Cytotoxicity Induced by a Redox-silent Analog of Tocotrienol in Human Mesothelioma H2452 Cell Line *via* Suppression of Cap-dependent Protein Translation

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Abstract. *De novo synthesis of proteins is regulated by cap-dependent protein translation. Aberrant activation of the translation is a hallmark of many cancer types including malignant mesothelioma (MM). We previously reported that a redox-silent analog of α–tocotrienol, 6-O-carboxypropyl-α-tocotrienol (T3E) induces potent cytotoxicity against human MM cells. However, the detailed mechanism of cytotoxicity of T3E remains unclear. In this study, we investigated if T3E induced potent cytotoxicity aganist MM cells. T3E reduced the formation of the cap-dependent translation complex and induced inactivation of oncogene from rat sarcoma virus (RAS). These events were associated with T3E cytotoxicity in MM cells. Furthermore, atorvastatin, an inhibitor of RAS function, had similar effects on MM cells. Moreover, 4EGI-1, a specific inhibitor of the cap-dependent translation complex, induced severe cytotoxicity in MM cells. Overall, T3E had a cytotoxic effect on MM cells via disruption of the activated cap-dependent translation complex through inactivation of RAS.*

Malignant mesothelioma (MM) arising from the serosal membranes of body cavities, such as the pleural cavity, is a particularly aggressive and treatment-resistant tumor caused primarily by asbestos exposure, and its prevalence is increasing a worldwide each year (1). Although many multimodal therapies for the treatment of MM have been attempted, the median patient survival is 8-18 months (2). In a phase III trial, a combination of pemetrexed and cisplatin was more effective than cisplatin alone, with a response ratio of 41.3% *versus* 16.3% (3), and new therapeutic agents for MM, such as epidermal growth factor receptor tyrosine kinase inhibitors, have been developed (4). However, most patients with MM experience relapse shortly after starting treatment. Thus, new effective therapies for MM are urgently required.

The pathogenesis of MM involves multiple-signaling pathways such as oncogene from rat sarcoma virus (RAS) signaling pathway. Among the signaling pathways, deranged cap-dependent protein translation is critical for malignancy of MM (5). The rate-limiting step in the initiation of mRNA translation is the binding of eukaryotic initiation factor 4E (eIF4E) to the 5'-cap structure (m7GpppN) of mRNA. In normal cells, eIF4E-binding protein 1 (4E-BP1) negatively regulates eIF4E functions, that is, 4E-BP1 blocks the interaction between eIF4E and the scaffolding protein eIF4G, inhibiting the formation of the active eIF4E complex and suppressing cap-dependent protein translation. The phosphorylation of 4E-BP1 protein by mitogenic signaling leads to a decrease of its affinity for eIF4E and finally to the activation of translation (6). In cancer, the increasing active eIF4E increases the translation of mRNAs with long structured 5'-untranslated regions, which typically encode growth regulatory factors and anti-apoptotic proteins, contributing to tumorigenesis (7). Thus, determining the mitogenic signaling pathway related to the activation of cap-dependent protein translation in MM may result in new effective targets for MM therapy.

In our previous studies, an ether derivative of α-tocotrienol (T3), 6-O-carboxypropyl-α-tocotorienol (T3E), had shown cytotoxicity against the human MM cell line H28 *in vitro*. The cytotoxicity of T3E was much stronger than that

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of T3 with a lesser toxic effect on non-tumorigenic cells (8, 9). The possible mechanism for the selective and strong cytotoxicity of T3E on MM cells remains unclear, however, we previously reported that T3E induced cytotoxicity in human lung cancer cells, partly due to the inactivation of the RAS signaling pathway (8). Another study indicated that RAS and known RAS effectors such as extracellular-regulated kinase (ERK) and c-Jun *N*-terminal kinase (JNK) were activated in most MM cell lines tested and that the increase of cap-dependent protein translation by the activation of RAS and its effectors contributed to MM cell proliferation (10). Thus, the inhibition of cap-mediated protein translation *via* the inactivation of RAS signaling may be a possible mechanism of T3E-induced cytotoxicity in MM cells. This study was undertaken to clarify this hypothesis.

