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Cadmium-Induced Germline Apoptosis in *Caenorhabditis elegans*: The Roles of HUS1, p53, and MAPK Signaling Pathways

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The transition metal cadmium (Cd) has been shown to induce apoptosis in a variety of cell lines and tissues. Caspase activation of the tumor suppressor gene p53 and mitogen-activated protein kinase (MAPK) signaling cascades have been reported to be involved in Cd-induced apoptosis. However, the underlying pathways of Cd-induced apoptosis have not been clearly elucidated in the *in vivo* systems, primarily for the lack of appropriate animal models. The nematode *Caenorhabditis elegans* has been shown to be a good model to study basic biological processes, including apoptosis. In this study, we used the mutated alleles of *C. elegans* homologs of known mammalian genes that are involved in regulation of apoptosis. Sublethal doses of Cd exposure increased *C. elegans* germline apoptosis in a dose- and time-dependent manner. The loss-of-function mutations of DNA damage response (DDR) genes HUS1 and p53 exhibited significant increase in germline apoptosis under Cd exposure, and the depletion of p53 antagonist ABL1 significantly enhanced apoptosis. Cd-induced apoptosis was blocked in the loss-of-function alleles of both c-Jun N-terminal kinase (JNK) and p38 MAPK cascades, which behaved normally under γ-irradiation. Our findings implicate that both JNK and p38 MAPK cascades participate in Cd-induced apoptosis. Together, the results of this study suggest the nonessential roles of the DDR genes *hus1* and p53 in Cd-induced germline apoptosis and that the apoptosis occurs through the ASK1/2-MKK7-JNK and ASK1/2-MKK3/6-p38 signaling pathways in a caspase-dependent manner. Finally, our study demonstrates that *C. elegans* is a mammalian *in vivo* substitute model to study the mechanisms of Cd-induced apoptosis.

Key Words: **cadmium; *Caenorhabditis elegans*; apoptosis; HUS1; p53; MAPK.**

Apoptosis is a highly controlled cellular process that occurs metabolically and genotoxically in all multicellular organisms. The cellular response to genotoxic stress involves the integration of multiple signals that dictate whether a cell lives or dies. Genotoxic exposure triggers DNA damage–dependent signals, such as the checkpoint proteins HUS1 and p53 that are involved in DNA damage repair and apoptosis. Such exposure can also trigger DNA damage–independent signals, such as the mitogen-activated protein kinase (MAPK) signaling cascades, that are involved in early cellular signal transduction (Roos and Kaina, 2006; Stergiou and Hengartner, 2004).

The transition metal cadmium (Cd) is commonly found in both the aquatic and terrestrial environments. Epidemiologic data suggest that chronic exposure to Cd is associated with multiple diseases, including cancer (Nawrot *et al.*, 2006). Cd has been shown to induce apoptosis in various experimental systems (Habeebu *et al.*, 1998; Harstad and Klaassen, 2002), but the mechanisms of Cd-induced apoptosis have not been well characterized. Caspase-dependent apoptosis induced by Cd was found in the human lymphoma cell line U937 (Galan *et al.*, 2000) and the promyelocytic cell line HL-60 (Kondoh *et al.*, 2002). However, pretreatment of primary cultures of rat hepatocytes with caspase-3–specific inhibitor, Ac-DEVD-CHO, did not prevent apoptosis induced by Cd, suggesting caspase-independent apoptosis (Pham *et al.*, 2006). Several reports have associated Cd-induced apoptosis with tumor suppressor gene p53 in primary epithelial lung cells (Låg *et al.*, 2002) and in normal human prostate epithelial cells (Achanzar *et al.*, 2000). Moreover, MAPK signaling cascades have also been shown to be involved in Cd-induced apoptosis in many experimental systems. It was found that c-Jun N-terminal kinase (JNK) and p38 are remarkably activated at high cytotoxic doses of Cd in the human CL3 cell line. Furthermore, the addition of SB202190, a p38 inhibitor, significantly suppressed Cd-induced cell death (Chuang *et al.*, 2000). Cd-activated p38 MAPK signaling pathways were observed in U937 human promonocytic leukemia cells (Galan *et al.*, 2000) and 9L rat brain tumor cells (Hung *et al.*, 1998). A recent report showed that JNK signaling pathways were required for caspase-3–dependent apoptosis induced by Cd due to less DNA fragmentation and caspase-3 activity detected in $jnk^{-/-}$ mouse embryonic fibroblasts cells incubated in 10 and 25μM

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Cd (Papadakis *et al*., 2006). These results provide us with useful information in understanding the mechanisms of Cd genotoxicity. However, most of the studies were based on *in vitro* experimental systems, which do not fully reflect the mechanisms of Cd-induced apoptosis in a living animal. To better understand the potential adverse effects of Cd exposure on both humans and wildlife, the employment of *in vivo* animal models is indispensable. Due to the lack of appropriate gene knockout animal models, the majority of the signaling pathways involved in Cd-induced apoptosis have not been reported in the *in vivo* animal systems.

