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Level of $HgCl_2$-Mediated Phosphorylation of Intracellular Proteins Determines Death of Thymic T-Lymphocytes With or Without DNA Fragmentation

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Abstract Exposure to Hg^{2+} at a wide range of concentrations (approximately 1–100 μM) more or less caused the death of murine thymic T-lymphocytes, and exposure to 1 μM but not 10 μM (or more) of Hg^{2+} induced DNA fragmentation. Exposure of cells to Hg^{2+} caused phosphorylation of multiple cellular proteins at the tyrosine residue in a concentration-dependent manner. We found that not only the DNA fragmentation induced by 1 μM Hg^{2+} but also the cell death bypassing DNA fragmentation caused by 10 μM or more Hg^{2+} was partly inhibited by protein kinase inhibitors such as staurosporine and herbimycin A. This result suggested the involvement of a protein phosphorylation-linked signal in the mechanism of the Hg^{2+}-mediated cell death with or without DNA fragmentation. Analysis of proteins by both one- and two-dimensional electrophoresis and immunoblot showed that a 52-kDa Shc protein was heavily phosphorylated by an early signal delivered by a high concentration of Hg^{2+}, which also phosphorylated extracellular signal-regulated kinase 1 (ERK1; p44) and ERK2 (p42) of the mitogen-activated protein kinase (MAPK) family in a concentration- and time-dependent manner. The c-Jun amino terminal kinase (p54), which is a distant relative of the MAPK family, was also phosphorylated by the treatment with Hg^{2+}. This eventually formed the signaling cascade that ended with a nuclear target by phosphorylating c-jun at the serine 73. This phosphorylation of c-jun was inhibited by staurosporine. These results suggest that a high level of Hg^{2+}-mediated protein phosphorylation-linked signal induces rapid cell death bypassing DNA fragmentation, whereas a lower level induces cell death accompanying DNA fragmentation. This conclusion in turn implies that DNA fragmentation is not always a prerequisite for the signal transduction-dependent cell death of T-lymphocytes. J. Cell. Biochem. 71:243–253, 1998.

Key words: T-lymphocyte; apoptosis; signal transduction; $HgCl_2$; tyrosine phosphorylation

The two common ways for cells to die are necrosis and apoptosis [reviewed by Cohen, 1993; Corcoran et al., 1994]. Cell death due to necrosis, also referred to as accidental cell death, occurs through passive mechanisms after physical or chemical trauma. In contrast, apoptosis or programmed cell death develops through active mechanisms during organ development [Cohen, 1993; Jacobson et al., 1997]. Apoptosis also underlies the nonspecific and ligand-specific death of thymocytes, the latter type of death being for negative selection. The nonspecific death of thymocytes is induced by glucocorticoid treatment [Cohen and Duke, 1984] or γ-irradiation [Sellins and Cohen, 1987], whereas ligand-specific death is induced by crosslinking CD3/T-cell receptor complex [Shi et al., 1989; Smith et al., 1989], co-crosslinking CD3 and Thy-1 [Nakashima et al., 1993], or stimulating with multireceptor reactive-agonist concanavalin A (Con A) [Akhand et al., 1997]. Apoptosis, not necrosis, generally requires a cascade of intracellular signal transductions that may include activation of nonreceptor tyrosine kinases and other signal delivery elements for gene activation [Cohen, 1993; Corcoran et al., 1994]. This signal transduction pathway for apoptosis is believed to involve activation of

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endonucleases for internucleosomal DNA fragmentation before cell death [Willie, 1980]. Some previous studies, however, have shown that the DNA fragmentation before membrane damage may not be always complete [Brown et al., 1993; Zakeri et al., 1993; Cohen et al., 1994]. The question arises as to whether, under some condition, cells would be subjected to intracellular signal transduction-dependent active cell death that might bypass DNA fragmentation.

Inorganic mercuric ions (Hg^{2+}) have been shown to act as inducers of multiple cellular events. These ions cause rapid tissue damage at high concentrations and induce autoimmune-related disorders at lower concentrations [Goldman et al., 1991]. Hg^{2+} is a well-known sulfhydryl reactive compound [Vallee and Ulmer, 1972], and we previously demonstrated that it induces aggregation of cell surface proteins such as CD4, CD3, CD45, and Thy-1 on thymic T-lymphocytes by bridging sulfhydryl groups of the respective proteins [Nakashima et al., 1994]. The aggregation of cell surface proteins accompanies intracellular aggregation and activation of nonreceptor protein-tyrosine kinases $p56^{lck}$ and $p60^{c\text{-}src}$ and phosphorylation of multiple cellular proteins at tyrosine residues [Rahman et al., 1993; Nakashima et al., 1994; Pu et al., 1996]. The Hg^{2+}-mediated tissue damage or cell death has been generally considered to be necrotic due to its direct cytotoxicity. In the present study, we demonstrate that Hg^{2+} induces two types of active cell death with or without DNA fragmentation, both of which require protein-tyrosine phosphorylation-linked cellular signal. The results also show that the Hg^{2+}-mediated cell death bypassing DNA fragmentation is linked to delivery of a high level of the Shc/mitogen-activated protein kinase (MAPK)/c-Jun phosphorylation-oriented signal.

METHODS

Cells and Reagents

Single cell suspensions of thymocytes in phosphate buffered saline (PBS) were prepared from 6–8-week-old C57BL/6 strain mice. Cell suspensions were incubated in the presence or absence of $HgCl_2$ (Sigma, St. Louis, MO) dissolved in PBS at 37°C before further analysis. Staurosporine was purchased from Sigma, genistein from Extrasynthese (Lyon, France), and herbimycin A was kindly donated by Dr. Y. Uehara, National Institute of Health, Tokyo.

Analysis of Cell Death and DNA Fragmentation by Flow Cytometry (FCM)

For measurement of the level of cell death, cells were incubated with 10 µg/ml propidium iodide (PI), which stains DNA of membrane-disrupted cells [Mangan et al., 1991]. The percentages of viable cells in 5,000 cells were then recorded by FCM (EPICS profile; Coulter, Hialeah, FL).