Materials and Methods

Chemicals. All cultures and chemicals were purchased from Invitrogen (Tokyo, Japan) and Sigma (St Louis, MO, USA), unless otherwise indicated. T3 was purchased from Tama Biochemicals (Tokyo, Japan). Atorvastatin (ATV) was purchased from LKT Laboratories, Inc. (St. Paul, MI, USA). 4EGI-1 was obtained from Tocris Bioscience (Minneapolis, MN, USA). All antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA), unless otherwise indicated.

T3E synthesis. T3E was synthesized from T3 according to a previously reported procedure (8). The purity of T3E was confirmed by ^{1}H NMR, ^{13}C NMR and IR. The NMR and IR spectra were consistent with the structure of T3E. 1H NMR (CDCl3) spectrum: 1.27 (3H, s), 1.59 (9H, s), 1.67 (3H, s), 2.00 (3H, s), 2.09 (3H, s), 2.12 (3H, s), 1.70-2.15 (16H, m), 2.57 (2H, t, *J*=7.8Hz), 2.65 (2H, t, *J*=6.5Hz), 3.68 (2H, t, *J*=7.7Hz), 4.95-5.25 (3H, m), 8.5 (1H, broad). IR (KBr) spectrum: 3200-3400 cm^{-1} (carboxylic OH) and 1710 cm^{-1} (C=O).

Cell culture and treatment. H2452 cells from the American Type Culture Collection (Manassas, VA, USA) were routinely grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 6.5 mg/ml glucose, 1 mM sodium pyruvate, 10 mM HEPES, 50 IU/ml penicillin and 50 μg/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. For experiments, exponentially growing cells were used. Cells were plated on culture plates and cultured for 24 hours to permit cell adhesion. After attachment, the cells were cultured in RPMI-1640 supplemented with 2% FBS, 6.5 mg/ml glucose, 1 mM sodium pyruvate, and 10 mM HEPES containing each agent (T3E, statin or 4EGI-1), and subsequently each parameter was determined.

Cell growth analysis. Cells (2×10^4) were seeded on a 96-well culture plate with culture medium, and after overnight culture, the cells were treated with each agent alone. Following this, cell growth was determined by a cell proliferation assay kit using WST-1 reagent (Roche Japan, Tokyo, Japan).

Estimation of ornithine decarboxylase (ODC) and B-cell CLL/lymphoma 2 (BCL2) level. Treated cells were lysed in cell lysis/extraction reagent including protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma). After centrifugation at 15,000 × *g* for 30 min at 4˚C, the supernatants were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane and subjected to immunoblotting with each antibody. Detection was performed using the ECL system (Amersham, Piscataway, NJ, USA) and a cooled CCD camera-linked Cool Saver System (Atto, Tokyo, Japan). Molecular sizing was estimated using Rainbow Molecular Weight Marker (Amersham). Protein concentration was determined by DC Protein Assay Kit (Bio-Rad, Tokyo, Japan).

RAS activation assay. RAS activation was evaluated using a RAS activation assay kit per manufacture's recommendation (Merck, Darmstadt, Germany). In brief, the cells were lysed in a lysis buffer (125 mM HEPES, pH 7.5, 750 mM NaCl, 5% NP-40, 50 mM MgCl2, 5 mM EDTA, and 10% glycerol). RAF1 RAS-binding domain agarose was added to the lysates, followed by incubation overnight at 4˚C. The beads were washed twice, and bound RAS-GTP (active form) was separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane and subjected to immunoblotting with a pan anti-RAS antibody (clone RAS 10).

Cap-binding assay. The cap-binding assay was carried out as previously described (11). In brief, the cells were lysed in a freeze-thaw lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10 mM tetrasodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 100 μM Na_3VO_4, and 20 mM β-glycerophosphate). The lysate (300 μg) was added to 50 μl of a 50% slurry of 7-methyl GTP-sepharose beads (Amersham BioScience UK Ltd, Buckinghamshire, UK) to isolate eIF4E and its binding molecules 4E-BP1 and eIF4G. The lysates were rotated at 4˚C for 4 h followed by three washes in the freeze-thaw lysis buffer. Proteins bound to the beads were then eluted by adding 50 μl of 3× loading buffer and boiling at 95˚C for 5 min. The eluate was then loaded onto 8% to 15% gradient SDS-PAGE gel electrophoresis, transferred to nitrocellulose membrane, and then immunoblotted with anti-eIF4E, anti-4E-BP1, and anti-eIF4G antibodies.

