The Polymerization Agent, 2-Methyl-4’-(methylthio)-2-morpholinopropiophenone Induces Caspases-3/7 in Human Blood Mononuclear Cells in Vitro

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Our previous studies detected the presence of a photoinitiator 2-methyl-4’-(methylthio)-2-morpholinopropiophenone (MTMP) in an intravenous (i.v.) injection bag solution. Importantly, MTMP has demonstrated cytotoxicity to normal human peripheral blood (PB) mononuclear cells (MNC). Cell death pathways have two well-known modes, apoptosis and necrosis. But it has not been clear whether MTMP induced apoptosis or necrosis in normal human PB MNC. In the present in vitro study, we examined normal human PB MNC for the frequencies of apoptosis and necrosis and changes upon exposure to MTMP. We also assessed the activity of caspases-3/7. The results demonstrated that MTMP induced apoptosis in normal human PB MNC after 24 h. In addition, MTMP induced caspases-3/7 in a time-dependent manner. In conclusion, we suggest that MTMP induces apoptosis in a caspase-dependent pathway in vitro.

Key words photoinitiator; apoptosis; caspase-3; caspase-7; cytotoxicity; human mononuclear cell

Photoinitiators are used in a broad range of commercial and biological applications such as printing,\textsuperscript{9} dentistry,\textsuperscript{10} encapsulation of pancreatic islet cells,\textsuperscript{11,12} and blood vessel adhesives.\textsuperscript{13} Thus, a high level of daily exposure to photoinitiators is possible. In previous in vitro studies, it was reported that photopolymerizing agents were cytotoxic. The photoinitiators Sarcat\textsuperscript{TM} CD 1012, Araidite\textsuperscript{TM} GY 281 and Epon\textsuperscript{TM} 825 were cytotoxic to L929 cells,\textsuperscript{14} and 4-octyphenyl phenyliodonioum-hexafluoroantimonate (OPIA) also was severely cytotoxic.\textsuperscript{15} In other studies, Irgacure 2959 was found to be the least toxic photoinitiator while 1-hydroxyyclohexyl phenyl ketone (Irgacure 184) and 2,2-dimethoxy-2-phenylacetophenone (Irgacure 651) were significantly more toxic to human fetal osteoblasts.\textsuperscript{16}

In our recent study, we detected the presence of the photoinitiator 2-methyl-4’-(methylthio)-2-morpholinopropiophenone (MTMP) at approximately 2.8 mg/bag (5.6 µg/mL) in an intravenous (i.v.) injection bag solution. We demonstrated that MTMP was cytotoxic to normal human peripheral blood (PB) mononuclear cells (MNC).\textsuperscript{17}

Cell death pathways are well conserved in all metazoans, from invertebrates to vertebrates.\textsuperscript{18} Apoptosis and necrosis represent two distinct modes of cell death. Apoptosis is a physiologic mode of programmed cell death. It represents a metabolically active process of cell suicide that occurs normally during embryogenesis, metamorphosis, and hormone-dependent tissue atrophy.\textsuperscript{19–21} Classical necrosis, however, is a non-physiologic, passive or accidental mode of cell death resulting, for example, from extremes in environmental conditions.\textsuperscript{19–21} Apoptosis is regulated by numerous modulators including some ions (e.g. calcium),\textsuperscript{22} genes (e.g., MYC, BCL2/BAX, FAS),\textsuperscript{23,24} proteins (e.g., p53, caspases)\textsuperscript{25} and even organelles (e.g., mitochondria).\textsuperscript{26} Fas ligand in T lymphocytes and natural killer cells induces cytotoxicity by initiating interaction with the Fas-associated death domain.\textsuperscript{27} Fas-associated death domain initiates the caspase cascade by activating pro-caspase-8 which in turn activates caspase-3.\textsuperscript{28}

Caspase activation can be regulated through an extrinsic or intrinsic signaling pathway. The intrinsic pathway, which may be the primary means of activating apoptotic caspases in mammals, triggers the mitochondrial release of cytochrome c, which oligomerizes with apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9 to form the apoptosome complex. Activated caspase-9 in this complex activates caspases-3 and -7 to execute apoptosis.\textsuperscript{29} Thus, caspases are the most important effectors of apoptosis.\textsuperscript{30}

The purpose of this study was to assess the frequencies of apoptosis and necrosis in normal human PB MNC exposed to MTMP. In addition, we examined the time-dependent changes in cell viability when normal human PB MNC were exposed to 250 µg/mL MTMP. Finally, we measured changes in the activities of caspases-3/7 as a function of time.

MATERIALS AND METHODS

Chemicals and Reagents MTMP was purchased from Tokyo Chemical Industry Co., Ltd. (Japan). MTMP was used as a solution in methanol. 3-(4,5-Dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) was purchased from Wako Pure Chemical Industries, Ltd. (Japan) and was dissolved in phosphate buffered saline (PBS). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (U.S.A.). Cell Event\textsuperscript{TM} Caspase-3/7 Green Detection Reagent was purchased from Life Technologies Co. (Japan).

