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Effect of Topical Dimethylarsinic Acid on the Expression of Apoptosis-Related Proteins in Mouse Skin

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Abstract We investigated the effect of the topical application of dimethylarsinic acid (DMA) on skin thickness and the expression of several apoptosis-related proteins in skin. After administration of DMA during pregnancy, skin thickness and skin expression of Bcl-2, Bcl-3, Bad, Bid, and caspases-3, -6, -8, -9, and -12 were examined in dams and their offspring. DMA treatment caused significant increases in skin thickness ($p < 0.05$) and the expression of Bcl-2, Bad, and capase-12 in the skin of dams at the mRNA and protein levels ($p < 0.01$). However, maternal exposure to DMA did not significantly alter the expression of the studied apoptosis-related factors in the skin of the offspring. These findings indicate that DMA may induce skin apoptosis, in part, by modulating the expression of Bcl-2, Bad, and caspase-12 in maternal skin. Additionally, our results suggest that maternal exposure to DAM during pregnancy may not induce apoptosis in the skin of the offspring.

Keywords Dimethylarsinic acid · Skin · Apoptosis · Offspring

Arsenic (As) is a naturally occurring metalloid found in pesticides, wood preservatives, pigments, and glass, from which it may be released into the environment (He et al. 2007). Human exposure to As typically occurs through the consumption of contaminated drinking water and foods, such as rice, grains, and fish (Brown et al. 2002). Exposure to As is known to cause numerous adverse health effects in many organs. While acute exposure to high levels of As produces gastrointestinal disturbances, such as nausea, diarrhea, and vomiting, central and peripheral neurotoxicity, bone-marrow suppression, and hepatic toxicity, chronic exposure to lower levels of As induces skin lesions, peripheral neuropathy, and cardiovascular disease (Orloff et al. 2009). Moreover, chronic exposure to As can also cause cancer in the skin, lungs, bladder and liver (Aposhian et al. 2003).

While As is a well-documented human carcinogen, several reports have shown that As, especially in the form of As_2O_3, can produce impressive chemotherapeutic effects when used to treat certain human cancers (Qu et al. 2009; Zheng et al. 2010). Although the mechanisms by which As induces cancer cell death are not well understood, the induction of apoptosis is known to play an important role in its chemotherapeutic effects (Dong 2002).

As accumulates in the skin, most likely due to binding to thiol groups in what is a protein-rich environment (Bailey et al. 2009; Yu et al. 2006). The skin is thus very sensitive to the effects of chronic As exposure, early symptoms of which include effects such as hyperpigmentation, hypopigmentation, and keratosis (Rossman et al. 2004; Vahter 2002). As can easily cross the placenta, and there is substantial evidence that exposure to As during pregnancy is associated with adverse pregnancy outcomes and infant mortality (DeSesso et al. 1998; Rahman et al. 2010; von Ehrenstein et al. 2006). Additionally, the results of experimental studies indicate that exposure to As during critical phases of fetal development may induce cancer and chronic diseases in early childhood (Gregory and Feusner 2009; Smith et al. 2006).

Methylation is a major and important step in the biotransformation of As (Ghosh et al. 2007). Most mammals

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metabolize inorganic arsenic by methylating it to methylarsonic acid and dimethylarsinic acid (DMA) (Vahter 2002). Thus, in this study, we investigated whether maternal exposure to topical DMA during pregnancy altered the expression of apoptosis-related signaling intermediates at the mRNA and protein level in the skins of dams and their offspring.

Materials and Methods

BALB/c mice (7-week-old females weighing 18.0 ± 0.6 g, $n = 10$/group) were assigned to one of three groups that were treated with vehicle (control), 1 mg/kg DMA, and 10 mg/kg DMA. Hundred milligram of DMA was mixed with a neutral cream vehicle, and then cream containing 0, 1, or 10 mg/kg of DMA was applied to a shaved area (2.5 × 2.5 cm) on the back of each mouse. Animals were treated once per week for 4 weeks. After the first treatment, all females were mated and received three further treatments during gestation. Female mice (n = 21) and live fetuses were euthanized 1 day post-delivery and skin tissues were rapidly removed and stored at −70°C until use. Before the female mice were euthanized, the bi-fold thickness of the skin in treated area of their backs was measured using digital calipers. Mice and DMA were purchased from Samtako (Osan, Korea) and Sigma (St Louis, MO, USA), respectively. Other reagents were obtained from commercial sources and were of the highest quality available. Experiments were approved by the Institutional Animal Care and Use Committee of Keimyung University, Korea. Experiments were conducted according to the NIH guidelines for the care and use of laboratory animals. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility, and had free access to food and water.

