and for 3 days at room temperature. For immunogold electron microscopy, thin section mounted on collodion-carbon-coated nickel grids were immersed in 0.01 mol/l PBS and then treated with anti-c-KIT for 12 hr. The sections were then treated with 15 nm colloidal gold (goat antirabbit Ig G Chemicon, U.S.A.) for c-KIT, diluted at an adequate concentration in 0.01 mol/l PBS containing 3% skim milk for 30 min in a moist chamber. The sections were rinsed in 0.01 mol/l PBS and stained with uranyl acetate and lead citrate. Control staining was performed without primary antibody and then treated with a secondary antibody. We did not detect the gold particles on control staining. The number of c-KIT molecules or cluster formed c-KIT was counted in 100 μmm in the 50 different area. The result was indicated as average ± standard deviation (S.D.).

Statistical Analysis —— Data are reported as mean ± S.D. Differences in groups were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc multiple comparisons test. A p-value of less than 0.01 was considered statistically significant.

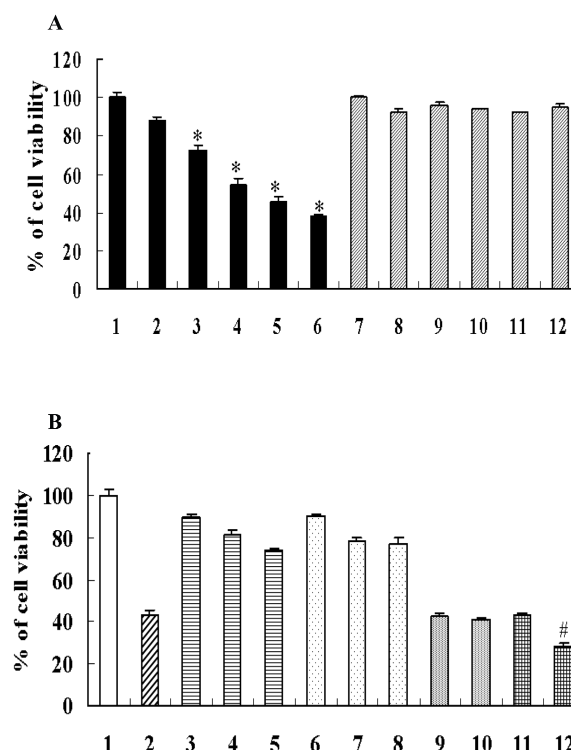

Fig. 1. Measurement of Cell Viability by MTT Assay

A: GIST-T1 and HS-sch-2 cells were treated with imatinib. Lanes 1–6, GIST-T1 treated with 0, 0.01, 0.02, 0.05, 0.1, 1 μg/ml of imatinib; Lanes 7–12, HS-sch-2 treated with 0, 0.01, 0.02, 0.05, 0.1, 1 μg/ml of imatinib. B: GIST-T1 was treated with 1 μg/ml of imatinib or 0.5, 1, 2 μg/ml of cisplatin or 10, 100, 300 nmol/l thapsigargin. Lane 1, non-treatment; 2, imatinib 1 μg/ml; 3, cisplatin 0.5 μg/ml; 4, cisplatin 1 μg/ml; 5, cisplatin 2 μg/ml; 6, thapsigargin 10 nmol/l; 7, thapsigargin 100 nmol/l; 8, thapsigargin 300 nmol/l; 9, imatinib 1 μg/ml + cisplatin 0.5 μg/ml; 10, imatinib 1 μg/ml + cisplatin 2 μg/ml; 11, imatinib 1 μg/ml + thapsigargin 10 nmol/l; 12, imatinib 1 μg/m + thapsigargin 100 nmol/l. Survival percentages are shown as the mean ± S.D. of triplicate experiments. *$p < 0.01$ *vs.* Lane 1; #$p < 0.01$ *vs.* Lane 2.