For determination of the level of apoptotic DNA fragmentation, cells were lysed in a hypotonic solution (0.1% sodium citrate, 0.1% Triton X-100) containing 10 µg/ml of PI, and the DNA content of the nuclei was analyzed by FCM [Nicoletti et al., 1991; Ohkusu et al., 1995]. To measure the fluorescence intensity of PI, both intact and fragmented nuclei were gated by forward versus side scatter. Apoptotic nuclei appeared as a wide hypodiploid DNA peak that was easily distinguished from the narrow peak of nuclei with normal diploid DNA content in the red fluorescence channel.

Analysis of DNA Fragmentation by Electrophoresis

Cells were lysed in 100 µl of hypotonic lysing buffer [50 mM Tris-HCl, 0.5% sodium dodecylsulfate (SDS), 10 mM EDTA], followed by the addition of 2 µl of proteinase K (20 mg/ml) and 6 µl of RNAse (10 mg/ml). The resultant mixture was incubated at 55°C for 1 h. The sample (10 µl) was mixed with 3 µl of 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose and was run on 1.5% agarose gel with 0.1 µg/ml ethidium bromide [Nakashima et al., 1991].

SDS–Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting

SDS-PAGE and immunoblot were performed as described elsewhere [Nakashima et al., 1991]. Briefly, cells were lysed by adding an equal volume of a twofold concentrated sample buffer [125 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercapto-ethanol (2-ME), 20% glycerol], and proteins thus obtained were subjected to SDS-PAGE with 10% gel. The proteins were then transferred to a polyvinylidene difluoride membrane. Subsequently, the membrane was stained with anti-phosphotyrosine (Ptyr) antibody (Transduction Laboratories, Lexington, KY) followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (Tago, Burlingame, CA). The protein bands were visualized by Western Blot Chemiluminescence Reagent (DuPont

NEN, Boston, MA) as directed by the manufacturer. For reprobing, the membrane was stripped (2% SDS, 62.5 mM Tris, pH 6.8, 100 mM 2-ME, 50°C, 30 min) and reprobed with anti-Shc antibody (Transduction Laboratories), followed by anti-rabbit IgG conjugated to horseradish peroxidase.

Detection of proteins with antibodies specific for phosphorylated MAPK and serine 73-phosphorylated c-Jun were done with kits supplied by New England Biolabs (Beverly, MA). The molecular sizes of the stained proteins were estimated by comparison with protein molecular mass standards (New England Biolabs).

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis was done as described elsewhere [Görg, 1991]. Briefly, 5×10^6 cells were lysed with 200 µl of sample solution (9 M urea, 2% 2-ME, 2% Pharmalyte, pH 3–10, and 0.5% Tryton X-100), and 100 µl of the lysate were loaded on Immobiline® DryStrip, pH 3–10 (Pharmacia, Uppsala, Sweden) for the one-dimensional electrophoresis. The two-dimensional electrophoresis and immunoblotting were done as described above.

RESULTS

Characterization of $HgCl_2$-Induced Cell Death

We counted viable cell numbers by using FCM after incubating thymocytes in vitro with 1–100 µM of $HgCl_2$ for 1–15 h (Fig. 1). After 1 h of incubation, 10 or 100 µM but not 1 µM of $HgCl_2$ induced cell death accompanying plasma membrane disruption: approximately 80% and 20% of the cells died when incubated with 100 µM and 10 µM of $HgCl_2$, respectively (Fig 1A). After a longer (15 h) incubation, a considerable decrease of cell viability was observed even when incubated with 1 µM of $HgCl_2$ (Fig. 1B).

We wondered whether the cell death due to incubation with $HgCl_2$ also involved DNA fragmentation. To investigate this question, we incubated cells in vitro for 15 h with or without 1–10 µM of $HgCl_2$, and the percentage of cells undergoing apoptosis with DNA fragmentation was evaluated by FCM. As shown in Figure 2, incubation with 1 µM $HgCl_2$ induced apoptosis as demonstrated by a wide peak displaying reduced DNA content. The absence of such a wide peak for cells incubated with 10 µM of $HgCl_2$ suggested that the cell death induced by this concentration of $HgCl_2$ was not due to typical apoptosis accompanying DNA fragmentation. Shorter (4–6 h) incubation with 1 or 10 µM of $HgCl_2$ failed to induce any pattern of DNA fragmentation in the histogram (data not shown). Agarose gel electrophoresis showed a ladder of fragments, typical for apoptosis, for the DNA isolated from cells cultured with 1 µM of $HgCl_2$ for 15 h (Fig. 3). Cells incubated with 10 µM did not show any trace of DNA fragmentation. This result corresponded well with that obtained by FCM (Fig. 2).

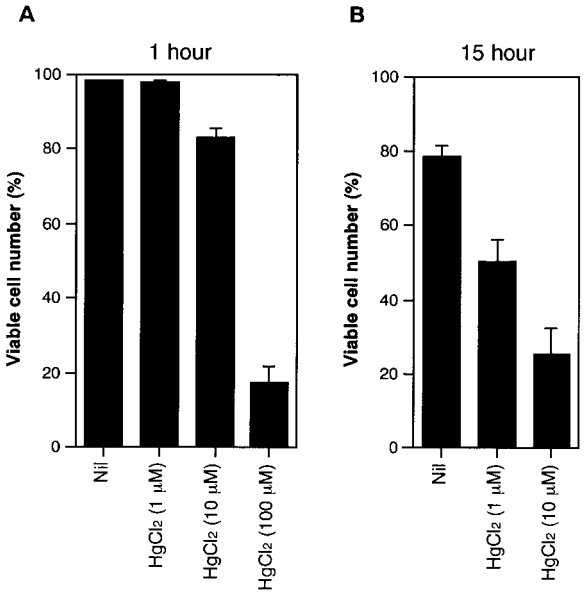

Fig. 1. $HgCl_2$ induces death of thymocytes. Thymocytes (10^6/100 µl) suspended in phosphate buffered saline were incubated at 37°C in vitro with or without 1–100 µM of $HgCl_2$ for 1 h (**A**) or 15 h (**B**). After 4 h, RPMI 1640 medium containing 10% fetal calf serum (100 µl) was added to the culture (B) to supplement essential components for cell survival. Numbers of viable cells were measured by flow cytometry after staining dead cells with propidium iodide. Each column represents the mean ± SD of triplicate assays.