The nematode *Caenorhabditis elegans* has been widely used in developmental biology and genetics studies because of its ease of use, short life span, cellular simplicity, and genetic manipulability. Previous studies have shown that *C. elegans* is a good model to study genotoxic and nongenotoxic stress–induced apoptosis (Hofmann *et al*., 2002; Salinas *et al*., 2006). We recently found that *C. elegans* is a good *in vivo* model to study the genotoxic effects of arsenite exposure and found that arsenite-induced germline apoptosis is mediated by the production of reactive oxygen species (Wang *et al*., 2007). In *C. elegans*, several distinct apoptosis pathways have been characterized in germline. Under genotoxic stress, germline apoptosis requires the checkpoint protein HUS-1 and the *C. elegans* p53 protein CEP-1, and the caspase protein CED-3 is absolutely required for the killing process (Stergiou and Hengartner, 2004). Phylogenetic analysis indicates that *C. elegans* MAPK signaling components are highly conserved in mammals (Caffrey *et al*., 1999). Moreover, some of the homozygous deletions of MAPK pathway components, such as p38, are embryonic lethality in mice (Ihle, 2000). Alternatively, most of the homozygous deletions of the checkpoint and MAPK pathways in *C. elegans* are viable. Thus, *C. elegans* provides the few accessible gene knockout *in vivo* models for analysis of the pathways that are involved in Cd-induced apoptosis. By using gene knockout *C. elegans*, we show here that Cd-induced germline apoptosis is dependent on the MAPK signaling pathways, while the checkpoint gene HUS1 and the tumor suppressor gene p53 were both nonessential.

MATERIALS AND METHODS

Worm strains and reagents. Strains used in this study were Bristol N_2 wild type, *ced-3*(*n717*), *cep-1(w40), hus-1(op141)*, *abl-1(ok171)*, *nsy-1*(*ag3*), *mek-1*(*ks54*), *jnk-1(gk7)*, *sek-1(ag1)*, *pmk-1(km25*), and *pmk-3(ok169)*, which were provided by the Caenorhabditis Genetics Center funded by the National Institutes of Health National Center for Research Resources. Worms were cultured at 20°C in petridishes on nematode growth medium (NGM) seeded with *Escherichia coli* strain OP50 as food. To obtain synchronized cultures, gravid hermaphrodites were lysed in an alkaline hypochlorite solution as described previously (Sulston and Hodgkin, 1988). $CdCl_2$ is a commercial product of Sigma Chemical (St Louis, MO), and acridine orange (AO) was purchased from Molecular Probes (Eugene, OR).

Worm treatment. The procedures for animal handling and chemical exposure were conducted as described previously (Williams and Dusenbery, 1990). Briefly, $CdCl_2$ was dissolved in distilled water and diluted to final concentrations as indicated in K medium (52mM NaCl and 32mM KCl), containing *E. coli* strain OP50 as a food source. Aliquots of the prepared K medium were dispensed to Costar 12-well tissue plates. For Cd exposure, 20 synchronized young adult hermaphrodites were picked and transferred into a plate containing K medium with or without test solutions. Worms were grown at 20°C and removed at 6, 12, and 24 h after exposure for further analysis. For different treatments, worms were exposed to 50μM dose of Cd for 12 h, and germ cell corpses were scored after AO staining. For ionizing irradiation, synchronized young adult hermaphrodites were picked into 3.5-mm NGM petri dishes and treated with a ^{60}Co irradiator, and germ cell corpses were scored 15 h after irradiation.

