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Proteomic Analysis and the Antimetastatic Effect of *N*-(4-methyl)phenyl-*O*-(4-methoxy) phenyl-thionocarbamate-Induced Apoptosis in Human Melanoma SK-MEL-28 cells

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We employed human SK-MEL-28 cells as a model system to identify cellular proteins that accompany *N*-(4-methyl)phenyl-*O*-(4-methoxy)phenyl-thionocarbamate (MMTC)-induced apoptosis based on a proteomic approach. Cell viability tests revealed that SK-MEL-28 skin cancer cells underwent more cell death than normal HaCaT cells in a dose-dependent manner after treatment with MMTC. Two-dimensional electrophoresis in conjunction with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis or computer matching with a protein database further revealed that the MMTC-induced apoptosis is accompanied by increased levels of caspase-1, checkpoint suppressor-1, caspase-4, NF-κB inhibitor, AP-2, c-Jun-N-terminal kinase, melanoma inhibitor, granzyme K, G1/S specific cyclin D3, cystein rich protein, Ras-related protein Rab-37 or Ras-related protein Rab-13, and reduced levels of EMS (oncogene), ATP synthase, tyrosine-phosphatase, Cdc25c, 14-3-3 protein or specific structure of nuclear receptor. The migration suppressing effect of MMTC on SK-MEL-28 cell was tested. MMTC suppressed the metastasis of SK-MEL-8 cells. It was also identified that MMTC had little angiogenic effect because it did not suppress the proliferation of HUVEC cell line. These results suggest that MMTC is a novel chemotherapeutic and metastatic agents against the SK-MEL-28 human melanoma cell line.

Key words: Apoptosis, Metastasis, *N*-(4-methyl)phenyl-*O*-(4-methoxy)phenyl-thionocarbamate, Proteome map, SK-MEL-26 cells

INTRODUCTION

The incidence of melanoma is increasing world-wide at a rate of about 5% per year (Morton *et al.*, 1996). Chemotherapy of late diagnosed and metastatic melanoma cases treated with dacarbazine and alkylating agents has important side effects. Moreover, melanoma has one of the worst rates of response to chemotherapy of all neoplasias (Schadendorf *et al.*,1994). These problems have led to searches for new types of treatment (Rodriguez-Vicente *et al.*, 1988) and for novel compounds with fewer side effects.

The major cause of death from melanoma is metastases resistant to conventional therapies (Fidler *et al.*, 1999), by the time a diagnosis of malignant melanoma is made the metastasis may have progressed to regional and distant lymph nodes, liver, lung, and the central nervous system. Also, it has been reported that brain metastases are clinically diagnosed in 40% to 60% of patients with metastatic melanoma (Takakura *et al.*, 1982). Melanoma develops from a series of architectural and phenotypically distinct stages and becomes progressively aggressive. Despite the progress that has been made in understanding the biological, pathological and immunological aspects of human melanoma, the precise biological and

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molecular determinants responsible for melanoma progression are not yet known (Satyamoorthy *et al.*, 1995).

Thionocarbamate derivatives were examined for fungicidal activity *in vitro* against gray mold (*Botrytis cinerea*) and capsicum phytophthora (*Phytophthora capsici*) (Sung *et al.,* 1999). One of the cabamates, pyrrolidine dithiocarbamate, was reported to have protective effect against oxidative stress in endothelial cells (Moellering *et al.*, 1999). In addition, thiuramdisulfide, a metabolite of dithiocarbamate, was found to induce apoptosis in the cell line, and prolinedithiocarbamate and diethyldithiocarbamate were revealed to be cancer chemopreventive agents (Liu *et al.*, 1998). Currently, the effect of disulfiram, a member of the dithiocarbamate family, on apoptosis of melanoma cells *in vitro* was explored (Cen *et al.*, 2002). 3D-QSAR/COMFA and HQSAR models were developed to guide the rational design of novel *N*-phenyl-*O*-(4-methoxy)phenylthionocarbamate analogs exhibiting improved biological property (Sung *et al.*, 2003).

Cancers such as melanoma arise due to accumulation of mutations in genes critical for cell proliferation, differentiation, and cell death. In addition, cancer cells acquire the ability to initiate and sustain angiogenesis, invade across tissue planes, and metastasize (Hanahan *et al.*, 2000). Also, because all melanomas may not have equal response to treatment modalities due to different mechanisms of tuorigenesis, the ability to perform complex genetic mutational analyses will allow for individually tailored patient progness and therapeutics (Rodolfo *et al.*, 2004). So, we identified other novel therapeutic targets in melanoma by proteomics screens using 2D-gel and MALDI-TOF MS analyses.

In this study, we studied the cytotoxic effects of a newly synthesized carbamate derivative, *N*-methyl-*0*-methoxyphenylthionocarbamate (MMTC) on SK-MEL-28 human melanoma cell line, and identified changes in the levels of apoptosis-related proteins in cells using MALDI-TOF MS. We also studied the metastasis suppressing effect of MMTC using migration assays and its inhibition of the proliferation of HUVECs to elucidate its angiogenic effect.

MATERIALS AND METHODS

Materials

Human melanoma cell line (SK-MEL-28), human keratinocytes (HaCaTs) and human umbilical vein endothelial cells (HUVECs) were obtained from the Korea Research Institute of Biology and Biotechnology (KRIBB, Daejon, Korea). MMTC was synthesized by Dr. Sung, N. D (Sung *et al.*, 1999). RPMI-1640, DMEM, Trypsin-EDTA solution, FBS, penicillin, streptomycin and PBS were commercially obtained from GIBCO (Gaithersburg, U.S.A.). Propidium iodide (PI), proteinase K, MTT, Tris-base, ethidium bromide, glutaraldehyde, glycerol, agarose, acetonitrile, trifluoroacetic acid (TFA), acrylamide, toluidine blue, ammonium bicarbonate, bovine serum albumin (BSA), bromophenol blue, DTT, eosin, HEPES, low-melting point agarose, RNasa A, SDS, silver, sucrose, trypsin, Triton X-100 and xylene were obtained from Sigma. Boric acid was from Amresco (U.S.A.). Isopropanol was from Oriental Chemical Ind. (Incheon, Korea). EDTA, methanol, HCl, sodium chloride, sodium phosphate, sodium diphosphate, paraformaldehyde, phosphate propylene oxide, potassium ferricyanide, sodium thiosulfate, sodium carcodylate and ethanol were from Ducksan Parmaceutical Co. (Korea). Uranyl acetate was purchased from Merck (Rahway, NJ, U.S.A.), and XTT Cell Proliferation kits were obtained from Roche Diagnostics (U.S.A.).