RESULTS

Cell Viability and Cell Death in GIST-T1 and HS-sch-2 Cells

GIST-T1 cells treated with imatinib alone showed a decrease in cell viability by MTT assay compared to the control HS-sch-2 cells. Viability rates in imatinib-treated GIST-T1 cells at concentrations of 0.01, 0.02, 0.05, 0.1 and 1 μg/ml were 88.23 ± 1.17, 72.27 ± 3.02, 54.43 ± 3.82, 45.44 ± 3.14, and 38.21 ± 0.95%, respectively. This decrease was dose–dependent (Fig. 1A). In contrast, imatinib did not affect the cell viability of HS-sch-2 cells (Fig. 1A). The cell viability of GIST-T1 was slightly decreased by thapsigargin treatment., but cell death was not induced by it. Thapsigargin might inhibit cell growth of GIST-T1. The combined treatment of 100 nmol/l thapsigargin and 1 μg/ml imatinib (28.50 ± 1.38%) further decreased GIST-T1 cell viability compared to treatment with 1 μg/ml imatinib alone (43.50 ± 1.87%) ($p < 0.01$) (Fig. 1B). Dead cells were demarcated by annexin V staining (Fig. 2). The cell death rate induced by the combination of 100 nmol/l thapsigargin and 1 μg/ml imatinib was significantly higher than that with 1 μg/ml imatinib alone ($p < 0.01$) (Fig. 2G and 2B). In contrast, the combination of cisplatin and imatinib had no significant effect on rate of cell death (Figs. 1B and 2E). We tested other anti-cancer drugs such as endoxan and mitomycin-C and found a similar effect to that seen for cisplatin (data not shown).

Quantitive Analysis of c-KIT on the Cell Surface

We analyzed the amount of c-KIT on the cell membrane using FACSCalibur. Treatment with