$HgCl_2$ Induces Intracellular Protein-tyrosine Phosphorylation in a Concentration- and Time-Dependent Manner

We previously showed that thiol-reactive Hg^{2+}, which induces aggregation of cell surface proteins, delivers a signal into the cells for phosphorylating multiple proteins at the tyrosine residues [Nakashima et al., 1994]. In previous experiments, however, we treated the cells with Hg^{2+} dissolved in minimum essential medium (MEM) containing cysteine, which should partly neutralize Hg^{2+}. Therefore, we retested the activity of various concentrations of $HgCl_2$ dissolved in PBS by treating the cells in PBS so as to induce the tyrosine phosphorylation of

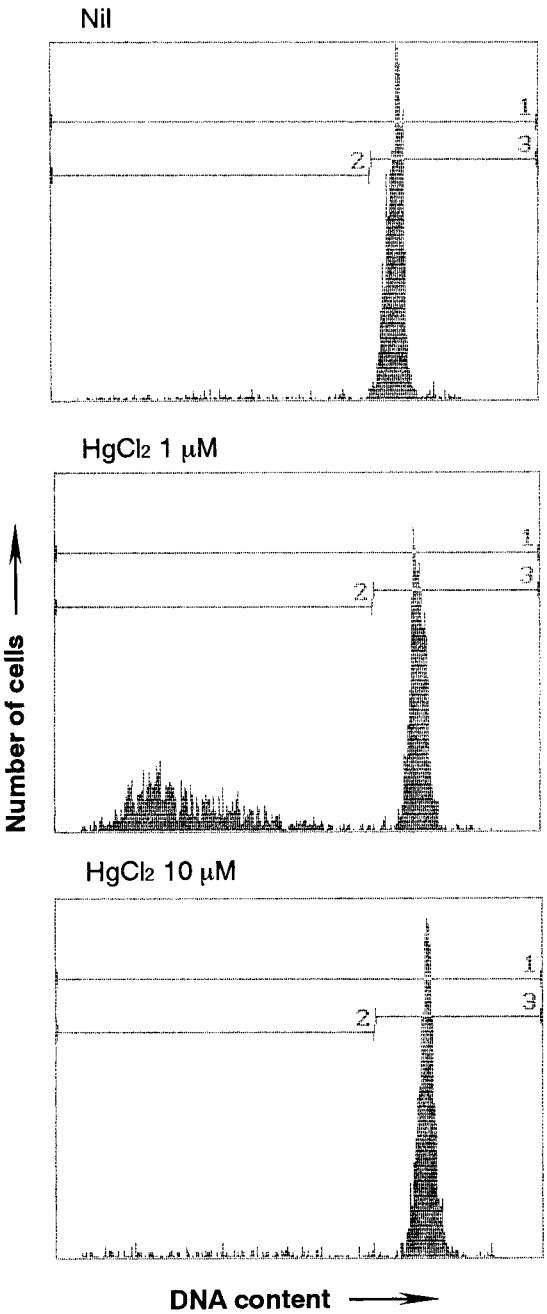

Fig. 2. Only low concentration of $HgCl_2$ induces DNA fragmentation of thymocytes. Thymocytes were incubated at 37°C with or without 1–10 μM of $HgCl_2$ for 15 h as described in Figure 1B. These cells were then lysed in hypotonic solution containing propidium iodide, and the DNA content of the nuclei was analyzed by flow cytometry. Nuclei with intact DNA are present in region 3 (under the middle horizontal line) and those with reduced DNA, due to fragmentation, are in region 2 (under the lower horizontal line). A representative of three experiments with consistent results is shown.

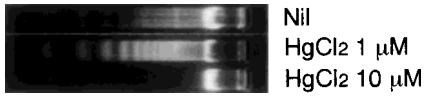

Fig. 3. Visualization of $HgCl_2$-induced DNA fragmentation by gel electrophoresis. Thymocytes were incubated at 37°C with or without 1–10 μM of $HgCl_2$ for 15 h as described in Figure 1B. These cells were lysed in hypotonic lysing buffer, and DNAs were then analyzed by agarose gel electrophoresis. A representative of three experiments with consistent results is shown.

cellular proteins. We detected a few protein bands in thymocytes without any stimulation by immunoblot with Ptyr antibody (Fig. 4A, nil). Only 2-min incubation with a wide range of $HgCl_2$ concentrations caused increased phosphorylation of multiple cellular proteins. From testing the actions of different concentrations, we found that 100 μM $HgCl_2$, which was barely active for cells suspended in MEM [Nakashima et al., 1994], induced the highest level of phosphorylation of 180-, 120-, 90-, 70/75-, 56/52-, 43-, and 40-kDa proteins. As the concentration was increased, the level of phosphorylation decreased. Lower concentrations (20–50 μM) of $HgCl_2$ developed low levels of protein-tyrosine phosphorylation. Addition of staurosporine, a broad-spectrum protein kinase inhibitor [Tamaoki and Nakano, 1990], before $HgCl_2$ greatly diminished the $HgCl_2$-mediated phosphorylation of cellular proteins (Fig. 4B), suggesting the involvement of staurosporine-sensitive protein kinase activity. We then performed a time course study with 10 μM of $HgCl_2$ (Fig. 4C). The intensity of protein-tyrosine phosphorylation increased up to 1 h and declined thereafter, nearly reaching the basal control level within 4 h. The time course study with 100 μM of $HgCl_2$ showed that phosphorylation started as early as 5 sec and reached a peak after 10-min stimulation (Fig. 4D). More than 50% of the cells died when the protein phosphorylation peaked, suggesting a linkage between heavy protein phosphorylation and rapid cell death.