Isolation and Culture of Normal Human PB MNC Normal human PB MNC were obtained from six healthy volunteers after informed consent. Thirty milliliter of PB was withdrawn from a vein in the forearm, and PB MNC were isolated from theuffy coat by centrifugation on a Ficoll-Paque system (Amersham Biosciences AB, Uppsala, Sweden). Cells were then washed three times with RPMI 1640 medium (Nissui Co., Ltd., Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma Chemical).
PB MNC were suspended at a final concentration of $1 \times 10^4$ cells/mL in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS.

The protocol was approved by the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences Ethics Committee (No. 1026).

Annexin V/Propidium Iodide (PI) Staining and Flow Cytometric Analysis Cells were treated with MTMP for 24 h. The detection of early apoptotic cells was then performed with annexin V-fluorescein isothiocyanate (FITC) (In-vitrogen, Tokyo, Japan) combined with PI by flow cytometry. Briefly, cells were plated in 6-well plates at a density of $1 \times 10^5$ cells/cm$^2$. Treated and untreated cells were incubated with FITC-conjugated annexin V and PI for 15 min at 20°C. They were immediately analyzed on a flow cytometer (Beckton Dickinson, FACSCalibur) in their staining solution. Data from 10000 cells were recorded on logarithmic scales. Data analysis was performed using FlowJo software on cells characterized by their forward/side scatter (FSC/SSC) parameters. Samples stained with annexin V-FITC and PI were represented by dot plots of PI versus annexin V intensity. The dot plots were divided into four regions as follows. The lower left region included cells that failed to stain with either annexin V or PI and were considered to be undamaged. The lower right region included cells stained with annexin V that were still PI negative and were considered to be early apoptotic. The upper right region included cells stained with both annexin V and PI; they were classified as late apoptotic or necrotic. The upper left region included cells that were annexin V-negative but PI-positive and were dead cells.

Cell Survival Assay with MTT Reduction Cell survival was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenase.$^{25}$ Cells ($1 \times 10^4$) were treated with MTMP for 1, 3, 6, 12 or 24 h in a humidified incubator at 5% CO$_2$ and 37°C. Medium (100 µL, including a final concentration of 2.5% methanol) was then incubated with 20 µL of 5 mg/mL MTT solution for 1 h in a humidified incubator at 5% CO$_2$ and 37°C. After centrifugation at 1500 rpm for 10 min, the culture medium was removed, and 100 µL of DMSO was added to each well to dissolve the formazan. The absorbance at 570 nm was measured using a microplate reader (Model 680 microplate reader; Bio-Rad Laboratories, Inc., Japan). Cell survival was expressed as a percentage of the absorbance value determined for control cultures using the following calculation:

$$\text{Cell survival rate} (\%) = \frac{\text{As} - \text{Ab}}{\text{Ac} - \text{Ab}} \times 100$$

where, As: Absorbance of sample, Ac: Absorbance of negative control, Ab: Absorbance of blank.

For negative control wells, methanol was added instead of MTMP.

Determination of Caspases-3/7 Apoptotic cells were determined using the CellEvent Caspase-3/7 Green Detection Reagent (Life Technologies Japan Ltd., Tokyo, Japan) as recommended by the manufacturer. In brief, CellEvent Caspase-3/7 Green Detection Reagent was added to a final concentration of 5 µM to the samples that were incubated for 30 min in a humidified incubator at 5% CO$_2$ and 37°C. Cells were examined by fluorescence microscopy (BIORIVO, BZ-9000; KEYENCE, Osaka Japan). The excitation/emission maxima for the CellEvent Caspase-3/7 Green Detection Reagent are 502 / 530. The control was defined as cells briefly exposed to 250 µg/mL MTMP.

Statistical Analysis The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett’s test. The significance level was set at $p<0.05$. 

Fig. 1. The Effect of Photoinitiators on Leukocyte Apoptosis

Normal human PB MNC were subjected to the photoinitiator at the indicated doses for 24 h. Cell were stained with annexin V-FITC and propidium iodide (PI). Figure shows representative flow cytometric patterns. The percentage of cells in each quadrant is indicated (C1: necrosis, C2: late apoptosis, C3: live cells, C4: early apoptosis).
RESULTS

Flow Cytometric Assay  Based on the finding that MTMP treatment inhibited cell proliferation, we investigated whether this effect was due to an increase in apoptosis or necrosis.

Normal human PB MNC were treated for 24h with a range of MTMP concentrations, stained for expression of annexin V and permeability to PI and analyzed by flow cytometry (Fig. 1). Figure 2 shows that MTMP significantly increased apoptosis at concentrations ≥62.5 µg/mL. MTMP induced a
significantly higher level of apoptosis than that observed in the controls (\(F(9, 30)=438.546, p<0.01\), Figs. 1, 2). Moreover, MTMP induced a significantly higher level of necrosis than that seen in the controls (\(F(9, 30)=21.603, p<0.01\), Figs. 1, 3).