Cell culture

SK-MEL-28 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal-bovine serum (FBS), 100 units of penicillin/mL, and 100 μg of streptomycin/mL, and maintained in a 5% CO_2 humidified atmosphere at 37°C. On the other hand, HaCaT cells was cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS, 100 units of penicillin/mL, 100 μg of streptomycin/mL, and maintained in a 5% CO_2 humidified atmosphere at 37°C. HUVECs were cultured in media-199 supplemented with 2 or 10% FBS, 50 μg/mL ECGS, 100 U of each of penicillin, streptomycin, and fungizone, 50 mg/mL gentamicin and 50 U/mL heparin and maintained in 75% N_2, 5% CO_2 and 20% O_2 at 37°C.

Cell viability assay

Cell viability was evaluated using microculture tetrazolium reduction assays using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Exponentially growing cells were inoculated at 1×10^4 cells/well into 96-well plates supplemented with 100 μL of RPMI-1640 for SK-MEL-28 cells and DMEM for HaCaT cells. After 24 h, cells were treated with various concentrations of MMTC. The treated cells were then incubated for 48 h, 50 μL of MTT (5 mg/mL) was added and plates were incubated at 37°C for 6 h. To dissolve formazan, media was replaced into 100mL of acidified-isopropanol. After adding 20 μL of 3% SDS, plates were measured at 540 nm using a microplate reader (THERMO max, Molecular Devices, U.S.A.).

TUNEL (terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling) assay

After SK-MEL-28 cells had been exposed to 10, 20, 30, or 40 μM of MMTC, the cells were incubated for different times. The cells were then harvested, and approximately 1×10^6 cells per sample were washed twice in PBS. Cell

pellets were then pre-fixed in 1 mL of 1% paraformaldehyde on ice for 15 min. After washing twice in PBS, pellets were fixed in 1 mL of 70% ethanol at -20°C for 24 h. The cells were then washed in PBS and resuspended in 50 μL of mixture of 0.2 M sodium carcodylate, 2.5 mM Tris-HCl (pH 6.6), 2.5 mM $CoCl_2$, 0.25 mg/μL BSA, 5 U terminal dideoxy nucleotidyl transferase and 0.5 mM dUTP-biotin (biotin-16-dUTP) and incubated at 37°C for 30 min. After washing twice in PBS, cell pellets were incubated in 100 μL of PBS containing 1 μg streptavidin-FITC for 30 min at room temperature in the dark, washed with PBS, and immediately analyzed using a FACS-can flow cytometer (Becton Dickenson, U.S.A.).

Electron microscopy

SK-MEL-28 cells were treated with 40 μM of MMTC and incubated for 48 h. The cells were then harvested, washed twice with PBS, and cell pellets obtained by centrifuging at 1500 rpm for 15 min were pre-fixed in 2.5% glutaraldehyde, and washed with 0.1M phosphate buffer (pH 7.4). Samples were post-fixed in 1% OsO_4, washed with 0.1M phosphate buffer (pH 7.4), dehydrated with absolute alcohol, and which was then with replaced propylene oxide. After permeabilizing samples with Epon mixture (polybed 812 kit; 4:6 ratio of Epon 812+DDSA and Epon 812+MNA) blocks were produced by sequential heat-induced polymerization (12 h at 37°C, 24 h at 45°C, followed by 48 h at 60°C). Blocks were sectioned at 0.5 μM, and section was dyed with 1% toluidin blue. Sections were then cut transversely 60-80 mmenRp using a diamond knife and placed on grids. Samples were stained with 2% uranyl acetate for 25 min, and washed with distillated water. After drying, samples were stained with 1% lead citrate for 6 min, and washed with distillated water again. The prepared samples were then observed by electronic microscopy (Hitachi H-600).

Two-dimensional gel electrophoresis

SK-MEL-28 cells were treated with 40 μM of MMTC and incubated for 24 and 48 h, treated with trypsin, harvested by centrifugation, and washed twice in PBS. After centrifugation at 1500 rpm for 15 min, cell pellets were lysed in lysis buffer [20 mM HEPES (pH 7.2), 1% Triton X-100, and 10% Glycerol]. For first dimension, pH 3-10 immobilized pH gradient (IPG) gel strips (13 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) were rehydrated overnight in rehydration solution (without DTT) containing 250 μg of protein sample in a IPGphor strip holder covered with cover fluid. IEF was conducted at 20°C using an IPGphor Iso-electric Focusing System (Amersham Pharmacia Bio-tech). A three-phase program was used for the analytical gels. The first phase was set at 1000V for 1 h, the second phase at 2000V for 2 h, and the third phase was a linear gradient from 2000 V to 8000 V for 8 h. Prior to 2-DE, IPG gel strips were equilibrated for 15 min in SDS equilibration solution. Second-dimension separation was carried out on 12% SDS-PAGE gel (16×20 cm) without stacking gels at 4°C. The IPG strips were embedded on top of the gels using 1% agarose. Electrophoresis was carried out at 30 mA/gel for 6 h until the bromophenol blue reached the bottom of the gel. Gel were fixed and stained with silver as recommended by the manufacturer (Amersham Pharmacia Bio-tech).

Destaining and enzymatic digestion of proteins in gel

Silver was removed from gel using chemical reducers. Briefly, a working solution was prepared by mixing 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (1:1 v/v). To cover the gel spots, 30 μL to 50 μL of working solution was added and vortexed. 200 mM of ammonium bicarbonate was then for 20 min and then discarded. Subsequently, gels were cut into small pieces, washed with water, and dehydrated repeatedly with acetonitrile until the gel pieces became opaque. The gel pieces were dried in a vacuum centrifuge for 30 min, and then subjected to trypsin digestion.