Statistical analysis. Data were analyzed by one-way analysis of variance followed by Dunnett's multiple-range test. A *p*-value of 0.05 or less was considered significant.

Results

Effect of T3E on cell growth, RAS activation, and cap-dependent protein translation in H2452 cells. Firstly, in order to evaluate the effect of T3E on cell growth, dose-dependent change in cell viability was determined. Viability of H2452 cells significantly decreased in a dose-dependent manner (Figure 1A). Next, we examined the effect of T3E on the activation status of RAS in H2452 cells, because a main target of T3E in cancer cell growth control is the suppression of RAS activation (8). As shown in Figure 1B, T3E drastically reduced the level of RAS–GTP; conversely, the treatment did not affect the total RAS level. Finally, we investigated if T3E treatment caused suppression of cap-dependent protein translation, an important downstream signal event of RAS essential for cell growth and

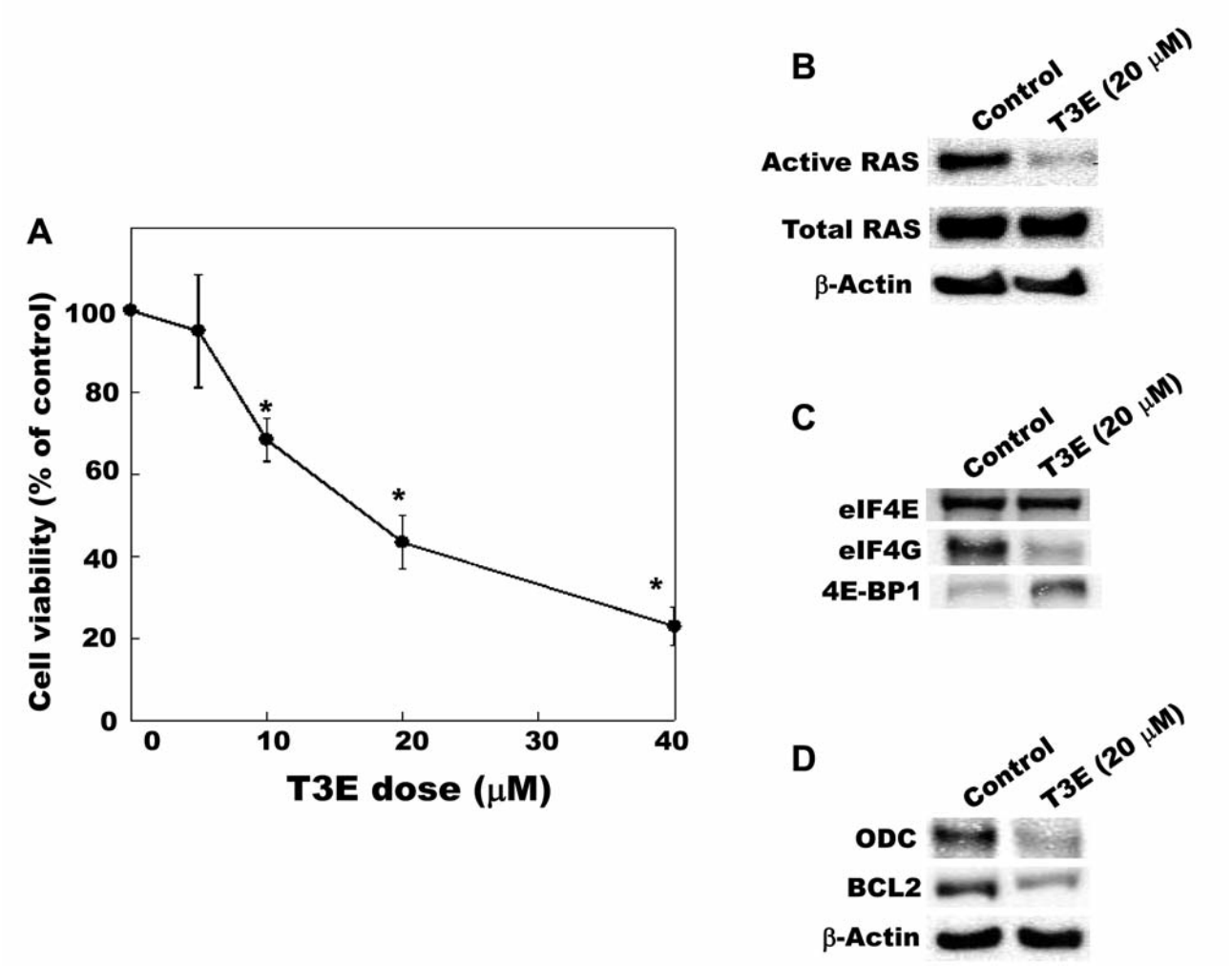

Figure 1. *Effects of 6-O-carboxypropyl-α-tocotrienol (T3E) on cell viability, activation of rat sarcoma virus (RAS) oncogene, and cap-dependent translation in H2452 cells. The cells were treated with indicated doses (A) and 20 μM T3E (B-D), respectively. The treatment periods were 48 (A) and 12 (B-D) h, respectively. A: After treatment, cell viability was determined by WST-1 assay. Each value is the mean from five determinants, and vertical lines indicate the SD. *Significant difference from 0 μM treatment group. B: RAS-GTP (active form) pull-down assay. The levels of total RAS and β−actin as the internal standard in