Apoptosis assay. Apoptotic germ cells were measured by AO vital staining using a modified procedure derived from Kelly *et al*. (2000). Briefly, worms at indicated time points were picked from test wells, transferred into a Costar 24-well plate containing 500 μl of 25 μg/ml AO and OP50 in M9 buffer, and then incubated for 60 min at 20°C. The addition of bacteria in the buffer facilitated the uptake of dye. Animals were allowed to recover for 45 min on bacterial lawns and then were mounted onto agar pads on microscope slides in 60 μg/ml levamisole in M9, after which they were examined with an Olympus 1×71 microscope. The apoptotic cells appeared yellow or yellow-orange, representing increased DNA fragmentation, while intact cells were uniformly green in color (Supplemental Fig. 1). In most conditions, only one gonad arm could be scored because the autofluorescence of the pharynx shaded the gonad arm near the pharynx.

Mitotic cell nuclei determination. For mitotic cell nuclei determination, 20 worms were picked from test wells at 24 h, suspended in 1 μl distilled water, fixed with Carnoy's fixative (six parts ethanol, three parts chloroform, and one part glacial acid), air dried, and stained with a small drop of 2 μg/ml 4′6-diamidino-2-phenylindole in M9 buffer as previously described (Gartner *et al*., 2000). The nuclei in the mitotic zone of the germline were counted under a fluorescence microscope (Supplemental Fig. 2).

Data analysis. All values were expressed as means ± SE, and statistical differences ($p < 0.05$) between different concentrations or different time points were tested using ANOVA followed by Turkey's multiple comparison test or tested by two-tailed Student's *t*-tests.

RESULTS

Cd Exposure Induces Germ Cell Death

In the *C. elegans* germline, two U-shaped gonad tubes join proximally at a common uterus. In the adult hermaphrodite, germ cells undergo mitotic proliferation distally and serve as stem cell population. During their passage through a "transition zone," they stop dividing and initiate meiosis. The so-called physiological apoptosis and stress-induced germline apoptosis occurs at the loop of the gonad (Gartner *et al*., 2000). To determine whether Cd exposure can induce germ cell apoptosis, we exposed synchronized young adult worms to 0.0, 1.0, 10.0, 50.0, and 100.0μM doses of $CdCl_2$ for 6, 12, or 24 h. As shown in Figure 1, the background apoptotic cells per gonad arm were 4.93 ± 0.30 and 5.07 ± 0.25 at 6 and 12 h, respectively. However, with Cd exposure, germ cell corpses per gonad arm increased significantly. At the dose of 10μM, apoptotic cells were 5.9 ± 0.28 and 7.3 ± 0.32 per gonad arm at 6 and 12 h ($p < 0.05$), respectively. With dosages increase, the apoptotic cells per gonad arm increased progressively