Total RNA was isolated from dorsal skin samples from mice treated with 10 mg/kg DMA or vehicle (control) using TRIzol (Invitrogen, Burlington, ON, Canada) and an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The protocol used included a DNase digestion step. RNA quantity and purity were determined using a 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada) and denaturing gel electrophoresis. Samples were amplified and labeled using a Quick Amp labeling kit and hybridized to Whole Mouse Genome Oligo Microarrays (Agilent Technologies), the probes of which represent 41,000 mouse genes and transcripts, in hybridization chambers (Agilent Technologies). Briefly, 1 μg of total RNA was reverse-transcribed, yielding cDNA labeled with Alexa Fluor 546 or Alexa Fluor 647. After hybridization and washing, the processed slides were scanned using a Revolution 4200 microarray scanner (Vidar systems, Herndon, VA, USA) with the settings recommended by the manufacturer. The resulting images were analyzed using GenePix Pro 6.0 software. Data normalization and analysis were performed using GeneSpring GX 7.3.1 software.

Samples were homogenized in RIPA buffer (Sigma) containing 1% protease inhibitor cocktail and a phosphatase inhibitor cocktail. The resulting homogenates were centrifuged (14,000 rpm, 40 min) and the supernatants collected. The protein concentration for each supernatant was estimated by the Bradford method, with bovine serum albumin as the standard. Aliquots of the samples, each containing 5 μg of protein, were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were then transferred to nitrocellulose membranes, which were probed with the following primary antibodies (all obtained from Santa Cruz Biotechnology): mouse anti-Bcl-2 monoclonal antibody (dilution 1:200), mouse anti-Bad monoclonal antibody (1:200), and rabbit anti-caspase-12 polyclonal antibody (1:300). After incubation with the appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology), immunoreactive bands were visualized using ECL Western Blotting Detection Reagents (Amersham Biosciences) and X-ray film. Band intensities were measured using the ImageJ software (NIH, Bethesda, MD, USA). GAPDH was used as an internal control.

Skin thickness and densitometric measurements of Bcl-2, Bad, and caspase-12 band intensities in DMA-treated and control animals were compared by one-way analysis of variance. Post hoc testing was performed using Duncan's test. Values of $p < 0.05$ or <0.01 were deemed to indicate statistical significance. The mean and standard error of the mean (SEM) were calculated for each treatment group. All statistical analyses were conducted using the SAS software (ver. 9.1; SAS Institute Inc., Cary, NC, USA).

Results and Discussion

As is widely distributed in the environment and exposure to it can result in the development of skin lesions. In this study, topical treatment with DMA at a dose of 1 or 10 mg/kg body weight resulted in a significant increase in bi-fold thickness skin. The percent change in skin thickness relative to that of control mice was calculated (Fig. 1). A significant increase in skin thickness was observed following topical treatment with DMA at a concentration of 1 or 10 mg/kg body weight ($p < 0.05$). Moreover, skin thickness increased in a dose-dependent manner in mice treated with 1 or 10 mg/kg DMA. As acts as an irritant, causing local skin inflammation. Moreover, some arsenicals may also function as contact allergens (Szymańska-Chabowska et al. 2002). Thus, we suggest that acute

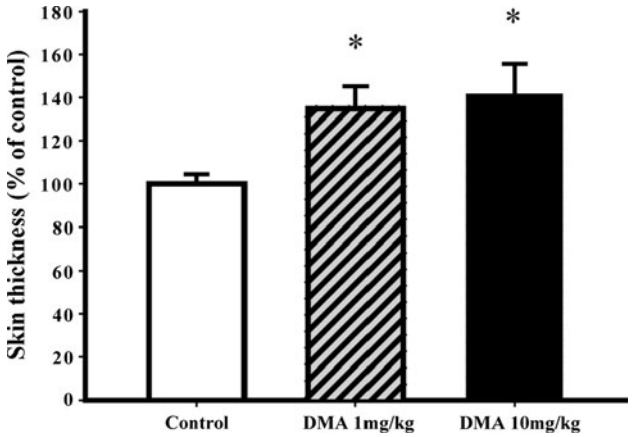

Fig. 1 Changes in skin thickness following topical administration of 1 or 10 mg/kg DMA. Skin thickness was measured using digital calipers. The percent change in skin thickness relative to vehicle-treated controls is shown. *$p < 0.05$. Data represent the mean ± -SEM ($n = 10$)

exposure of the skin to DMA may cause local irritation and/or dermatitis.