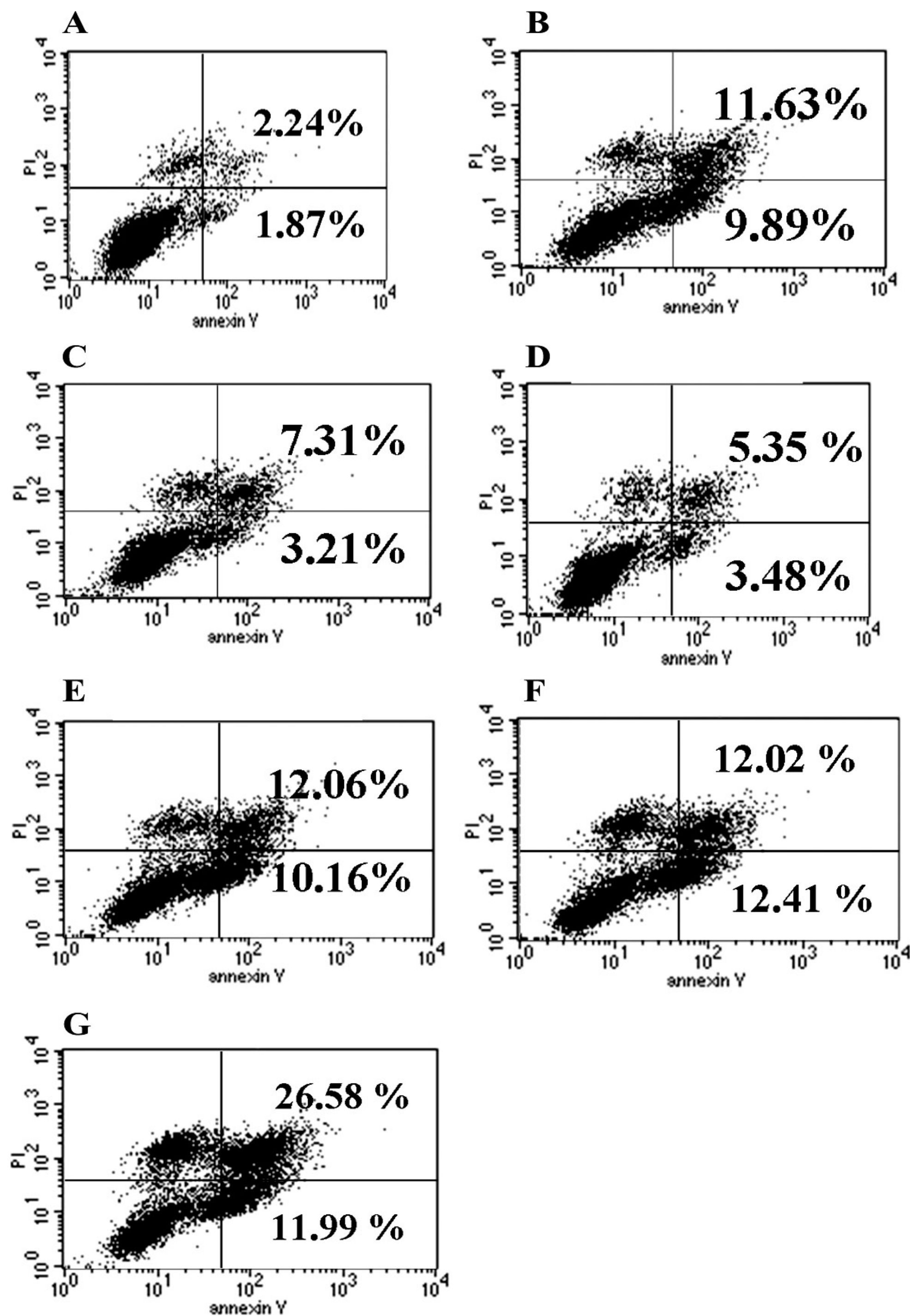

Fig. 2. Distribution of Cell Death Counted by Flow Cytometry

A: non treatment; B: imatinib 1 μg/ml; C: cisplatin 2 μg/ml; D: thapsigargin 100 nmol/l; E: imatinib 1 μg/ml + cisplatin 2 μg/ml; F: imatinib 1 μg/ml + thapsigargin 10 nmol/l; G: imatinib 1 μg/ml + thapsigargin 100 nmol/l.

thapsigargin for 5 hr decreased the cell-surface expression of c-KIT (Fig. 3). Control cells labeled with normal mouse IgG and a FITC-labeled secondary antibody showed no difference between no treatment and thapsigargin treatment (data not shown).

Western Blot Analysis of Activated c-KIT

We tested whether the c-KIT activation was inhibited in cells treated with imatinib or thapsigargin. Western blotting showed that c-KIT in the GIST-T1 cells was auto-phosphorylated without ligand stimulation (Fig. 4, Lane 1). Following treatment with

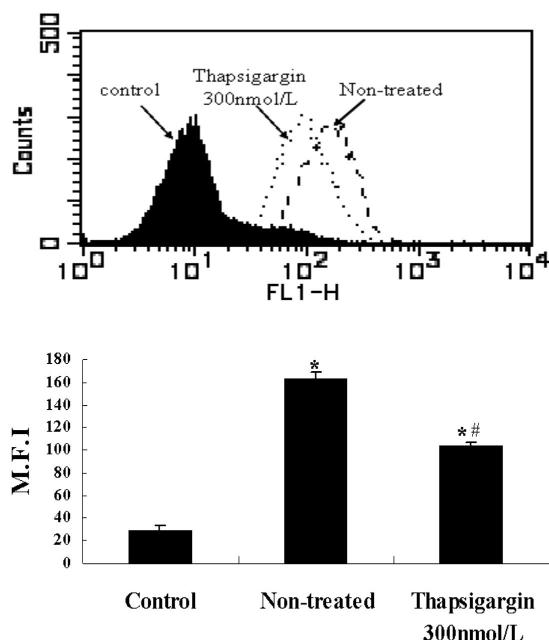

Fig. 3. Quantitive Analysis of c-KIT Expression on the Cell Surface

Amount of c-KIT molecules on the cell surface was determined by flow cytometry. Control: non-treated cells reacted with normal mouse IgG; Non-treated: non-treated cells reacted with anti-c-KIT antibody; Thapsigargin 300 nmol/l: treated with thapsigargin (300 nmol/l) cells reacted with anti-c-KIT antibody. Mean fluorescent intensities (M.F.I.) represented amount of c-KIT on the cell surface of the GIST-T1 cells. M.F.I. is shown as the mean ± S.D. of triplicate experiments. $*p < 0.01$ *vs*. Control; $\#p < 0.01$ *vs*. Non-treated.