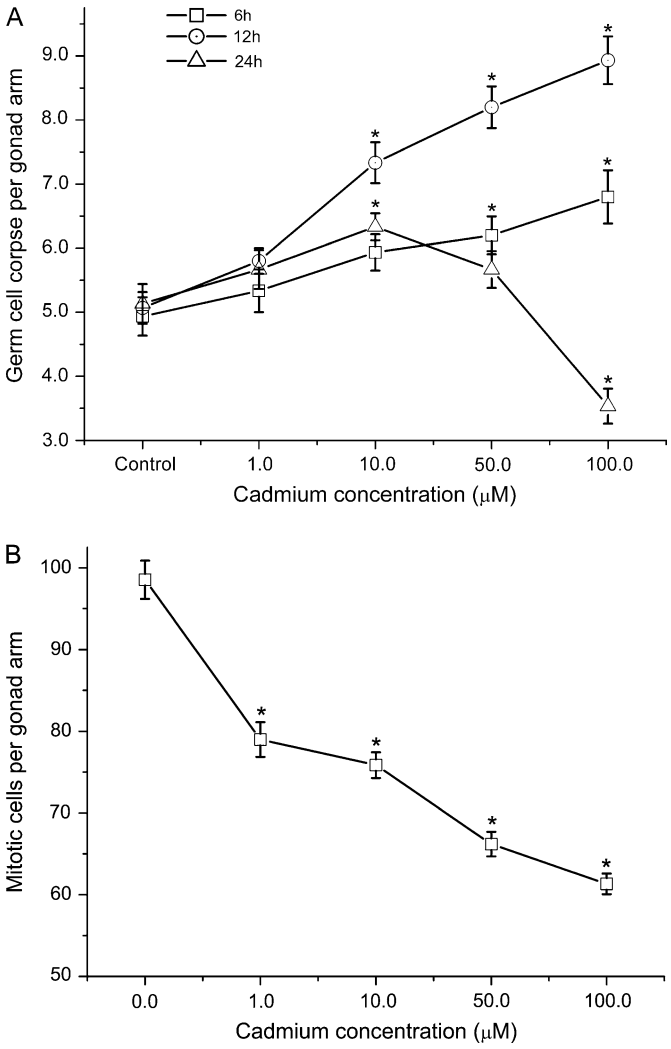

FIG. 1. Cd induces *C. elegans* germline cell apoptosis and inhibits germ cell proliferation. (A) Synchronized young adult hermaphrodites were treated in K medium with the indicated concentrations of Cd for 6, 12, and 24 h; apoptotic cells were scored after staining with AO. (B) Synchronized young adult hermaphrodites were treated in K medium with the indicated concentrations of Cd for 24 h; mitotic cells were scored after 4′6-diamidino-2-phenylindole staining. All values were represented mean ± SE, $n = 15$, *represents $p < 0.05$.

during exposure. These results indicated that Cd-induced *C. elegans* germline apoptosis increased in a dose- and time-dependent manner. At the time point of 24 h, it was interesting to observe that the apoptotic cells stopped increasing at the dose of 50μM and decreased significantly at the dose of 100μM when compared to the untreated control (Fig. 1A; $p < 0.05$). At the dose of 100μM for 24 h exposure, the decrease in germ cell corpses seemed to be the cause of decreased germ cells. To confirm our hypothesis, we counted the number of germ cells after 24 h of Cd exposure. At the dose of 100μM, as expected, the mitotic nuclei per gonad arm (Fig. 1B; $p < 0.05$) and total germ cells (data not shown) decreased dramatically.

The Roles of CEP-1 and HUS-1 in Cd-Induced Germline Apoptosis

To confirm whether Cd-induced cell death was programmed cell death, we exposed the worms containing the loss-of-function allele *ced-3* (*n717*), a caspase mutant that blocks all of the programmed cell death in *C. elegans*, to 50μM of Cd for 12 h. The germ cell corpse did not increase after Cd treatment (Table 1; $p > 0.05$), implying that Cd-induced germ cell death was programmed cell death nature. Since *hus-1* is a conserved checkpoint gene that is required for DNA damage–induced apoptosis (Hofmann *et al.*, 2002), we exposed the strain *hus-1(op241)* to 50μM of Cd for 12 h. Table 1 showed that germline apoptosis increased significantly in the loss-of-function mutation *hus-1* (Table 1; $p < 0.05$), and the apoptotic cells per gonad arm increased 1.24-fold in *hus-1(op241)* and 1.63-fold in N2 compared to the untreated control. Two strains containing the loss-of-function alleles *cep-1(w40)*, a *C. elegans* p53 homolog, and *abl-1(ok171)*, a conserved nonreceptor tyrosine kinase that antagonizes *cep-1* (Deng *et al.*, 2004), were exposed to 50μM of Cd for 12 h. Both strains showed a significant increase in germline apoptosis (Table 1; $p < 0.05$). The apoptotic cells per gonad arm increased 1.45-fold and 2.1-fold in *cep-1(w40)* and *abl-1(ok171)* compared to the untreated control, respectively. Furthermore, germline apoptosis induced by Cd in the strains *hus-1* and *cep-1* were lower than that of N2, and the loss-of-function of *abl-1* exhibited the promoting effects (Table 1; $p < 0.05$). These results implicate the nonessential roles of *hus-1* and *cep-1* in Cd-induced germline apoptosis. For a comparison, we irradiated the worms with 120 Gy γ-ray and scored germ cell corpse after 15 h. As shown in Table 1, γ-irradiation caused significant germline apoptosis in both N2 and *abl-1(ok171)* but was blocked in the mutated strains *cep-1(w40)*, *hus-1(op241)*, and *ced-3(n717)*. These results indicate that the functions of *hus-1* and *cep-1* in Cd- and ionizing irradiation–induced apoptosis are different, while the *ced-3* is essential for both of the processes.