$HgCl_2$-Mediated Cell Death at Least in Part Requires Protein Phosphorylation-Linked Signal

To investigate the possible involvement of protein kinase activity in the mechanism of $HgCl_2$-mediated cell death, we added staurosporine before $HgCl_2$ to the cells. Figure 5 shows the viable cell number, measured by FCM, after incubating the cells with $HgCl_2$ plus or minus staurosporine. Less than half (46.60 ± 2.33%)

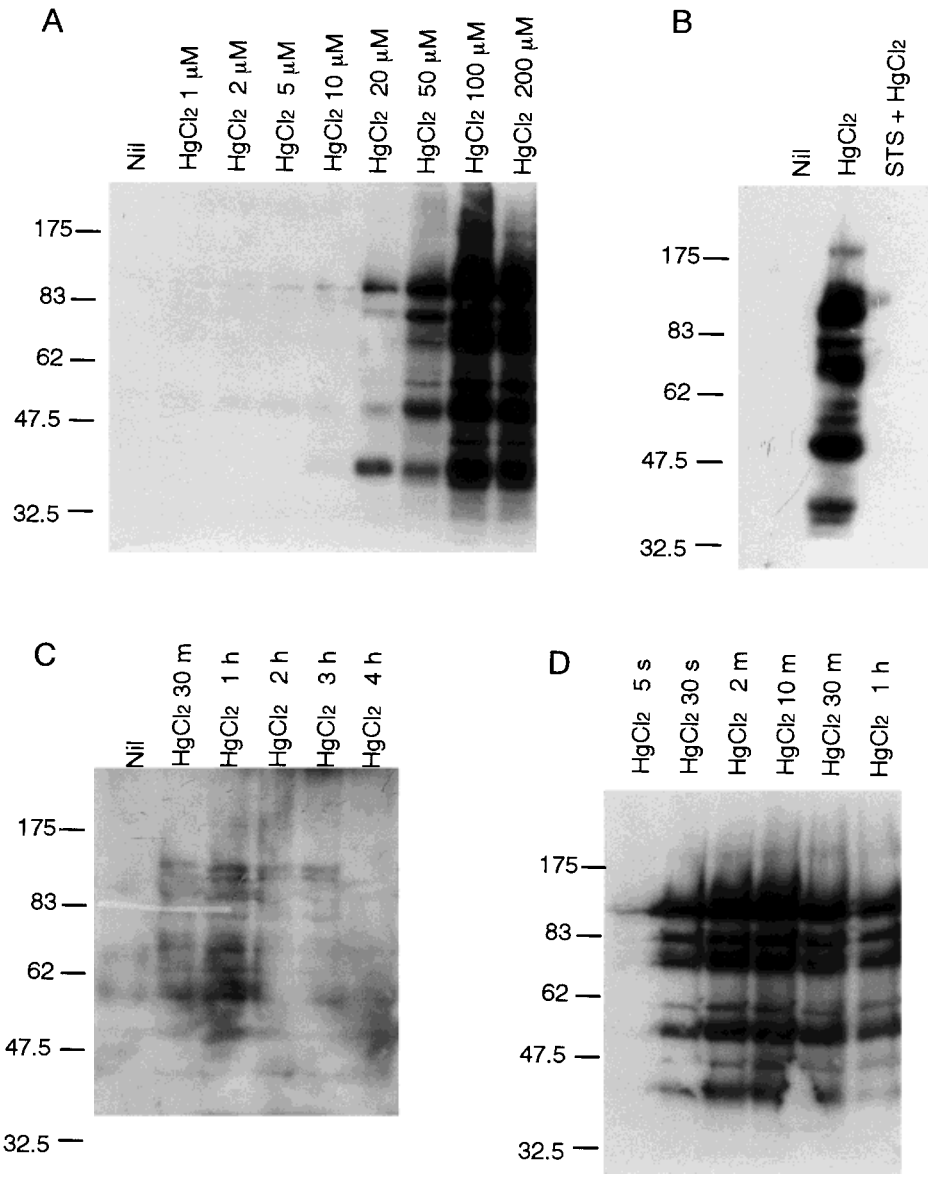

Fig. 4. $HgCl_2$ induces tyrosine phosphorylation of cellular proteins of thymocytes. Thymocytes (10^7/100 µl phosphate buffered saline) were incubated at 37°C with or without 1–200 µM of $HgCl_2$ for 2 min (**A**), 100 µM of $HgCl_2$ for 2 min (**B**), 10 µM of $HgCl_2$ for 30 min–4 h (**C**), or 100 µM of $HgCl_2$ for 5 sec–1 h (**D**). Some cultures were added with staurosporine (STS; 10 µg/ml) and preincubated for 5 min before addition of $HgCl_2$ (right lane in B). The cells were then lysed and subjected to immunoblot assay with anti-phosphotyrosine antibody. Positions of molecular mass markers (kDa) are shown at left.

of the cells remained viable after 10-min incubation with 100 µM of $HgCl_2$ (Fig. 5A), but cell viability was increased to 73.17 ± 3.12% when staurosporine was added prior to $HgCl_2$. From this result it is assumed that there could be a linkage between staurosporine-sensitive kinase activity and $HgCl_2$-mediated rapid cell death. We then performed a time course study to determine the effect of staurosporine in preventing cell death caused by 10 µM of $HgCl_2$ (Fig. 5B). After 30-min incubation, most cells treated with this concentration of $HgCl_2$ were viable. As the incubation period increased, the extent of cell death increased. Staurosporine acted in part to prevent the progression of $HgCl_2$-mediated cell death and maintained a higher cell viability (58.50 ± 6.25%) than did $HgCl_2$ alone (30.80 ± 4.73%) after 4-h incubation. It should be mentioned, however, that staurosporine failed to prevent $HgCl_2$-mediated cell death when cells were incubated for more than 6 h with 10 µM of $HgCl_2$ (data not shown)