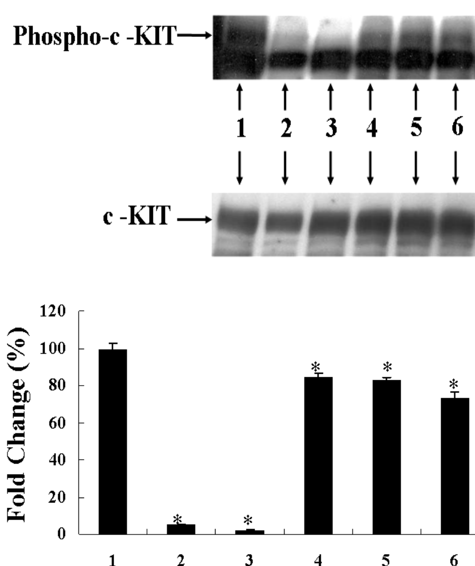

Fig. 4. Assessment of Activated c-KIT by Western Blotting

GIST-T1 cells were treated with imatinib or thapsigargin at the indicated concentrations for 5 hr. Total cell lysate (50 μg) was loaded in each lane. Western blotting was performed using an anti-phosphotyrosine antibody (upper panel) and reprobed with anti-c-KIT (lower panel). Lane 1: non-treatment; 2: imatinib 0.1 μg/ml; 3: imatinib 1 μg/ml; 4: thapsigargin 100 nmol/l; 5: thapsigargin 200 nmol/l; 6: thapsigargin 300 nmol/l. Fold change was shown by dividing the phospho-c-KIT value by c-KIT value using densitometric analysis. The bars represent the mean of three independent experiments. $*p < 0.01$ *vs*. Lane 1.

imatinib or thapsigargin, c-KIT activation was obviously and slightly inhibited respectively compared with non-treated cells (Fig. 4, Lanes 2–6). The total amount of c-KIT was equal in each lane.

Observation of c-KIT on the Cell Surface in the GIST-T1 Cells

The functional part of the c-KIT protein is localized on the cell surface and allows transduction of downstream signaling to the nucleus *via* phosphorylation of tyrosine residues in the signaling proteins. In our study, we observed c-KIT molecules on the cell surface using immunoelectromicroscopic analysis. c-KIT molecules were assembled on the cell surface of non-treated GIST-T1 cells (Fig. 5A). The number of the cluster formed c-KIT was 2.13 ± 0.25 and total c-KIT molecules was 8.93 ± 1.25 (100 μm^2 of section). Imatinib inhibited the clustering of c-KIT on the cell surface membrane (Fig. 5B). The number of the cluster formed c-KIT was 0.78 ± 0.38 and c-KIT molecules was 6.28 ± 1.76 by treatment with imatinib. In the case of thapsigargin treatment, the number of the cluster formed of c-KIT was 0.72 ± 0.33 and the number of c-KIT molecules was 5.28 ± 1.15 (Fig. 5C). These results indicated that activated c-KIT molecules were assembled on the cell surface membrane and amount of c-KIT molecules were decreased by thapsigargin.

DISCUSSION

Inhibitors of c-KIT kinase have recently been developed for clinical use. The compound, imatinib was developed as an ATP competitive inhibitor of ABL tyrosine kinase. At concentrations required for inhibition of Bcr-Abl, imatinib also inhibits other tyrosine kinase receptors such as PDGFR and c-KIT.[10] Advanced GISTs are invariably fatal and unresponsive to conventional chemotherapy. Imatinib has been used successfully in patients with c-KIT-positive GIST,[7,8] although a complete response (CR) has not been achieved. Therefore it is important to investigate whether other drugs can enhance the cytotoxity to GIST cells that is induced by imatinib.