MALDI-TOF mass spectrometric analysis and database search

All mass spectrometric analyzes were performed using a PerSeptive Biosystems MALDI-TOF Voyager DE-RP mass spectrometer (Applied Biosystems, Framingham. MA, U.S.A.) operated in delayed extraction and reflector mode. Peptide mixtures were analyzed using a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile 0.1% TFA. The search programs, PeptIdent of ExPASY, MS-Fit, and ProFound-Peptide Mapping were used for database searches.

Migration assay

After SK-MEL-28 cells had been separated into pre-treated cells and non-pretreated cells, only pre-treated cells were treated with 40 μM of MMTC and incubated for 24 h. For cell migration assay, the lower portion of transwell membranes were coated with 10 μL of 0.5 μg/mL chemotactic fibronectin, and dried. Eight hundred μL of RPMI-1640 media was added to 24 well and transwell was set onto 24-well. The cells from two groups were harvested with trypsin-EDTA solution and washed in PBS. Cells were then centrifuged at 1500 rpm for 15 min, and 5×10^4 cells were suspended in 0.1 mL of 0.1% BSA-supplemented media containing 40 μM of MMTC and added to the transwell and incubated for 6 h. Cells were then fixed in methanol for 1 min, stained with hematoxylin for 3 min. And after rinsing with water, stained with eosin

for 10 sec. After rinsing with water again, the remaining cells on the upper membrane were carefully removed with cotton swabs. Membranes were removed carefully from transwells, and washed with xylene. Canadian balsam was then dropped onto membranes and migrated cells were counted under a microscope .

Assay of HUVEC cell proliferation

HUVECs (5×10^3 cells) in EGM-2 containing 100 μL of VEGF (10 ng/mL) and bFGF (10 ng/mL) were seeded into 96 well culture plates, treated with 50 mM of MMTC, and incubated at 37°C for 24 h. Cell proliferation was assayed using an XTT Cell Proliferation Kit I, by following the manufacturer's instructions.

RESULTS

To study cytotoxic the effects of MMTC on SK-MEL-28 cells and HaCaTs, SK-MEL-28 cells and HaCaTs were treated with MMTC at various concentrations (0, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} or 10^{-3} M, incubated for 48 h, and then MTT assays were performed. The survivals of SK-MEL-28 cells exposed to MMTC are shown Fig. 1. After treating cells with 10^{-4} M, exposure for 48 h caused a marked (69.7%) decrease in survival compared to controls (0 M). Nearly all cells died at 10^{-3} M. In contrast, treating HaCaTs increased survival by 115.07% at 10^{-5} M, and then decreased it by 68.36% at 10^{-4} M and by more than 80% at 10^{-3} M. IC_{50} values of MMTC were 4.49×10^{-5} M for SK-MEL-28 cells and 1.53×10^{-4} for HaCaT, showing that MMTC kill cancer cells (SK-MEL-28) more effectively than normal cells (HaCaT) and has low side effect.

We also examined whether cell death induced by MMTC occurs via apoptosis or necrosis. TUNEL assays are based on the principle that dUTP is attaches at the terminal 3'-OH groups of DNA cleaved during apoptosis and that the FITC-fluorescence is conjugated at dUTP combinated at the 3'-OH group. This fluorescence is detected using a flow cytometer. TUNEL assays were performed to confirm the result of DNA fragmentation on agarose gel.

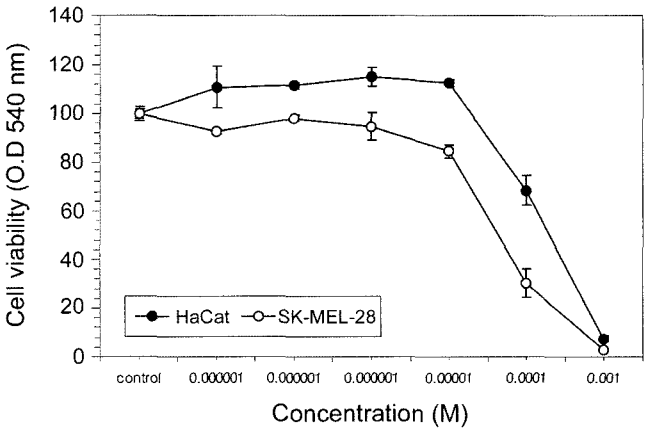

Fig. 1. Effects of MMTC on cell viability in SK-MEL-28 cells (open circle) and HaCaT cells (closed circle). Cells were incubated with various concentrations of MMTC for 48 h. Cell viability was measured by MTT assay. Results are expressed as the percent change of control conditions (media without drug). Data points represent the mean values of three replicates, with bars indicating SEM.

SK-MEL-28 cells were treated with 0, 10, 20, 30, or 40 μM of MMTC and incubated for 24, 48 or 72 h, and the sample obtained were analyzed by flow cytometry, after performing the above-described method. It was found that the fluorescence intensities of cells treated with the substituents were stronger than those of non-treated cells, and that fluorescence intensities increased in time and concentrations dependent manner. After treating MMTC, although the DNA of cells cultured for 24 h showed some degradation, DNA of cells cultured for 72 h showed marked degradation (Fig. 2A). The TUNEL assay results well matched the DNA fragmentation results on agarose gel. These findings confirmed that MMTC induces apoptosis in SK-MEL-28 cells through DNA fragmentation.

Other apoptotic characters include chromatin condensation, cell shrinkage, membrane blebbing, and the formation of apoptotic bodies, and one of the ways of identifying these characteristics is to use EM photographs. To take EM photographs, SK-MEL-28 cells were treated with 40 μM MMTC, and incubated for 48 h, and then worked-up as described above (Fig. 2B).

It was confirmed that SK-MEL-28 apoptosis was induced by MMTC. The difference between apoptotic and non-apoptotic cells (control) was evident. The untreated control cells had a clear plasma membrane, a nuclear membrane and an intact nucleus, but MMTC treated cells had apoptotic bodies on their plasma membranes, nuclear membranes had collapsed and chromatin was condensed. In particular, cells treated with MMTC showed fractured nuclear membranes. The above results demonstrate that MMTC induces SK-MEL-28 cell apoptosis.

In order to evaluate which specific proteins were changed by MMTC apoptosis, proteins were separated by 2-DE. Over 300 proteins were clearly visible (Fig. 3). The total number of proteins visible by silver staining in the separated cellular extracts was consistent in the control after 24°C and 48 h. Of these proteins, only 18 proteins were identified by MALDI-TOF (Fig. 4). And Table I presents that physico-chemical characteristics of the polypeptides differentially expressed in SK-MEL-28 cells by MMTC.