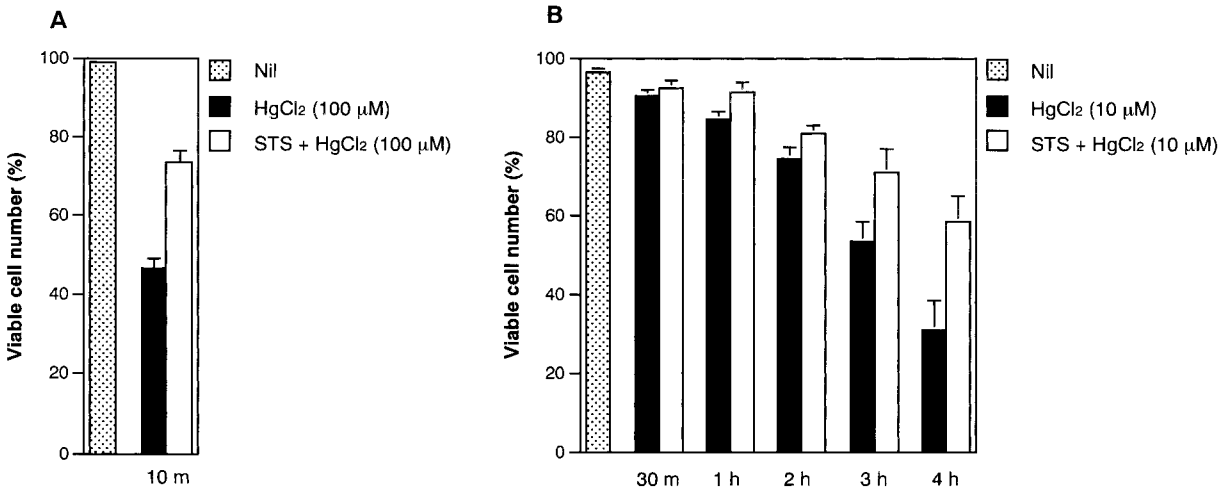

Fig. 5. Partial prevention of $HgCl_2$-induced cell death by staurosporine. Thymocytes (10^6/100 µl phosphate buffered saline) were incubated at 37°C in vitro with or without 100 µM of $HgCl_2$ for 10 min (**A**) or 10 µM of $HgCl_2$ for 30 min–4 h (**B**). The indicated cultures were added with staurosporine (10 µg/ml) before $HgCl_2$. Numbers of viable cells were measured by flow cytometry after staining dead cells with propidium iodide. Each column represents the mean ± SD of triplicate assays. The protective effect of staurosporine on cells exposed to 10 µM of $HgCl_2$ was statistically significant ($P < 0.05$) from 1 h to 4 h. The statistical analysis was done using the Mann-Whitney *U*-test.

because staurosporine itself was cytotoxic under that condition. The protein-tyrosine kinase (PTK) inhibitor genistein [Akiyama et al., 1987] and herbimycin A [Uehara et al., 1985] were much less apt to prevent $HgCl_2$ (10–100 µM)-mediated cell death than was staurosporine (data not shown). Correspondingly, these two inhibitors only marginally reduced the extensive tyrosine phosphorylation of proteins caused by the high concentrations of $HgCl_2$ (data not shown). The similar low effectiveness of these two inhibitors against the high concentrations of Con A-induced signal for protein-tyrosine phosphorylation has been reported previously [Akhand et al., 1997].

We further investigated whether protein kinase inhibitors could also prevent DNA fragmentation induced by 1 µM of $HgCl_2$. Cells were cultured with $HgCl_2$, staurosporine, or both, and the DNAs of the cells were analyzed by gel electrophoresis. In this experiment, however, staurosporine could not prevent $HgCl_2$-induced DNA fragmentation as it itself induced fragmentation (data not shown), corresponding to previous observations [Bertrand et al., 1994; Weil et al., 1996]. This made it difficult to evaluate the effect of staurosporine on the $HgCl_2$-mediated signaling for DNA fragmentation. We then tested the effect of herbimycin A on the $HgCl_2$-induced DNA fragmentation. Herbimycin A effectively inhibited the $HgCl_2$-mediated DNA fragmentation demonstrated by agarose gel electrophoresis (data not shown). These results suggested the common involvement of different levels of protein kinase activity in the two types of $HgCl_2$-mediated active cell death with or without DNA fragmentation.

Partial Characterization of Intracellular Signals Possibly Involved in $HgCl_2$-Mediated Cell Death

We next attempted to characterize in part those intracellular signals that could ultimately cause cell death bypassing DNA fragmentation. The anti-Ptyr immunoblot picture of $HgCl_2$-treated and -untreated cell lysates demonstrated a 52-kDa heavily phosphorylated protein (Fig. 6A-1). Assuming that this could be a 52-kDa Shc protein, we reprobed the same membrane with anti-Shc antibody after stripping out the anti-Ptyr antibody. Two sharp Shc protein bands (p52 and p46) were stained in both $HgCl_2$-treated and -untreated cell lysates (Fig. 6A-2). The 52-kDa Shc protein band completely overlapped the heavily phosphorylated 52-kDa protein that was induced by the treatment with $HgCl_2$. This result suggested that $HgCl_2$ induced heavy phosphorylation of a 52-kDa Shc protein. To confirm that the 52-kDa Shc protein was phosphorylated by treating with $HgCl_2$, we next performed two-dimensional electrophoresis of $HgCl_2$-treated cell lysate and then immunoblotted with anti-Ptyr/anti-Shc antibodies. A dark spot (arrowhead in Fig. 6B-1), showing a 52-kDa molecular mass,