Cisplatin is well known as an anti-cancer regent

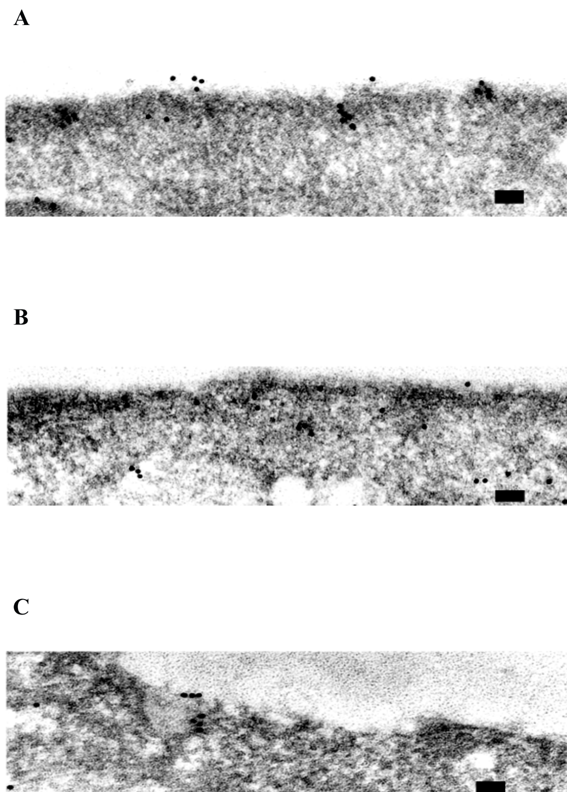

Fig. 5. Immunoelectromicroscopic Analysis

Cells were treated with/without imatinib (1 μg/ml) or thapsigargin (300 nmol/l). A: non-treated; B: imatinib treated; C: thapsigargin treated. Samples were reacted with anti-c-KIT and 15-nm-gold labeled secondary antibody. Bar: 100-nm.

for several cancers and it is often used with another anti-cancer drugs.[18] In this study, we used cisplatin with/without imatinib to assess the cell viability and cell death of the GIST-T1 cells. Cisplatin did not significantly reduce the cell viability and did not induce cell death of the GIST-T1 cells compared with imatinib. In the case of combination with imatinib, effect to both cell viability and cell death was not enhanced compared with imatinib alone. These results suggested that cisplatin was not useful compound for GIST therapy.

Next we used thapsigargin with/without imatinib to assess the cell viability and cell death of the GIST-T1 cells. Thapsigargin is important in regulating the calcium homeostasis of the ER as it inhibits calcium influx into this compartment.[11] Newly synthesized proteins undergo folding in the ER, and high luminal calcium is necessary for the proper folding of several proteins. A protein will only be exported from the ER if it is correctly folded.[12–14] Calcium might also be necessary for the functions of many calcium-binding proteins, such as ER-resident chaperones, that are involved in protein folding.[13,14] In this study, we assessed how thapsigargin acted on the GIST-T1 cells or how worked on the c-KIT in the GIST-T1. Thapsigargin did not induce the cell death, but in the case of combination with imatinib, thapsigargin enhanced cell death of GIST-T1. If c-KIT was not correctly folded in the ER, c-KIT could not exported from ER to cell surface membrane. The functional part of the c-KIT protein is localized on the cell surface and allows transduction of downstream signaling to the nucleus *via* phosphorylation of tyrosine residues in the signaling proteins. FACSCalibur analysis revealed that thapsigargin reduced the number of c-KIT molecules on the cell surface and western blot analysis indicated that thapsigargin attenuated activation of c-KIT. These results implied that thapsigargin may act on ER-resident chaperones and disrupts the proper maturation processing of c-KIT through the ER. Inhibition by combination of thapsigargin and imatinib to activation of c-KIT strongly induced cell death in the GIST-T1 cells. Further studies are required to identify the ER-resident chaperones that are involved in the maturation of c-KIT.

Thapsigargin is known to activate the cell death pathway in response to ER stress.[11] However, the percentage of the dead cells in the GIST-T1 was not significantly increased in the case of thapsigargin alone (~300 nmol/l) for 24 hr compared with imatinib. ER stress induces expression of glucose-regulated protein 78 (GRP78) and activates caspase-12.[19,20] In our study, expression of GRP78 was not induced by incubation with thapsigargin alone (~1 μmol/l), but it was highly expressed following co-treatment with imatinib and thapsigargin for 24 hr (data not shown). Caspase-3, a component of the apoptotic pathway, is activated following ER stress. Caspase-3 was highly activated in cells treated with both thapsigargin and imatinib (data not shown), suggesting that treatment with imatinib and thapsigargin might induce cell death *via* the ER stress response.

The faithful translocation and subsequent cell signaling roles of c-KIT also depend on its proper maturation. We have now confirmed that inhibition of factors that are involved in the maturation process of oncogenic proteins has potential for use in cancer therapy. Further studies are required to further characterize potential regulators of oncogenic protein maturation and to clarify the effect of such reagents on cell survival in health and disease states.

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