It is interesting to note that the levels of expression of apoptotic proteins differ. EMS1, ATP synthase beta chain, tyrosin-phosphatase, Cdc25c, specific structure of nulear receptor were decreased, and caspase-1, checkpoint

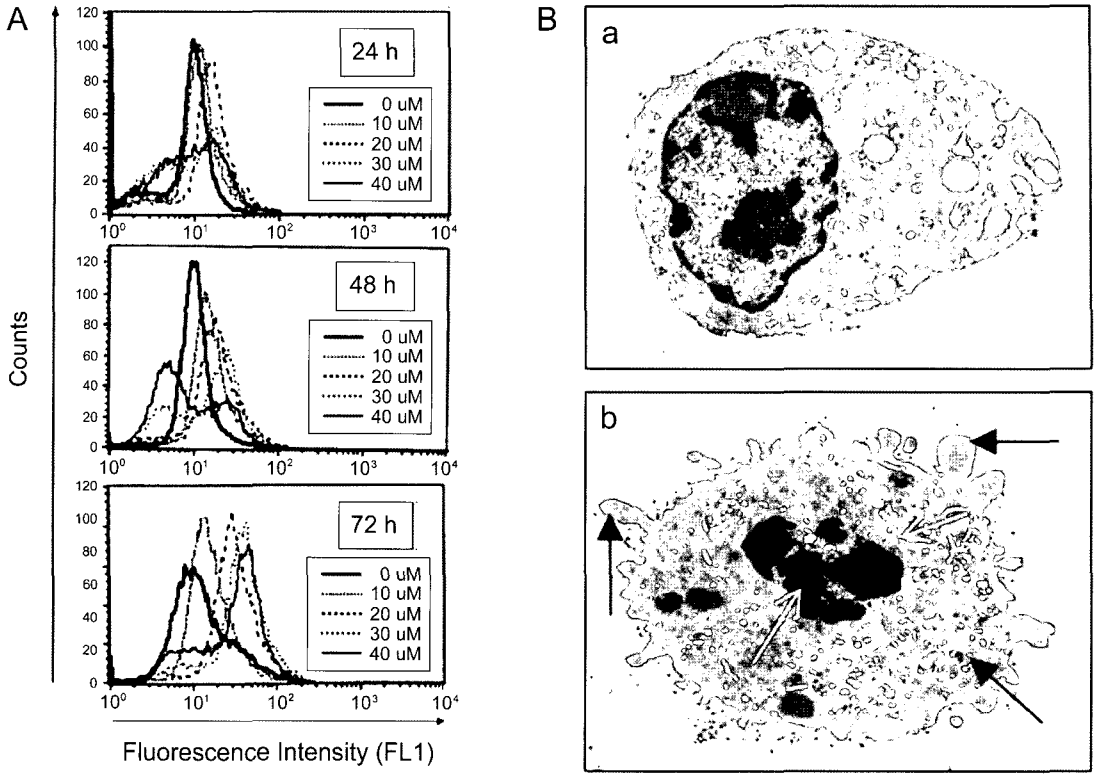

Fig 2. Apoptotic effect of MMTC on SK-MEL-28 cells. (A) TUNEL assays were performed to identify SK-MEL-28 cell apoptosis induced by MMTC; SK-MEL-28 cells were treated with 0, 10, 20, 30 or 40 μM of MMTC and incubated for 24, 48 or 72 h. Samples was analyzed by flow cytometry. (B) Electronic microscopic photographs of SK-MEL-28 cells undergoing apoptosis after MMTC treatment; Electronic microscopic photographs showing SK-MEL-28 cells undergoing apoptosis. SK-MEL-28 cells were treated with 40 μM MMTC and incubated for 48 h. : apoptotic body, : chromatin condensation, a : control cell, b : cell treated with MMTC.

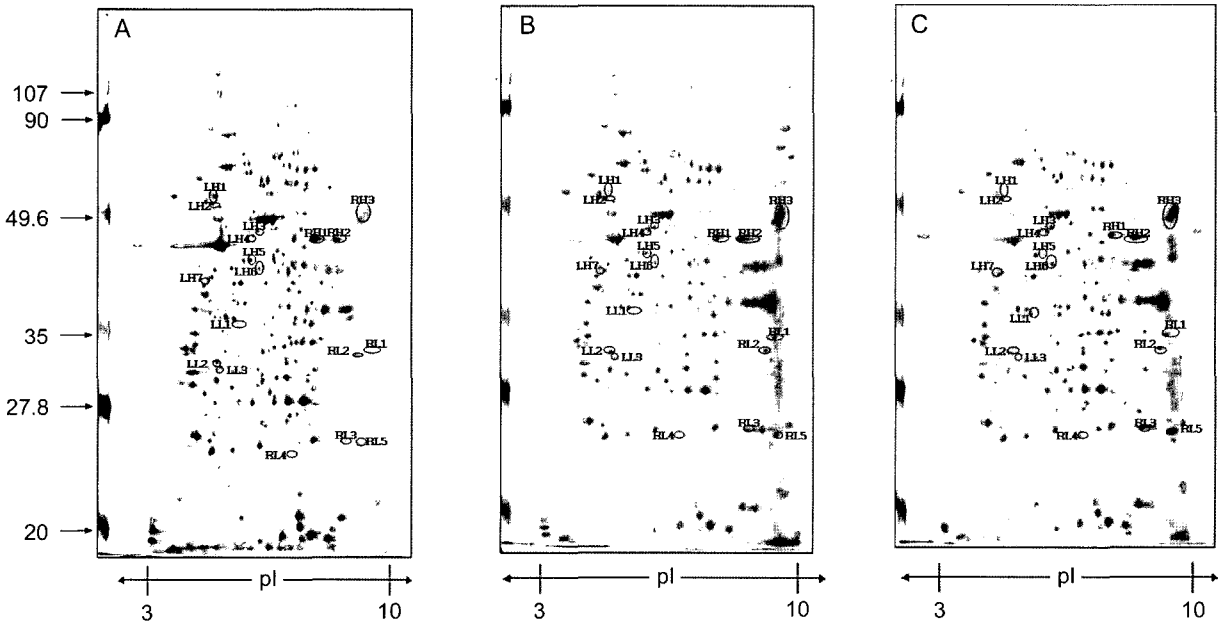

Fig. 3. 2-D gel image of proteins extracted from SK-MEL-28 cells exposed to MMTC for 28 h (B) or 48 h (C) and proteins extracted from untreated cells (A). After SK-MEL-28 cells were cultured for 28 and 48 h in RPMI-1640 supplemented with 10% FBS including 40 μM MMTC, cells were lysed in lysis buffer [20 mM HEPES (pH7.2), 1% Triton X-199, 10% Glycerol]. Lysates were electrophoresed on 2D gels. Left panel indicates the molecular weight (kD) marker, and the right panel indicates the pI range.