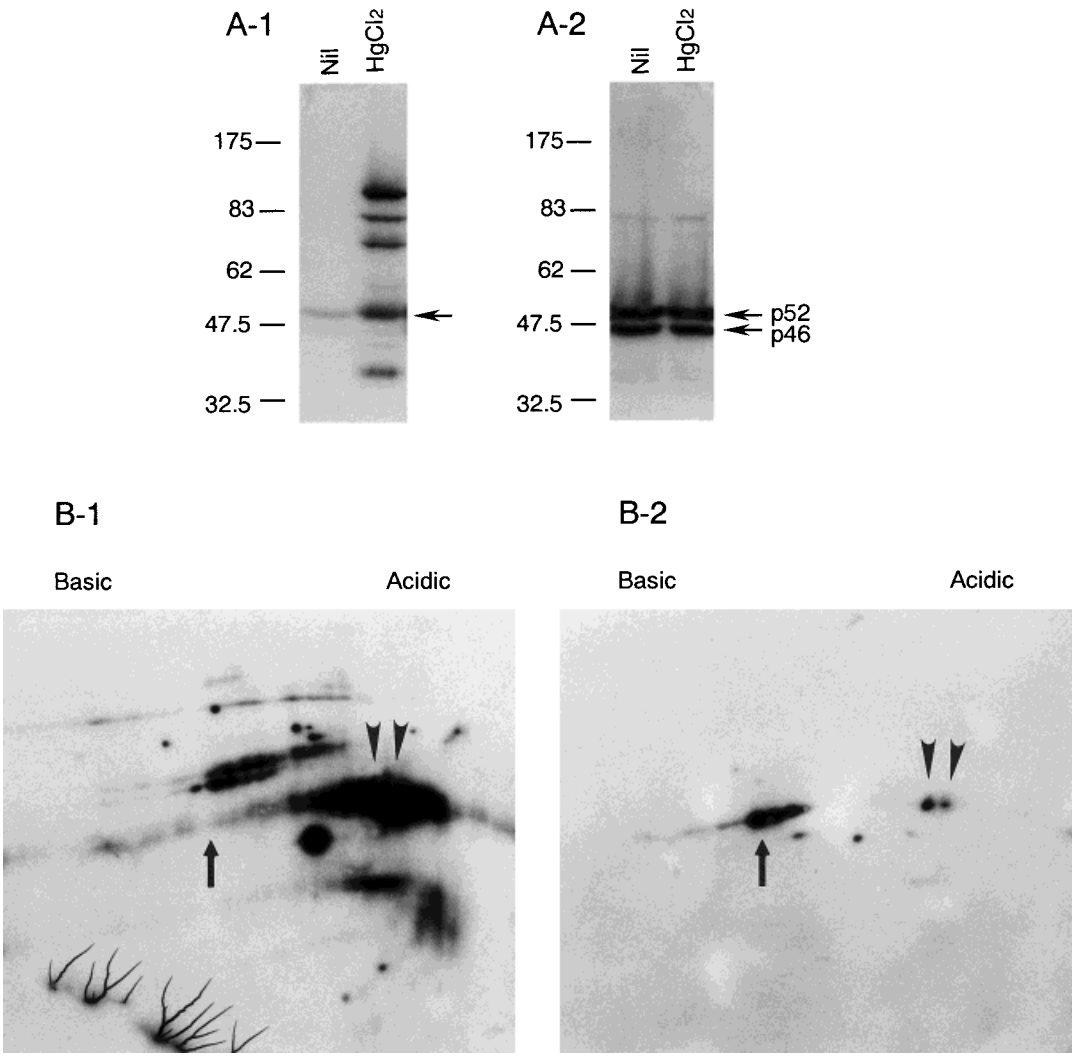

Fig. 6. The $HgCl_2$-generated signal phosphorylates Shc protein. Thymocytes (10^7/100 µl phosphate buffered saline) were incubated at 37°C with or without 100 µM of $HgCl_2$ for 2 min. They were then lysed and subjected to immunoblot assay with anti-phosphotyrosine (anti-Ptyr) antibody (**A-1**), and the same membrane was reprobed with anti-Shc antibody (**A-2**). $HgCl_2$-stimulated cell lysates were also analyzed by two-dimensional electrophoresis for immunoblot assay with anti-Ptyr antibody (**B-1**), and the same membrane was reprobed with anti-Shc antibody (**B-2**). Positions of molecular mass markers (kDa) are shown at the left. A representative of three experiments with consistent results is shown.

developed at the acidic end of the anti-Ptyr blot. The membrane was then reprobed with anti-Shc antibody. The 52-kDa Shc protein was separated according to the pI values and developed two spots (arrowhead in Fig. 6B-2) at the acidic end and one elongated dark spot (arrow in Fig. 6B-2) at the relatively basic end. The two spots at the acidic end completely overlapped the dark spot of the anti-Ptyr blot, suggesting that the 52-kDa Shc protein consists of molecules with different pI values and that the acidic portion of the protein was definitely phosphorylated.

Tyrosine phosphorylation of Shc and its association with the Grb2–Sos complex in response to extracellular signals are thought to be an important mechanism of Ras activation, which in turn regulates MAPK cascades (Blenis, 1993). Therefore, we next examined whether $HgCl_2$ could also promote tyrosine phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) as MAPK family members. Cell lysates obtained from $HgCl_2$-treated and -untreated cells were analyzed by immunoblotting with anti-phospho-MAPK antibody. Figure 7A shows that $HgCl_2$ induced phosphorylation