lysates before the pull-down assay were evaluated by immunoblot analysis. This result is representative of two independent experiments. C: Cap-binding assay after treatment with T3E. This result is representative of two independent experiments. D: After treatment, the level of each protein was determined by immunoblot analysis. This result is representative of two independent experiments.*

proliferation (12). A cap-binding assay showed that T3E reduced the binding of eIF4E to eIF4G and, in turn, increased the binding of eIF4E to 4E-BP1 (Figure1C). Linked with this result, the levels of ODC and BCL2 proteins were suppressed by T3E treatment (Figure 1D). These results suggest that T3E-induced cytotoxicity in H2452 cells involves the suppression of cap-dependent translation *via* the inactivation of RAS.

Effect of atorvastatin on RAS activation and cap-dependent translation. It is well-known that statins, inhibitors of 3-hydroxymethyl-3-methylglutaryl coenzyme A reductase (HMGR), exhibit anticancer activity due to their inactivation of RAS (13). Thus, we determined if a statin (atorvastatin) would suppress cap-dependent translation *via* inactivation of RAS. As shown in Figure 2A, statin treatment had a cytotoxic effect on H2452 cells in a dose-dependent manner. On an adequate treatment dose of statin, as shown in Figure 2A, the level of RAS activation was clearly reduced (Figure 2B). Under these same treatment conditions, atorvastatin induced a decrease in binding of eIF4E to eIF4G and an increase in binding of the former protein to 4E-BP1 (Figure 2C), indicating that atorvastatin suppresses cap-dependent translation.