Cd-Induced Apoptosis is Dependent on JNK Signal Cascades

In *C. elegans*, the *nsy-1* encodes a MAPK kinase kinase (MAPKKK), *mek-1* is the member of MAPK kinase (MAPKK), and *jnk-1* is the member of the JNK homologs. The loss-of-function of these genes in *C. elegans* exhibits hypersensitivity to heavy metals and starvation (Koga *et al.*, 2000; Kim *et al.*, 2004). To test the roles of JNK signal cascades in Cd-induced germline apoptosis, the *nsy-1*, *mek-1*, and *jnk-1* loss-of-function strains *nsy-1(ag3)*, *mek-1(ks54)*, and *jnk-1(gk-7)* were exposed to 50μM of Cd for 12 h. Under Cd exposure, germ cell corpses in *nsy-1(ag3)*, *mek-1(ks54)*, and *jnk-1(gk-7)* strains were 3.6 ± 0.27, 3.2 ± 0.27, and 3.4 ± 0.27 per gonad arm versus the baseline 3.3 ± 0.23, 3.0 ± 0.17, and 2.9 ± 0.18 (Table 2; $p > 0.05$), respectively. In comparison to the 1.63-fold increase in germ cell apoptosis in N2 after Cd

TABLE 1
Roles of *ced-3*, *hus-1*, *cep-1*, and *abl-1* in Cd-Induced Germline Apoptosis

Genotype	Control	Cd	–IR	+IR
N2	4.4 ± 0.18 (25)	7.09 ± 0.26 (22), $p = 1.73 \times 10^{-8}$	4.6 ± 0.26 (21)	7.1 ± 0.33 (22), $p = 7.86 \times 10^{-6}$
ced-3(n717)	0.14 ± 0.08 (22)	0.15 ± 0.09 (24), $p = 0.43$	0.1 ± 0.07 (20)	0.16 ± 0.12 (19), $p = 0.72$
hus-1(op241)	3.9 ± 0.24 (21)	4.8 ± 0.18 (20), $p = 5.40 \times 10^{-3}$	3.3 ± 0.2 (18)	3.9 ± 0.25 (19), $p = 0.11$
cep-1(w40)	4.5 ± 0.21 (17)	6.5 ± 0.33 (25), $p = 1.09 \times 10^{-4}$	3.5 ± 0.26 (19)	3.8 ± 0.31 (15), $p = 0.52$
abl-1(ok171)	6.0 ± 0.30 (22)	12.5 ± 0.78 (24), $p = 8.95 \times 10^{-7}$	6.1 ± 0.28 (23)	9.2 ± 0.37 (20), $p = 2.18 \times 10^{-6}$

Note. Synchronized young adult hermaphrodites were treated in K medium with 50μM of Cd for 12 h, and apoptotic cells were scored after AO staining. For γ-irradiation, synchronized worms were irradiated with a ^{60}Co irradiator, and germ cell corpses were scored 15 h after irradiation. Apoptotic cells were shown in mean ± SE per gonad arm. *p* values were derived from comparison with untreated control under same conditions. Number of gonads observed (sometimes two per animal) was shown in parenthesis. –IR, without irradiation; +IR, with 120 Gy γ-irradiation. As there were no statistical differences in the number of apoptotic cells for N2 or *nsy(ag3)* in the separate experiments, for comparison convenience, the N2 values presented in the three tables and *nsy-1* values presented in Tables 2 and 3 were the same.

exposure, the germline apoptosis in *nsy-1(ag3)*, *mek-1(ks54)*, and *jnk-1(gk-7)* increased only 1.09-, 1.06-, and 1.17-fold, respectively. As it has been described above, the elevated dose of Cd inhibited germline apoptosis, but we failed to observe germline apoptosis increase at the less toxic doses of 10μM after a 12-h exposure (data not shown). These results indicated that the JNK signal cascades were required for Cd-induced germ cell death, and the loss-of-function of any of the three genes conferred the apoptosis blockage.

Cd-Induced Apoptosis is Dependent on p38 Signaling Pathways with the Exception of pmk-3

The *C. elegans sek-1* is a member of MAPKK, and *pmk-1* and *pmk-3* are the p38 MAPK homologs. To test whether Cd-induced germ cell apoptosis was p38 dependent, we scored germ corpses after exposing the worms to 50μM of Cd for 12 h. We failed to score an increase in germ cell corpses in the strains *sek-1(ag1)* and *pmk-1(km25)* after Cd exposure (Table 3, $p > 0.05$). The germ cell corpses were 5.0 ± 0.32 and 4.3 ± 0.29 per gonad arm compared with the untreated control (4.5 ± 0.27 and 4.4 ± 0.28, respectively) and N2 (7.09 ± 0.26 at 50μM of Cd) (Table 3). These results suggested that *sek-1* and *pmk-1* were required for Cd-induced germline apoptosis. As shown in Table 3, the loss-of-function of *pmk-3(ok169)* does not prevent Cd-induced germline apoptosis ($p < 0.05$), and germ cell corpses increase 1.26-fold compared to the untreated control. We then compared the germline apoptosis induced by Cd with those that were subjected to 120 Gy γ-irradiation. All the JNK and p38 MAPK cascades loss-of-function strains showed normal germline apoptosis similar to that of N2 after irradiation, indicating that the roles of intracellular signaling pathways in Cd-induced apoptosis are different from that of ionizing irradiation (Tables 2 and 3; $p < 0.05$).