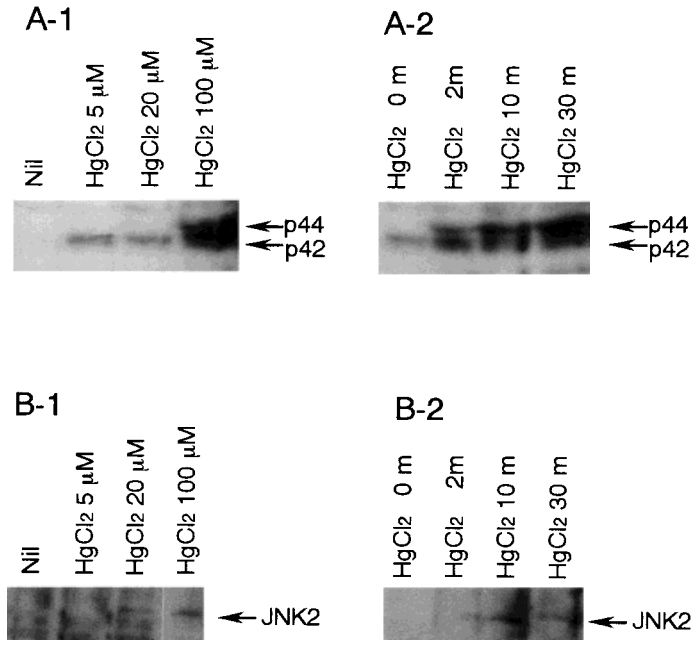

Fig. 7. The $HgCl_2$-generated signal phosphorylates mitogen-active protein kinase (MAPK) family. Thymocytes (10^7/100 µl phosphate buffered) were incubated at 37°C with or without 5–100 µM of $HgCl_2$ for 2 min (**A-1, B-1**) or with 100 µM of $HgCl_2$ for 0–30 min (**A-2, B-2**). They were then lysed and subjected to immunoblot assay with anti-phospho-MAPK extracellular signal-regulated kinases (ERKs; A-1, A-2) and anti-phospho-c-Jun amino terminal kinase (JNK; B-1, B-2) antibody. Positions of ERK1 (p44), ERK2 (p42), and JNK2 are shown at right. A representative of three experiments with consistent results is shown.

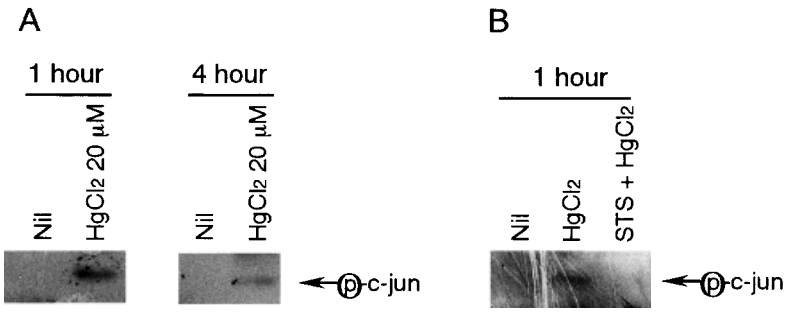

Fig. 8. The $HgCl_2$-generated signal phosphorylates c-Jun at serine 73. Thymocytes (10^7/100 µl phosphate buffered saline) were incubated at 37°C with or without 20 µM of $HgCl_2$ for 1 or 4 h (**A**) or 10 µM of $HgCl_2$ for 1 h (**B**). Staurosporine (10 µg/ml) was added to the right lane before addition of $HgCl_2$ (B). They were then lysed and subjected to immunoblot assay with anti-phospho-c-Jun antibody. Position of phospho-c-Jun protein is shown at right. A representative of three experiments with consistent results is shown.

of both ERK1 (p44) and ERK2 (p42) in a concentration- and time-dependent manner. High concentration (100 µM) of $HgCl_2$ induced phosphorylation of both ERK1 and ERK2 within 2 min of stimulation, and the intensity of phosphorylation increased up to 30 min (Fig. 7A-2). In contrast, lower concentrations (5–20 µM) of $HgCl_2$ preferentially caused phosphorylation of ERK2 (Fig. 7A-1). We then tested the effect of $HgCl_2$ on c-Jun amino terminal kinase (JNK), a distant relative of MAPK group. As shown in Figure 7B, $HgCl_2$ induced low-level phosphorylation of JNK2 (p54). Compared with the no-stimulation control, 100 µM and 20 µM (less evidently) but not 5 µM of $HgCl_2$ induced detectable phosphorylation of JNK2 in 20 min (Fig. 7B-1). The time course study with 100 µM of $HgCl_2$ showed a phosphorylation peak of JNK2 at 10 min (Fig. 7B-2).

It has been reported that MAP family kinases may ultimately phosphorylate c-Jun at specific serine residues and thus regulate the activity of c-Jun [Pulverer et al., 1991; Hibi et al., 1993; Kyriakis et al., 1994]. To investigate the possible role of $HgCl_2$ in c-Jun phosphorylation, we treated the cells with or without $HgCl_2$, and the cell lysate was immunoblotted with antibody specific to serine 73 phosphorylated c-Jun. As shown in Figure 8, 10–20 µM of $HgCl_2$ induced phosphorylation of c-Jun at serine 73 in 1 and 4 h of incubation, and the level of phosphorylation at 1 h was higher than the level at 4 h. We also tested the effect of staurosporine on the $HgCl_2$-mediated c-jun phosphorylation. The result (Fig. 8B) showed that staurosporine completely inhibited $HgCl_2$-mediated c-Jun phosphorylation.

DISCUSSION

This study reports the two types of signal level-dependent cell death with or without DNA fragmentation in Hg^{2+}-treated thymic T-lym-

phocytes. We found that high concentrations (10–100 μM) of $HgCl_2$ caused rapid cell death without DNA fragmentation, whereas a low concentration (1 μM) induced typical apoptosis with DNA fragmentation. Different concentrations of Hg^{2+} were shown to provoke corresponding levels of tyrosine phosphorylation of cellular proteins (Fig. 4). In the present study, we have demonstrated that the Hg^{2+}-induced cell death without DNA fragmentation, which was apparently necrotic due to direct cytotoxicity of Hg^{2+}, also required a protein phosphorylation-linked signal. This finding was demonstrated by the partial prevention of the Hg^{2+}-mediated cell death with a powerful protein kinase inhibitor (staurosporine; Fig. 5), which greatly reduced the level of Hg^{2+}-mediated, otherwise extensive protein-tyrosine phosphorylation (Fig. 4B). Together with the fact that induction of DNA fragmentation by a low concentration (1 μM) of Hg^{2+} was inhibited by a milder protein-tyrosine kinase inhibitor (herbimycin A), it is likely that qualitatively similar but quantitatively different protein phosphorylation-linked signals operate for inducing both types of cell death with or without DNA fragmentation.