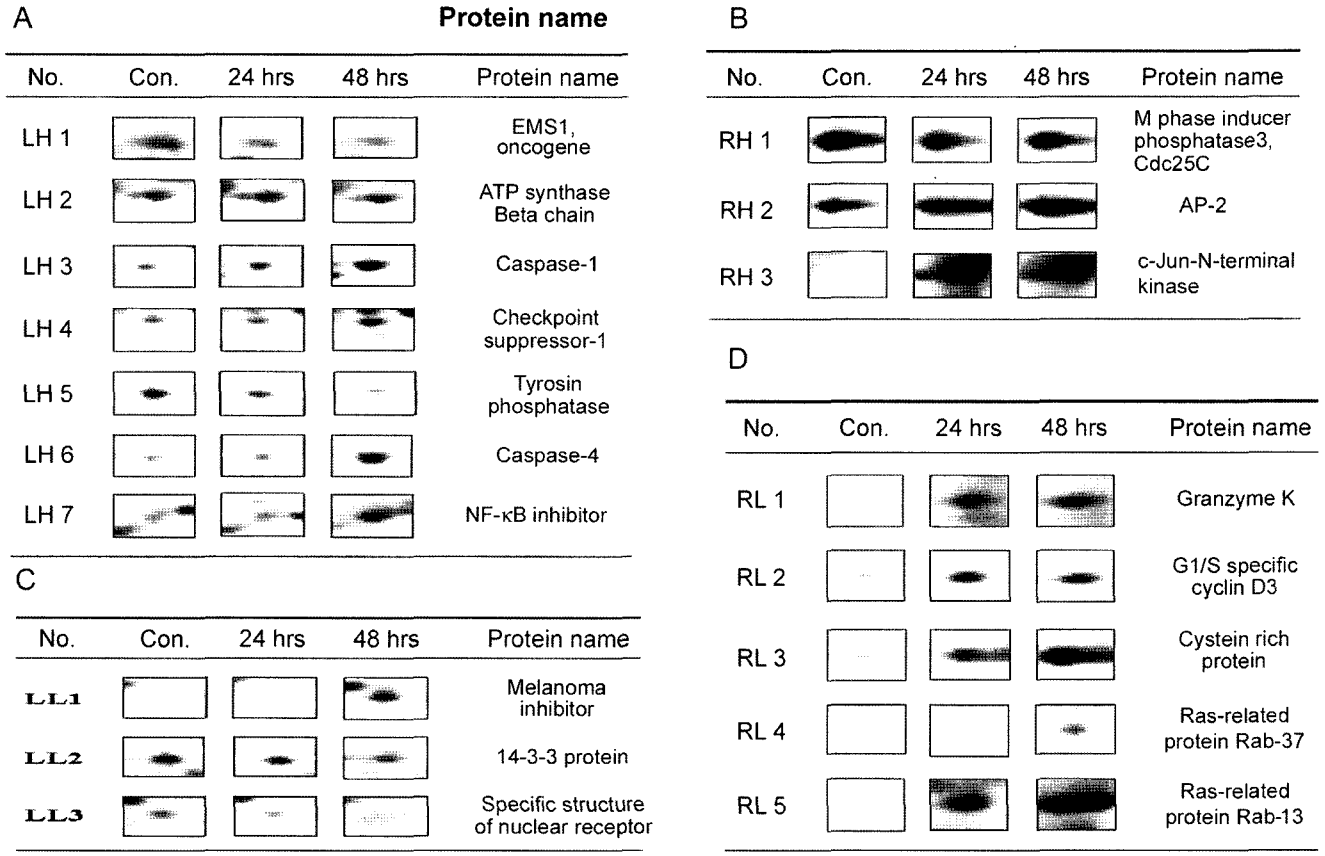

Fig. 4. Enlargement of the area of the 2-D gel from SK-MEL-28 cells treated with MMTC for 24 or 48 h. (A) Left-higher quarter of 2D gels. (B) Right-higher quarter of 2D gels. (C) Left-lower quarter of 2D gels. (D) Right-lower quarter of 2D gels (cells were treated with 40 mM MMTC and incubated for 0, 24 or 48 h. The cells were loaded on 2D gel, and stained with silver. The numbers in the oval indicate analyzed protein spots).

Table I. Physico-chemical characteristics of the polypeptides differently expressed in the SK-MEL-28 cell line by MMTC

Spot no.	Protein name	pI	M.W. (kDa)
LH1	EMS, oncogene	5.24	61.64
LH2	ATP synthase	5.00	51.77
LH3	Caspase-1	5.86	42.89
LH4	Checkpoint suppressor-1	6.19	53.83
LH5	Tyrosine Phosphatase	6.20	47.45
LH6	Caspase-4	5.70	43.24
LH7	NF-κB inhibitor	5.56	48.20
RH1	Cdc25c	7.48	48.00
RH2	AP-2	8.10	48.06
RH3	c-Jun-N-terminal kinase	9.26	47.48
LL1	Melanoma inhibitor	5.53	32.80
LL2	14-3-3 protein	4.76	27.95
LL3	Specific structure of nuclear receptor	5.4	29.36
RL1	Granzyme K	9.90	28.86
RL2	G1/S phase specific cyclin D3	6.70	32.52
RL3	Cystein rich protein	8.11	25.50
RL4	Ras-related protein Rab-37	6.00	24.82

suppressor-1, caspase-4, NF-κB inhibitor, AP-2, *c*-Jun-*N*-terminal kinase, granzyme K, 14-3-3 protein, G1/S specific cyclin D3, cystein rich protein, Ras-related protein Rab-37°C, Ras-related protein Rab-13 and melanoma inhibitor were increased by MMTC treatment.