DISCUSSION

It has been suggested that *C. elegans* could substitute for vertebrate organisms in predicting mammalian acute lethality from metals. Exposing *C. elegans* to Cd causes reduced brood size, shortened life span, and abnormal behavior (Anderson *et al*., 2004; Harada *et al*., 2006). In the present study, we show that Cd exposure causes germline apoptosis in a time- and dose-dependent manner, indicating that *C. elegans* could be used as an *in vivo* model to study the pathways involved in Cd-induced apoptosis. Cd-induced apoptosis has been shown in a variety of cell lines and tissues, but the underlying pathways involved in the process have not been clearly defined in the *in vivo* mammalian systems. To address this problem, we used the mutated alleles of *C. elegans* of known mammalian genes

TABLE 2
Roles of JNK Cascades in Cd-Induced Germline Apoptosis

Genotype	Control	Cd	–IR	+IR
N2	4.4 ± 0.18 (25)	7.09 ± 0.26 (22), $p = 1.73 \times 10^{-8}$	4.6 ± 0.26 (21)	7.1 ± 0.33 (22), $p = 7.86 \times 10^{-6}$
nsy-1(ag3)	3.3 ± 0.23 (21)	3.6 ± 0.27 (21), $p = 0.45$	3.2 ± 0.17 (22)	4.7 ± 0.25 (26), $p = 1.41 \times 10^{-4}$
mek-1(ks54)	3.0 ± 0.17 (23)	3.2 ± 0.27 (23), $p = 0.45$	3.7 ± 0.21 (25)	4.5 ± 0.23 (25), $p = 5.72 \times 10^{-4}$
jnk-1(gk7)	2.9 ± 0.18 (23)	3.4 ± 0.17 (23), $p = 0.12$	2.3 ± 0.26 (22)	3.2 ± 0.22 (19), $p = 0.025$

Note. Worms were treated as described in the ''Materials and Methods'' section. Apoptotic cells were shown in mean ± SE per gonad arm. *p* values were derived from comparison with untreated control under same conditions. Number of gonads observed (sometimes two per animal) was shown in parenthesis. –IR, without irradiation; +IR, with 120 Gy γ-irradiation.

TABLE 3
Roles of p38 Cascades in Cd-Induced Germline Apoptosis

Genotype	Control	Cd	–IR	+IR
N2	4.4 ± 0.18 (25)	7.09 ± 0.26 (22), $p = 1.73 \times 10^{-8}$	4.6 ± 0.26 (21)	7.1 ± 0.33 (22), $p = 7.86 \times 10^{-6}$
nsy-1(ag3)	3.3 ± 0.23 (21)	3.6 ± 0.27 (21), $p = 0.45$	3.2 ± 0.17 (22)	4.7 ± 0.25 (26), $p = 1.41 \times 10^{-4}$
sek-1(ag1)	4.5 ± 0.27 (20)	5.0 ± 0.32 (21), $p = 0.26$	3.7 ± 0.21 (19)	4.5 ± 0.23 (24), $p = 3.27 \times 10^{-3}$
pmk-1(km25)	4.4 ± 0.28 (22)	4.3 ± 0.29 (20), $p = 0.83$	2.4 ± 0.18 (19)	3.4 ± 0.18 (23), $p = 4.78 \times 10^{-4}$
pmk-3(ok169)	5.0 ± 0.20 (22)	6.2 ± 0.37 (23), $p = 7.16 \times 10^{-3}$	4.4 ± 0.3 (21)	5.9 ± 0.30 (23), $p = 1.27 \times 10^{-3}$

Note. Worms were treated as described in the ''Materials and Methods'' section. Apoptotic cells were shown in mean ± SE per gonad arm. p values were derived from comparison with untreated control under same conditions. Number of gonads observed (sometimes two per animal) was shown in parenthesis. –IR, without irradiation; +IR, with 120 Gy γ-irradiation.

that are involved in apoptosis regulation. We found that DNA damage response (DDR) genes *hus-1* and p53 are not essential in Cd-induced germline apoptosis, the depletion of p53 antagonist ABL1 promotes apoptosis, and apoptosis occurs through both the ASK1/2-MKK7-JNK and ASK1/2-MKK3/6-p38 signaling pathways in a caspase-dependent manner.