**The Time-Dependence of 250μg/mL MTMP Cytotoxicity Determined by the MTT Assay** In cell viability studies with the MTT assay, increased cytotoxicity was observed at later times. The negative control gave an average cell survival of 100±3.94%. In treatment with 250 μg/mL MTMP, we observed cell survival of 104.55±15.57% after 1 h, 106.23±11.41% after 3 h, 105.53±11.98% after 6 h, 74.90±31.29% after 12 h and 28.8±16.24% after 24 h. At the last time point, the attenuation of cell viability was dramatic [\(F(5, 30)=15.969, p<0.01\)]. The post hoc comparisons showed that a significant effect was observed after 24 h (\(p<0.01\)) (Fig. 4).

**The Time-Dependent Activities of Caspases-3/7 with 250μg/mL MTMP** After six h of treatment with 250 μg/mL MTMP, increased activities of caspases-3/7 were observed. Thus, at the last time point, the change in caspase-3/7 activity was highly significant [\(F(5, 30)=109.403, p<0.01\)]. The post hoc comparisons showed that significant effects were observed at 24 h (\(p<0.01\)) (Figs. 5, 6).

**DISCUSSION**

In this in vitro study, we demonstrated that MTMP induced apoptosis in normal human PB MNC. In addition, MTMP induced apoptosis via caspases-3/7. Moreover, the cellular response was dramatic after 24 h. Therefore, we suggested that MTMP occurred in normal human PB MNC, and may be involved in adverse events.

It was reported that photoinitiators induce free radicals, and an antioxidant (free radical scavenger), ascorbic acid, added to the cell medium significantly improved relative cell survival. However, in this in vitro study, MNC were not exposed to UV and cells were maintained under dark conditions in the presence of MTMP. Therefore, this raises the possibility that the observed cytotoxicity of MTMP was not induced by free radical formation in this study. Rather, toxicity appeared to be due to MTMP itself.

Apoptosis is a specific process that leads to programmed cell death through the activation of an evolutionarily conserved intracellular pathway. Apoptosis is distinct from necrosis, and it is essential in the homeostasis of normal tissues, such as the immune system and skin. Apoptosis can be divided into early and late phases. In our results, we showed that MTMP induced late apoptosis after 24 h of exposure. We believe that our data demonstrated that MTMP induced apoptosis via caspases-3/7 in vitro.

At a dose of 250 μg/mL, MTMP decreased cell survival after 12 h, and significantly lowered cell survival after 24 h. Consistent with that observation, caspase-3/7 activities were significantly increased after 24 h. Therefore, it is possible that apoptosis induced by MTMP could also occur via a caspase-independent pathway. In the caspase-dependent pathway, the extrinsic pathway is activated through trimerization of death receptors, followed by recruitment of adaptor molecules and pro-caspase-8 to form the DISC. Subsequent cleavage of caspase-8 leads to cleavage of downstream executioner caspases, caspases-3 and -7, and subsequently cell death. In contrast, the intrinsic pathway is initiated by the formation of pores in the mitochondrial membrane, and subsequent release of mitochondrial inner membrane proteins into the cytosol. These proteins, along with pro-caspase-9, form the apoptosisosome that cleaves caspase-9. Activated caspase-9 then goes on to cleave the downstream executioner caspases-3 and -7, leading to cell death.

One limitation of this study is the fact that it was conducted in vitro. Moreover, in a clinical setting, MTMP that is present in an infusion bag slowly enters the body in small amounts over an extended period of time. With regard to the potential significance of photoinitiators including MTMP, a report published by the European Food Safety Authority (EFSA) stated that photoinitiators were present in milk and juice. Published by the European Food Safety Authority (EFSA) stated that photoinitiators were present in milk and juice. According to the EFSA, although no fully convincing data on photoinitiator toxicity are currently available, the presence of photoinitiators in food is still considered undesirable. Therefore, whether photoinitiators are generally considered to be a human health concern, is not clear.

The incorporation of the photoinitiator benzophenone into sunscreen may induce an allergic skin reaction similar to
skin irritants that cause photoallergies, allergic contact dermatitis, and facial erythema. Many photoinitiators have been identified as the sources of possible adverse cellular events such as histamine release. The cytotoxicity of compounds such as photoinitiators may be due in part to their hydrophobicity since permeability through the phospholipid bilayer of cellular membranes increases with compound hydrophobicity. Momo et al. reported that 2-isopropylthioxanthone (2-ITX), a highly lipophilic photoinitiator, was involved in possible interactions with the lipid moieties of biological membranes that increased the fluidity of the bilayer. They hypothesized that 2-ITX underwent a relaxation process when the lipid matrix passed from the gel to the fluid state. Similarly, MTMP might accumulate in cells and induce allergic reactions in the body even in small amounts. Future in vitro studies will clarify the mechanisms involved in the adverse events caused by photoinitiators.

In conclusion, we demonstrated that activation of caspases-3/7 was significantly increased in normal human PB MNC following exposure to MTMP. It is not clear at this time whether the caspase-dependent pathway or the caspase-independent pathway is more critical to MTMP-mediated apoptosis.

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