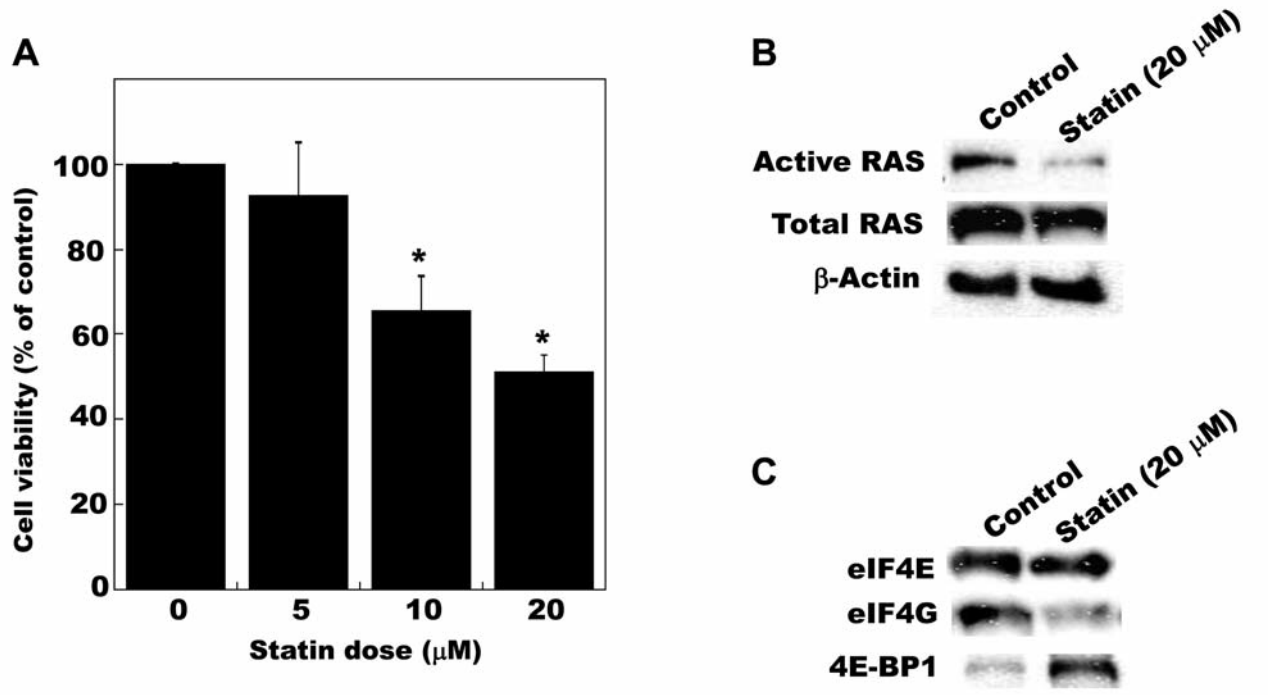

Figure 2. *Effects of atorvastatin on cell viability, activation of rat sarcoma virus (RAS) oncogene, and cap-binding translation in H2452 cells. The cells were treated with the indicated doses (A) and 20 μM atorvastatin (B and -C), respectively. The treatment periods were 48 (A) and 12 (B and C) hours, respectively. A: After treatment, cell viability was determined by WST-1 assay. Each value is the mean from five determinants, and vertical lines indicate the SD. *Significant difference from 0 μM treatment group. B: RAS-GTP (active form) pull-down assay. This result is representative of two independent experiments. C: Cap-binding assay after the treatment with atorvastatin. This result is representative of two independent experiments.*

Effect of 4EGI-1 on growth and cap-dependent translation in H2452 cells. Finally, in order to confirm if inhibition of cap-dependent translation contributes to growth of H2452 cells, we estimated the influence of 4EGI-1, a specific inhibitor of the interaction between eIF4E and eIF4G (14), on cell growth. As shown in Figure 3A, 4EGI-1 treatment led to a dose-dependent change in cytotoxicity towards H2452 cells. Cytotoxic treatment with 4EGI-1 disrupted the interaction between eIF4E and eIF4G and enhanced the binding of eIF4E to 4E-BP1 (Figure 3B). Linked with this result, the levels of ODC and BCL2 proteins, two proteins typically related to cap-dependent translation, were clearly reduced by 4EGI-1 treatment (Figure 3C). These results indicate that the inhibition of cap-dependent translation induces a cytotoxic effect on H2452 cells.

Discussion

MM cells are resistant to several chemotherapies and other conventional treatments (4), thus strongly necessitating the development of new potential therapy to establish effective MM treatment protocols. In our previous studies, T3E, a redox-silent analog of T3, exhibited a potential cytotoxic effect against several kinds of cancer cells (9, 15). In particular, T3E has a potential cytotoxic effect on MM cells at pharmacological doses and it is less toxic to non-tumorigenic cells than other treatments (9). Thus, we tried to clarify a possible mechanism of the cytotoxic effect of T3E. As a result, we have previously reported that the inactivation of a non-receptor tyrosine kinase (SRC) is an event leading to effective cytotoxicity in MM cells treated with T3E (16). Since SRC plays a critical role in survival, proliferation, invasion, and metastasis of solid tumors (17), it seems that T3E-dependent inactivation of SRC is a main event leading to the severe cytotoxicity in MM cells. However, SRC inhibition alone had only a modest anti-cancer effect on MM cells (9), indicating that inhibition of other signaling molecules in addition to SRC is associated with the potential anticancer effect of T3E on MM cells. Thus, the present study was undertaken to further clarify the possible mechanisms of the cytotoxic effect of T3E on MM cells.