When MMTC was treated, cell migration was suppressed (Fig. 5). The migration of cells treated with 40 μM was suppressed by over 50% and that of cells pre-treated for 24 h was suppressed by over 75%. Therefore, it was found that the MMTC could suppress migration of SK-MEL-28 cells.

HUVECs were treated with 50 μM MMTC and incubated for 24 h, and vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were added to experimented cells to promote the sprouting of blood vessels. Results showed that although HUVEC proliferation of EGM-2, controlled by growth factors, increased, that the proliferations of cells treated with MMTC were only slightly decreased (Fig. 6). This experiment showed that MMTC has little effect about angiogenesis.

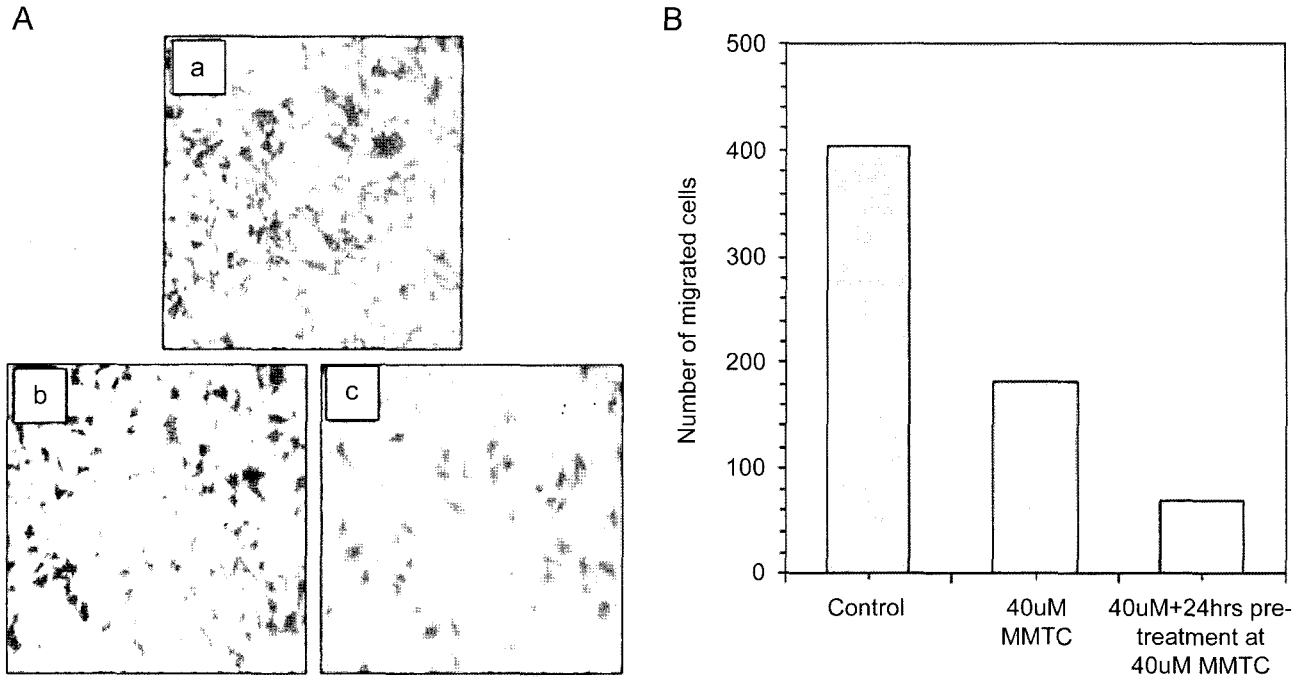

Fig. 5. Migration assays on SK-MEL-28 cells treated with MMTC. A) Cells were incubated for 6 h with 40 μM MMTC after injection into transwell chambers. The markers show that; a : control cell cultured in media only, b : cells cultured for 6 h after treated with 40 μM of MMTC in a transwell, c : cells pre-cultured for 24 h with 40 μM MMTC before being cultured for 6 h in a transwell. B) Result showing that MMTC can suppress the migration of SK-MEL-28 cells.

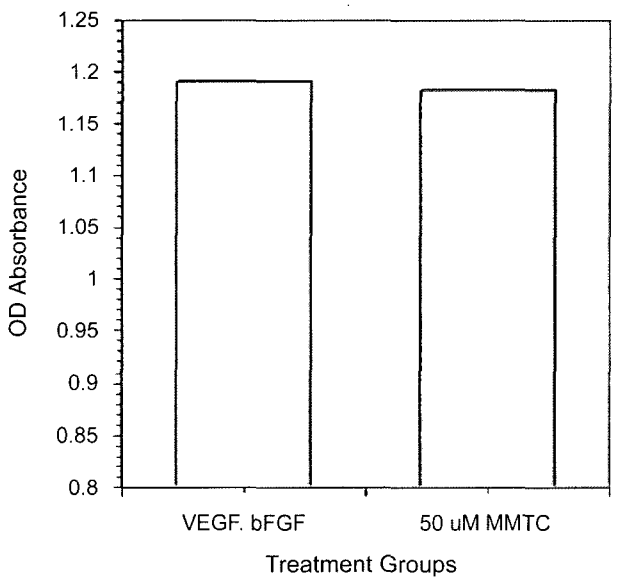

Fig. 6. Proliferation of HUVECs affected by MMTC. HUVECs (5×10^3 cells) in EGM-2 containing 100 μL of VEGF (10 ng/mL) and bFGF (10 ng/mL) were seeded into 96 well culture plates, treated with 50 μM of MMTC, and incubated at 37°C for 24 h.

DISCUSSION

MMTC kills SK-MEL-28 cells (a human skin cancer cell), but functions as a growth factor in normal cells (HaCaT), and thus, is a potential cancer chemotherapeutic agent. Fourteen proteins of a total of 18 proteins evaluated by MALDI-TOF were identified as apoptosis-related proteins.