In *C. elegans*, although the adult somatic cells are invariant, the germ cells are malleable under environmental stress. To maintain germline homeostasis, it has been estimated that approximately 50% female germ cells are doomed to die by physiological programmed cell death during normal development (Gumienny *et al*., 1999). HUS-1 is a conserved DNA checkpoint protein that acts as the DNA damage sensor and is required for DNA damage–induced germ cell cycle arrest and apoptosis (Hofmann *et al*., 2002). In the present study, we observed a significant increase in germ cell corpses in the *hus-1*–mutated allele *op241* exposed to 50μM of Cd. CEP-1 is a homolog of the mammalian tumor suppressor gene, p53, that promotes DNA damage–induced apoptosis (Derry *et al*., 2001). Several reports have implicated that p53 is involved in Cd-induced apoptosis in mammalian cell lines (Achanzar *et al*., 2000; Låg *et al*., 2002). p53-independent germline apoptosis in *C. elegans* was observed under oxidative, osmotic, and heat shock stress conditions (Salinas *et al*., 2006). We found that the loss-of-function of *cep-1* behaved similarly to that of wild-type strain under Cd exposure. Taking into consideration our present data and the results reported previously, it is reasonable to deduce that HUS1 and p53 are not essential for Cd-induced apoptosis. However, as the absolute number of germ cell corpses in *cep-1(w40)* and *hus-1(op241)* were less than that of N2, and moreover, the loss-of-function of the *cep-1* antagonist, *abl-1*, enhanced Cd-induced apoptosis (Table 1), the roles of *hus-1* and p53 in Cd-induced apoptosis should not be negligible. It is known that CED-3 is the one of the four caspase-like proteins that is required for germline apoptosis in *C. elegans* (Gumienny *et al*., 1999). In the present study, the *ced-3(n717)* strain showed a deficiency in germline apoptosis under both physiological and Cd-exposed conditions. This result implicates the pivotal roles of caspase in Cd-induced apoptosis, even in the intact animal models.

MAPKs are evolutionally conserved from yeast to mammalians. Three classes of MAPK pathways have been defined: the extracellular signaling-regulated protein kinase (ERK), the JNK, and the p38 MAPK. Each of the pathways is activated by its counterpart MAPK kinase that in turn is activated by MAPKKK. MEK1/2, MKK3/6, MKK4, and MKK7 are identified as the members of MAPKK. The MEK1/2 has been shown to activate ERK, the MKK4/7 to activate JNK, and the MKK3/6 to activate p38 MAPK. These MAPKKs are in turn activated by the members of MAPKKK, such as Raf and ASK (Johnson and Lapadat, 2002). MAPKs phosphorylate specific serines and threonines of target protein substrates and regulate many diverse physiological processes, including development, growth, proliferation, stress responses, and apoptosis. The *C. elegans nsy-1* gene encodes a mammalian homolog of ASK1, and the *mek-1* and *jnk-1* are the homologs of mammalian MKK7 and JNK, respectively. The loss-of-function mutant of *nsy-1* conferred enhanced susceptibility to killing by pathogens (Kim *et al*., 2004), while the mutants of *mek-1* and *jnk-1* exhibited hypersensitivity to heavy metals (Koga *et al*., 2000). ASK1-mediated Cd-induced apoptosis was observed in SH-SY5Y human neuroblastoma cells, and the overexpression of dominant-negative ASK1 reduced JNK phosphorylation and blocked apoptosis induced by Cd (Kim *et al*., 2005). MKK7 was the specific activator of JNK in mammals, and Cd-induced JNK phosphorylation was MKK7 dependent in CL3 cells and SH-SY5Y cells (Chuang *et al*., 2000, Kim *et al*., 2005). The roles of JNK in Cd-induced apoptosis are controversial: JNK-dependent apoptosis was observed in CL3 cells and murine macrophage cells, but it seemed unnecessary in HT4 neuronal cells (Chuang and Yang, 2001; Kim *et al*., 2004). We failed to observe germline apoptosis increase in the mutated strains *nsy-1(ag3)*, *mek-1(ks54)*, and *jnk-1(gk7)* under Cd stress, and the loss-of-function of any of the genes would prevent Cd-induced apoptosis. Similar results were observed in osmotic and heat shock stress, in which germline apoptosis was *mek-1* dependent (Salinas *et al*., 2006). JNK can be activated by ionizing irradiation and UV radiation (Pearson *et al*., 2001; Wang *et al*., 2000), and in our cases, the loss-of-function mutants *nsy-1*, *mek-1*, and *jnk-1* showed normal germline apoptosis under