As mentioned in the introduction section, MM cells have severe malignant phenotypes based on the aberrant regulation of multiple signaling pathways such as the RA signaling pathway (18). RAS mutations are found in as many as 30% of human cancers (19), leading to characteristic activation of

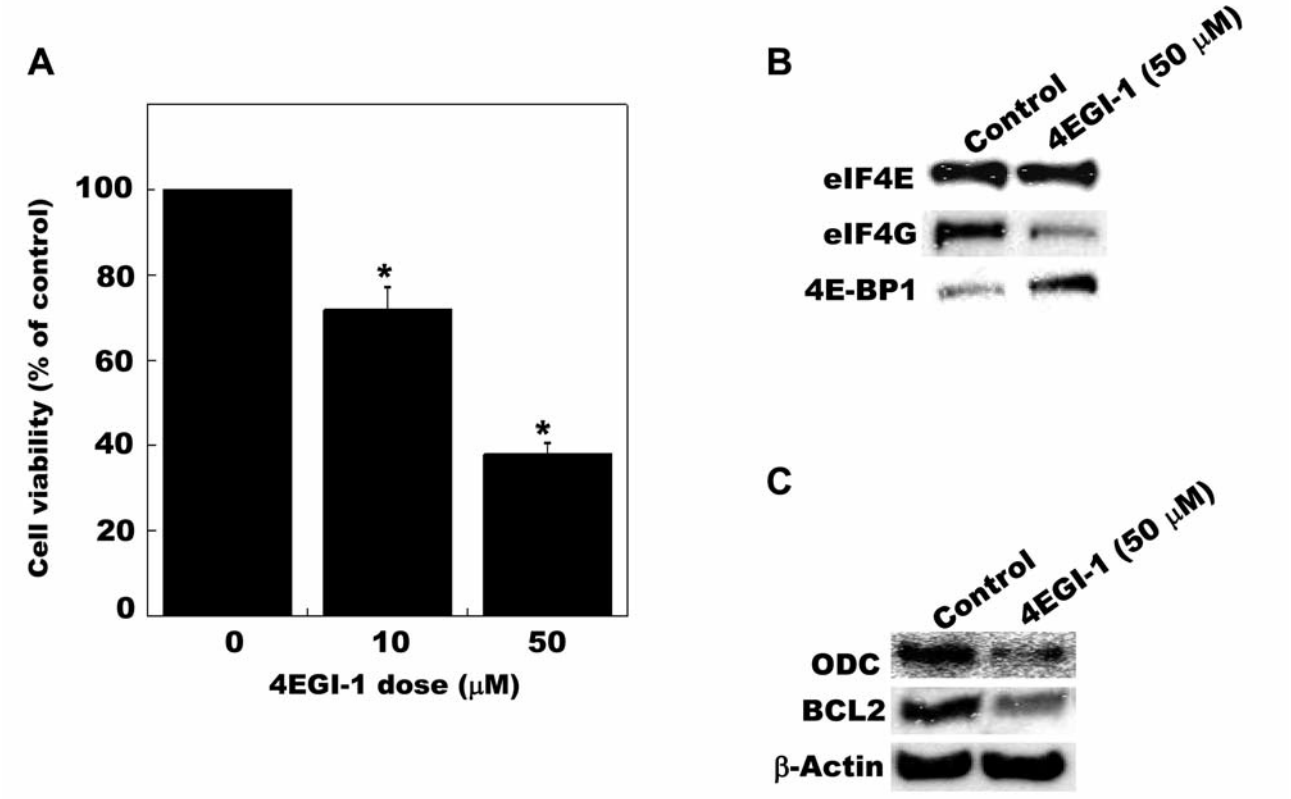

Figure 3. *Effects of 4EGI-1 on cell viability, activation of rat sarcoma virus (RAS) oncogene, and cap-binding translation in H2452 cells. The cells were treated with indicated doses (A) and 50 μM 4EGI-1 (B and C), respectively. The treatment periods were 48 (A) and 12 (B-C) h, respectively. A: After the treatment, cell viability was determined by the WST-1 assay. Each value is the mean from five determinants, and vertical lines indicate the SD. *Significant difference from 0 μM treatment group. B: Cap-binding assay after the treatment with 4EGI-1. This result is representative of two independent experiments. C: After treatment, the level of each protein was determined by immunoblot analysis. This result is representative of two independent experiments.*