The signal triggered by high concentrations (10 μM or more) of Hg^{2+} was shown to induce heavy tyrosine phosphorylation of Shc protein and MAP family kinases (ERK1 and ERK2 and less extensively JNK2) and serine 73 phosphorylation of c-Jun. The protein phosphorylation-linked signal has been shown to play a key role in controlling cellular proliferation and differentiation [Weiss, 1993; Hunter, 1995; Appleby et al., 1996], but its role in cell death is not always clear. Several lines of evidence suggest that phosphorylated Shc protein associates with Grb2–Sos and thus is implicated in the Ras signaling pathway in mammalian cells [Rozakis-Adcock et al., 1992; Egan et al., 1993; Hibi et al., 1993]. Shc has also been shown to function in signaling pathways through T- and B-cell antigen receptors [Ravichandran et al., 1993; Saxton et al., 1994], interleukin-2 (IL-2) receptors [Burns et al., 1993; Ravichandran and Burakoff, 1994], and G-protein-coupled receptors [van Biesen et al., 1995], all of which lead to Ras activation. Activation of Ras through intracellular protein–protein interactions between phosphorylated Shc and Grb2–Sos can directly influence the downstream serine/threonine kinases, or MAPKs [Blenis, 1993]. In the present study, we have shown that ERK1 and ERK2 of the MAPK family are phosphorylated at the tyrosine residue by the action of Hg^{2+} in a concentration- and time-dependent manner (Fig. 7). JNK2, which is a distant member of the MAPK family, was also phosphorylated by Hg^{2+} less extensively. ERK1, ERK2, and JNK2 could be involved in the phosphorylation of the specific serine residue of c-Jun [Pulverer et al., 1991; Kyriakis et al., 1994]. We also found that c-jun was phosphorylated at serine 73 when cells were stimulated with Hg^{2+}, and both c-jun phosphorylation and cell death were prevented by the treatment of cells with staurosporine before Hg^{2+}. Thus, our results suggest the involvement of high-level phosphorylation of Shc–MAPK–c-Jun in the Hg^{2+}-mediated cell death that bypasses DNA fragmentation. The molecular mechanism of cell death bypassing DNA fragmentation following such signal transduction, however, remains to be studied further.

Nakashima et al. [1994] showed that a very low quantity of signal triggered by 1 μM of Hg^{2+} in MEM (because of the presence of cysteine in MEM, the free form of Hg^{2+} is calculated as less than one-tenth the total amount of added Hg^{2+}) affected mature splenic T-lymphocytes for enhanced proliferation and IL-2 production. That result is contrast to the present result that 10–100 μM of Hg^{2+} in PBS and 1 μM of Hg^{2+} in PBS cause rapid and delayed types of death of immature T-lymphocytes, respectively. It is not known at present how the higher concentration of Hg^{2+}-mediated signals for cell death are involved in the mechanism of Hg^{2+}-induced autoimmune disorders in rodents [Goldman et al., 1991; Nakashima et al., 1994]. Cell death caused by a higher concentration of Hg^{2+} may remove some regulatory cells that prevent autoimmune disorder induction.

Ligand-mediated crosslinkage or dimerization of receptors may transduce the signal for apoptosis in thymic T-lymphocytes [Shi et at., 1989; Smith et al., 1989]. Nakashima et al. [1993] also demonstrated that co-crosslinking of CD3 and Thy-1 delivers the signal for DNA fragmentation in thymic T-lymphocytes, thereby enhancing tyrosine phosphorylation of p40 and other cellular proteins. Hg^{2+} has a high affinity for sulfhydryl groups of proteins, and Nakashima et al. [1994] reported that Hg^{2+} aggregated cell surface CD4, CD3, CD45, and Thy-1 on thymic T-lymphocytes by bridging sulfhydryl groups of the respective molecules and activated nonreceptor protein-tyrosine kinases

such as $p56^{lck}$ in T-lymphocytes and $p60^{c\text{-}src}$ in NIH3T3 cells [Nakashima et al., 1994; Pu et al., 1996]. Therefore, the Hg^{2+}-mediated signals for the two types of active cell death could relate to some signals transduced by crosslinking the receptors with natural ligands to different extents. Supporting this view are reports indicating that the overall pattern of tyrosine phosphorylation of cellular proteins in the Hg^{2+}-treated T-lymphocytes was similar in quality to that in anti-CD3-stimulated or Con A-treated T-lymphocytes [Nakashima et al., 1991, 1993, 1994; Akhand et al., 1997].

Positive and negative selections of immature T-lymphocytes occur in the thymus following the signal transduction guided by TCR and other receptors [Nossal, 1994; von Boehmer, 1994]. Difference in quantity rather than quality of the delivered signal, which depends on the avidity of T-cell receptor (TCR) for its ligand, may play a key role in switching between positive and negative selections [Allen 1994; Ashton-Richardt et al., 1994; Sebzda et al., 1994]. Akhand et al. [1997] showed that the level of the protein phosphorylation-linked signal triggered by Con A, a multireceptor crosslinker, determines growth versus death of thymic T-lymphocytes. In accordance with that report, our present results suggest that the level of the Hg^{2+}-mediated protein phosphorylation-linked signal may also determine the fate of thymic T-lymphocytes for either of the two types of cell death with or without DNA fragmentation. Only a lower-level signal, which should still be higher than that needed for cell growth induction, induces DNA fragmentation before cell death, whereas a high-level signal causes rapid cell death bypassing DNA fragmentation. The results of the present study imply that DNA fragmentation is not a necessary prerequisite for all receptor-mediated cell death linked to negative selection of thymic T-lymphocytes. Thus, there may be an atypical apoptosis, in which cell death occurs without DNA fragmentation as in typical apoptosis, and it is this occurrence that may trigger rapid negative selection of extensively autoreactive thymic T-lymphocytes into which a high-level signal would be delivered.

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