120-Gy γ-ray irradiation (Table 2). These results indicate that the intracellular signaling pathways of Cd-induced germline apoptosis are distinct from that of ionizing irradiation and that Cd-induced germline apoptosis is dependent on the *C. elegans* NSY-1-MEK-1-JNK-1 cascades. On the other hand, Cd-induced germline apoptosis was dependent on the mammalian MAPK cascades, ASK1/2-MKK7-JNK.

In general, the p38 MAPK pathways have been associated with diverse cellular processes including cell fate determination, immunity, apoptosis, and stress responses (Johnson and Lapadat, 2002; Ono and Han, 2000). Three *C. elegans* p38 MAPK homologs have been identified, *pmk-1, pmk-2*, and *pmk-3*, which are activated by mammalian MKK3/6 homolog *sek-1*, which in turn is activated by *nsy-1*. The *C. elegans* p38 MAPK pathways are involved in stress response and innate immunity, although the role of *pmk-3* is unclear but might be associated with osmotic response (Kim *et al.*, 2002). Like osmotic and heat shock–induced germline apoptosis (Salinas *et al.*, 2006), the loss-of-function allele of *sek-1* showed a blockage in Cd-induced germline apoptosis. The roles of p38 MAPK in Cd exposure were pleiotropic: p38-dependent apoptosis was observed in U937 cells (Galan *et al.*, 2000), and in 9L cells (Hung *et al.*, 1998), meanwhile, the activation of p38 MAPK might contribute to mitotic arrest and genomic instability (Chao and Yang, 2001). Because the *pmk-2* mutant was reported to be L1 larval lethal, we only tested the *pmk-1* and *pmk-3* mutants of *km25* and *ok169*, in which the *pmk-1* was required for Cd-induced apoptosis. Thus, the *C. elegans* NYS-1-SEK-1-PMK-1 cascades are required for Cd-induced apoptosis. Unlike Cd exposure, γ-ray irradiation can induce germline apoptosis in the strains of *sek-1(ag1)*, *pmk-1(km25)*, and *pmk-3(ok169)* (Table 3). These results strengthen the idea that Cd-induced apoptosis and ionizing irradiation–induced apoptosis share different intracellular signaling pathways.

In *C. elegans*, it appears that MEK-1 and SEK-1 act coordinately in response to heavy metal exposure (Mizuno *et al.*, 2004), and the requirement of both MEK-1 and SEK-1 in stress-induced apoptosis has been reported by Salinas *et al.* (2006). Kim *et al.* (2004) have demonstrated that MEK-1 is required for full physiological activation of PMK-1 in *C. elegans* pathogen resistance. In our case, the loss-of-function of both *mek-1* and *sek-1* prevented Cd-induced germline apoptosis and implied their indispensable roles in Cd exposure. Depending on the cell type, MAPKs are diversely involved in stress-induced apoptosis. Cd-induced apoptosis mediated by JNK was reported in murine macrophages, SH-SY5Y cells, and mouse embryonic fibroblasts (Kim and Sharma, 2004; Kim *et al.*, 2005; Papadakis *et al.*, 2006), and p38-mediated apoptosis was seen in CL3 cells and primary epithelial lung cells (Galan *et al.*, 2000; Låg *et al.*, 2005). Other evidences indicate that JNK and p38 participate synergistically in Cd-induced apoptosis in CL3 cells (Chuang *et al.*, 2000; Chuang and Yang, 2001). In our study, Cd exposure failed to induce germline apoptosis in both *jnk-1(gk7)* and *pmk-1(km25)* strains (Table 3). Our findings provide new evidence that JNK and p38 both participate in Cd-induced apoptosis. Since the *C. elegans* caspase CED-3 is absolutely required for programmed cell death (Gumienny *et al.*, 1999), it is convincing that Cd-induced germline apoptosis is mediated by JNK and p38 MAPK signaling pathways via the activation of caspase and that the DDR genes HUS1 and p53 are not essential in the processes.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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