the downstream signalling pathway. However, *RAS* has not been found to be mutated in MM cells and MM tissues (20, 21). In the absence of *RAS* mutation, the activation of receptor tyrosine kinases (RTKs) occurs as upstream signal and induces RAS activation (22). Actually, several types of RTKs can activate RAS in MM cells (23). In such a case, one of the most effective RAS-inhibitory approaches may be the inactivation of RAS itself. The critical process for the activation of RAS is prenylation of the C-terminal of the RAS protein (24), hence the inhibition of this process is considered a potentially useful procedure to induce RAS inactivation. HMGR plays a key role in controlling RAS activation by generating prenyl intermediates, particularly farnesyl and geranyl-geranyl moieties (25). Thus, a potential HMGR inhibitor may act as a potential RAS inactivator. In a previous report, T3 clearly down-regulated the level of HMGR (26), and linked with that report, this study clearly showed T3-induced inactivation of RAS in MM cells. In addition, we confirmed this result by the fact that a statin, an inhibitor of HMGR, induced the inactivation of RAS.

The RAF/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT signaling pathways are important downstream targets regulated by activated RAS, and MAPK and AKT molecules are essential for stimulating cell proliferation and maintaining cell survival. A recent study indicated that the activation of eIF4E is absolutely required for cell proliferation and survival, and is induced by the above two proto-oncogenic signaling pathways (27). That is, the phosphorylation of eIF4E by MAPK and AKT increases the binding of this protein to the cap-binding translation complex, resulting in the activation of cap-binding translation and subsequent increase of molecules related to cell proliferation and survival of cells (28). Finally, this translation activation leads to aberrant cell proliferation and survival in cancer cells. Thus, eIF4E may be a potential target for development of anticancer therapies. In fact, T3E disrupted the interaction of eIF4E with the cap-dependent translation complex *via* the inactivation of RAS, finally leading to the cytotoxicity of T3E towards MM cells. This result is supported by the finding that the disruption of the cap-dependent translation complex by

atorvastatin and 4EGI-1 caused effective cytotoxicity in MM cells. With respect to the downstream signal of RAS that might contribute to T3E-dependent disruption of the cap-binding complex, we speculated that the RAS/MAPK signal is more closely related to the disruption than the PI3K/AKT signal, because our previous report clearly indicated that inactivation of RAS by statin induced the suppression of activation of MAPK, but not that of AKT (29). Overall, it seems that T3E-dependent cytotoxicity essentially depends on the inactivation of cap-dependent translation *via* the inhibition of RAS/MAPK signal.

In mammalian cells, the activity of eIF4E is the rate-limiting component in the cap-dependent translation complex (30), and its overexpression is closely associated with several types of cancer and clearly related to poor clinical outcome in patients (31, 32). As mentioned, the translation of select mRNAs with long, complex 5'UTRs (*i.e.* those involved in malignancy) is stimulated by the activation of eIF4E (7). Thus, it is assumed that inhibition of eIF4E activity leads to severe cytotoxicity *via* the reduction of the translation of the specific mRNAs. In MM cells, a growth-regulatory protein, ODC, and a main anti-apoptotic protein, BCL2, were both shown to be regulated at the level of cap-dependent translation (33). Our study demonstrated that T3E induced the reduction of ODC and BCL2 due to reduction of cap-dependent translation, leading to cytotoxicity against MM cells. In addition, in a recent study, the suppression of eIF4E by several approaches led to enhanced chemosensitivity (34). From these reports, it is speculated that the attenuation of eIF4E-regulated cap-dependent translation activity by T3E sensitizes MM cells to low doses of several cancer-therapeutic agents. If this is confirmed, T3E may be an ideal MM therapeutic agent. Furthermore, in addition to this advantage, T3E has multiple target points in MM cells, hence it may be difficult for MM cells to acquire resistance to T3E. Taken together, T3E appears to be a promising treatment agent for MM.

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