It was reported that caspase-1 induces apoptosis when it is overexpressed in healthy cells (Kamens *et al.*, 1995) and that thymocytes from ICE-deficient mice do appear to be resistant to the apoptosis induced by anti-Fas antibody. By binding FADD (Fas-associated death domain) (Chinnaiyan *et al.*, 1995), caspase-8 is recruited to the cytoplasmic death domain of fas and then activated (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Caspase-8 is a member of a growing family of cystein proteases that are involved in Fas-mediated apoptosis and apoptosis triggered by another mechanism (Henkart, 1996). Caspase-1 is a member of this family and is probably involved in the apoptosis pathway downstream of caspase-8 (Hofman *et al.*, 1997). Caspase-1 enhances Fas-mediated apoptosis by facilitating caspase-8 activation (Toru *et al.*, 2000).

It was found that checkpoint suppressor-1 was increased after treating SK-MEL-28 cells with MMTC. When DNA is damaged, cells activate cell cycle checkpoints at various stage of the cell cycle. These DNA damage-inducible cell cycle arrests presumably give cells the chance to repair DNA prior to the replication of damaged DNA or the segregation of damaged DNA chromosomes. The selectivity of existing drugs for tumor cells over normal cells can be attributed, at least in part, to a loss of cell cycle checkpoint

control in cancer cells (Elledge, 1996). These checkpoint controls normally cause cells with damaged DNA to arrest until repair mechanisms can correct drug-induced injury, and if drug-induced DNA damage presents arrest continues and leads to apoptosis. Therefore, it is supposed that increases in checkpoint suppressor-1 by MMTC do not allow the repair of damaged DNA, and that finally SK-MEL-28 cells are committed to apoptosis.

The expression of tyrosine phosphatase was decreased by MMTC. Tyrosine phosphatase is a critical regulator of cellular phosphorylation function in processes such as cell growth, differentiation, and adhesion (Wheeler *et al.*, 2002). Also, accumulating evidence shows that protein phosphorylation and dephosphorylation as carried out by protein kinases and protein phosphatases are fundamental biochemical events in intracellular signal transduction. In particular, tyrosine phosphatase is widely involved in a variety of signaling pathaway triggered by cytokines and growth factors. Recent studies have demonstrated that this phosphatase plays an important role in transducing signals relayed from the cell surface to the nucleus, and that it is a critical intracellular regulator in cytokine and growth factor-induced cell survival, proliferation, and differentiation (Qu, 2002). Thus, it is suggested that after treating SK-MEL-28 cells with MMTC, reduced levels of tyrosine phosphatase inhibited cell proliferation, reduced cell survival, and promoted cell death.

Caspase-4 levels were increased by MMTC, and it is known that caspase-4 belongs to same group as caspase-1, and affect on activation of cytokines including IL-1β, and does not participate directly on apoptosis.

NF-κB inhibitor expression was increased by MMTC. NF-κB is released from its cytoplasmic retainer IkB and translocates to the nucleus, where it binds to specific DNA sequence motifs to activate gene transcription. It has been reported that the NF-κB sites in the ICE protease promoter, p53, and TNF promoters activated by NF-κB are consistent with the role of NF-κB in the transcription-dependent induction of cell death (Casano *et al.*, 1994; Trede *et al.*, 1995; Wu *et al.*, 1994). However, NF-κB activation is generally considered to be a cell proliferation signal rather than a cell death signal, and NF-κB has been reported to suppress apoptosis (Ashkenazi, 1998). Moreover, cell type-specific activities and multiple distinct NF-κB related transcription factors indicate that the role of NF-κB in cell death is not straightforward. The above suggests that the expression of NF-κB inhibitor increased by MMTC suppresses the proliferation of SK-MEL-28 cell and induce apoptosis.

AP-2 was increased, and the expression of the cell surface adhesion molecule, MCAM/MVC18, correlates directly with the metastatic potential of human melanoma cells. Moreover, the expressions of both genes are regulated by the AP-2 transcription factor. Re-expression of AP-2 in highly metastatic A375SM cells reduced their tumorigenicity and inhibited their metastatic potential in nude mice. In addition, the expression of MCAM/MUC18 mRNA and protein expression was significantly down-regulated while c-KIT (Tyrosin-kinase receptor) expression was regulated in AP-2 transfected cells, and AP-2 also regulates other genes that are involved in the progression of human, melanoma such as F-cadherin, MMP, $p2^{WAF-1}$, HER-2, BCL-2 and insulin like growth factor, receptor-1. Also loss of AP-2 is a crucial event in the development of malignant melanoma (Menashe, 1999). Thus, increased levels of AP-2 in cells treated with MMTC suppresses the tumorigenicity and metastasis of melanoma.

Increased *c*-Jun-*N*-terminal kinase (JNK) expression was also identified. JNK is activated by stress and is implicated in the regulation of apoptosis in several tissues (Estus *et al.*, 1994; Ham *et al.*, 1995; Mesner *et al.*, 1995). It has been reported that activation of JNK in PC12 cells contributes to apoptosis caused by nerve growth factor (NGF) withdrawal (Xia *et al.*, 1995). Also, JNK is activated through TNF and activated JNK stimulates AP1 and then causes apoptosis. Thus, increased expression of JNK by MMTC is directly associated with the induction of apoptosis in SK-MEL-28 cells.

Cytotoxic lymphocytes (CTLs) invoke the apoptotic suicide of other cells by two distinct mechanisms that can be distinguished by their dependence on calcium. The calcium-independent (nonsecretory) pathway involves the Fas receptor and its ligand (Hanabuchi *et al.*, 1994; Suda *et al.*, 1993; Takahashi *et al.*, 1994) and appears to involve a cascade of ICE/CED3 cystein proteases (Tewari *et al.*, 1995). On the other hand, the calcium-dependent pathway utilizes a discrete subset of cytotoxic granule proteins that invade the target cell and trigger apoptosis (Berke, 1995; Henkart, 1994; Smyth *et al.*, 1995). The proposed model for granule-mediated CL cytotoxicity proceeds *via* the synergistic interaction between perforin (cytolysin) and a family of serine proteases, the granzymes. Both perforin and the granzymes are contained within cytoplasmic granules. The binding of CL to an appropriate target cell leads to a calcium-dependent degranulation process, which results in the discharge of these granule constituents into the inter-cellular space. The secreted perforin then undergoes calcium-dependent polymerization into transmembrane channels that form 10 to 20 nm pores in the target cell membrane. Although there is no direct evidence, these pores are believed to facilitate the entry of granzymes into the target cell cytoplasm, where they trigger an apoptotic response. The principal granzyme involved in provoking apoptosis appears to be granzyme B, which is by far the most abundant of the granule proteases and also the most efficient at initiating a suicide response (Shi *et*

al., 1992). Although granzyme K is not as abundant as granzyme B, the proteases can induce apoptosis. Granzyme K was found to be increased in SK-MEL-28 cell treated with MMTC, which suggests that granzyme K was activated by MMTC and then affected apoptosis.

G1/S phase specific cyclin D3 was increased by MMTC. Cyclin D3 is combined specifically with Cdk4 and Cdk6, and operates in the G1 phase. P15 activated by TGF-β blocks the activation of cyclin D, as do p16 and p18. Cell cycles progress from the G1 to the S phase through G1/S phase specific cyclin D3. But, because of increasing cyclin D3, the percent of S phase was increased more so in MMTC-treated cells than nontreated cell. On the other hand, the percentage in the ~~of~~ G1 phase was reduced more in MMTC-treated cells than nontreated cells. Therefore, it is believed that increasing G1/S phase specific cyclin D3 induced S phase arrest.

14-3-3 protein is known as an apoptosis suppressor protein. Moreover, Bad which is upstream of 14-3-3 protein combines with bcl-2, an apoptosis suppressor gene, and then Bad blocks bcl-2 activation and induces apoptosis. In addition, phosphorylated Bad combines with 14-3-3 protein instead of bcl-2, in a process that is initiated by the activation of tyrosine-kinase receptor. If the receptor combines with its ligand, then PI3K is activated. Also, PI3K activates Akt, and Akt phosphorylates Bad, which then combines with 14-3-3 protein. This mechanism plays a role in cell survival, but if the ligand does not combined with receptor, Bad combines with bcl-2, which blocks the apoptotic suppression of, and thus, the cell undergoes apoptosis. The expression of 14-3-3 protein by cells treated with MMTC was reduced, suggesting that Bad is not phosphorylated or the ligand did not combine with tyrosine-kinase receptor related with the cell survival pathway.

It was found that proteins large amount of cystein were upregulated in cells treated with MMTC. From three to six homologous repeating cystein-rich extracellular domains are known as TNFR1, TNFR2, NGFR, CD27, CD30, CD40, OX40, 4-1BB, VV-A53R, and SFV-T2 and others including Fas. Also, caspase-1 and other caspases have abundant cystein. Therefore, it is believe that although this spot cannot indicate directly any extracellular receptor or any caspase, fragment of apoptosis associated protein was increased by MMTC.

Ras is an essential component of the signal transduction pathway that control cell proliferation, differentiation, and survival. Also, Ras is well established as modulator of apoptosis. Increasing the expression of oncogenic human Ras result in a dose-dependent induction of apoptosis in both primary and immortalized cells (Tom *et al.*, 1999). Outcomes in response to Ras are also dictated by the relative levels of activation of different effector pathways and by the timing of activation. For example, expression of activated Ras typically promotes mitogenesis in fibroblasts. In contrast, the activation of Ras induces apoptosis in these cells if protein kinase C activity is suppressed (Chen *et al.*, 1995). Moreover, the preferential activation by Ras of the PI3-kinase effector pathway protects fibroblasts from *c*-Myc-induced apoptosis, where the selective activation of the MAP kinase effector pathway potentiates *c*-Myc-induces apoptosis (Kauffmann-Zeh *et al.*, 1997). High levels of Ras activity induce an apoptotic response which is p53 independent and this requires the activations of JNK and ERK/MAP kinase cascades. Ras-mediated signals are necessary and sufficient to protect against Ras-induced apoptosis through a pathway that involves NF-κB activation (Tom *et al.*, 1999). It has also been suggested that NF-κB inhibitor can induce apoptosis. The expressed proteins, Ras-related protein-Rab-37 and Ras-related protein-Rab-13 were upregulated by MMTC treatment, which suggests that the apoptosis induced by MMTC is regulated by Ras. The case of melanoma inhibitor was increased by exposure at MMTC, suggesting that melanoma inhibitor is expressed in melanoma cell by MMTC and that melanoma can be treated using MMTC.

After it was identified that MMTC can induce apoptosis on SK-MEL-28 cell, of the investigated proteins, the expression of AP-2 was considered because this is known to be associated with the metastasis of melanoma. It has been reported that the re-expression of AP-2 in highly metastatic A375SM cells decreased their tumorigenicities and inhibited their metastatic potentials in nude mice. Our MALDI-TOF results that the expression of AP-2 was increased after treating 40 μM MMTC. Therefore we investigated whether MMTC could suppress SK-MEL-28 cell metastasis. SK-MEL-28 cells were divided into two groups. One group was pre-treated with MMTC before 24 h, and the other group was untreated. Then, SK-MEL-28 cells were treated with 40 μM MMTC, and incubated for 6 h in the transwell using for migration experiments. It was found that MMTC can suppress the migration of SK-MEL-28 cells. This result shows that MMTC may be valuable for treating skin cancer, in which metastasis is the major cause of death.

In addition, in this study, we tested whether MMTC has an inhibitory effect on angiogenesis. Angiogenesis is the process of new vascular capillary channel growth from pre-existing vessels, and is of fundamental importance in a number of physiological processes such as embryonic development, reproduction, wound healing and bone repair. Uncontrolled angiogenesis is often associated with tumor growth, rheumatoid arthritis, diabetic retinopathy and hemangiomas (Singh *et al.*, 2002). Three decades of intensive research strongly indicate the involvement of angiogenesis in the progression of primary tumors and

their metastasis to distant organs (Folkman *et al.*, 1992).

Cancer chemopreventive agents have been shown to inhibit cell growth, proliferation and to induce apoptosis in a variety of cancer cell lines. Moreover, the blocking of angiogenesis provides a novel therapeutic target against tumor spread. Thus, the identification of chemopreventives with multiple biological activities that act in various steps of the angiogenic cascade would be of great clinical significance. However, it was found that MMTC does not have an effect on angiogenesis, although after MMTC treatment HUVEC proliferation was reduced slightly.

In conclusion, the present study suggests that MMTC is a potential novel chemotherapeutic and antimetastatic agent against human melanoma cells, SK-MEL-28.

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