To understand the effects of DMA on apoptosis-related gene expression in dams and their offspring, the mRNA expression of several apoptosis-related genes was compared in control and DMA-treated mice using microarrays (Table 1). Expression of Bcl-2, Bad, and caspase-12 was respectively 1.91-, 1.84- and 1.51-fold higher in DMA-treated skin than in vehicle-treated skin; these differences exceeded the pre-established cut-off values (≥1.5- and ≤0.5-fold). However, of the nine apoptosis-related genes studied, only caspase-12 displayed significantly increased skin expression in the offspring of DMA-treated dams. In addition, the most strongly up-regulated and down-regulated genes identified in the skin of dams treated with DMA and their offspring are listed in Tables 2 and 3.

Table 1 Quantitative analysis of apoptosis-related gene of mRNA expression in the skin of dams treated with DMA and their offspring

Gene	NCBI RefSeq no	Fold change*	
		Dam	Offspring
Bcl-2	NM_009741	1.91	0.66
Bcl-3	NM_033601	0.59	0.95
Bad	AK029400	1.84	1.21
Bid	NM_007544	0.93	0.64
Caspase-3	NM_009810	1.36	1.05
Caspase-6	NM_009811	1.07	0.78
Caspase-8	NM_009812	0.80	0.92
Caspase-9	NM_015733	1.33	1.47
Caspase-12	NM_009808	1.51	1.62

* Target mRNA expression levels were normalized to those for GAPDH mRNA and fold changes in expression calculated relative to the control group

In view of the mRNA expression data described above, we next assessed Bcl-2, Bad, and caspase-12 protein levels in the skin of dams by Western blotting. Representative Western blots and the results of quantitative densitometric analysis for skin exposed to 1 or 10 mg/kg DMA are shown in Fig. 2. Consistent with the mRNA data, Bcl-2, Bad, and caspase-12 protein levels increased significantly in skin treated with 1 or 10 mg/kg DMA ($p < 0.01$). Moreover, the expression of Bcl-2 and Bad increased in a dose-dependent manner in dam skin treated topically with DMA. Although the expression of Bcl-2 was significantly increased with the treatment of DMA, Bcl-2/Bax ratio was not significantly changed (Fig. 2d).

Table 2 Genes most strongly up-regulated and down-regulated in the skin of dams treated with DMA, compared to control

Gene	NCBI RefSeq no	Fold change*
Up-regulated		
Krtap14	NM_013707	14.23
Krtap8-1	AK133727	10.41
Neo1	AK052439	9.15
Krt25	NM_133730	8.37
Lba1	XM_983780	8.30
Down-regulated		
Fibcd1	NM_178887	0.03
Npy5r	NM_016708	0.10
Agtrap	NM_009642	0.12
Fut11	NM_028428	0.15
Mas1	NM_008552	0.17

* Target mRNA expression levels were normalized to those for GAPDH mRNA and fold changes in expression calculated relative to the control group

Table 3 Genes most strongly up-regulated and down-regulated in the skin of offspring

Gene	NCBI RefSeq no	Fold change*
Up-regulated		
Eif2s3y	NM_012011	16.66
2210421G13Rik	NM_175391	12.20
Jarid1d	NM_011419	9.49
Uty	NM_009484	9.36
Ddx3y	AK020213	8.10
Down-regulated		
Olfr1404	NM_146881	0.04
Xist	NR_001463	0.07
Ube2g2	NM_019803	0.07
Shq1	NM_181590	0.09
Olfr1352	NM_147071	0.11

* Target mRNA expression levels were normalized to those for GAPDH mRNA and fold changes in expression calculated relative to the control group

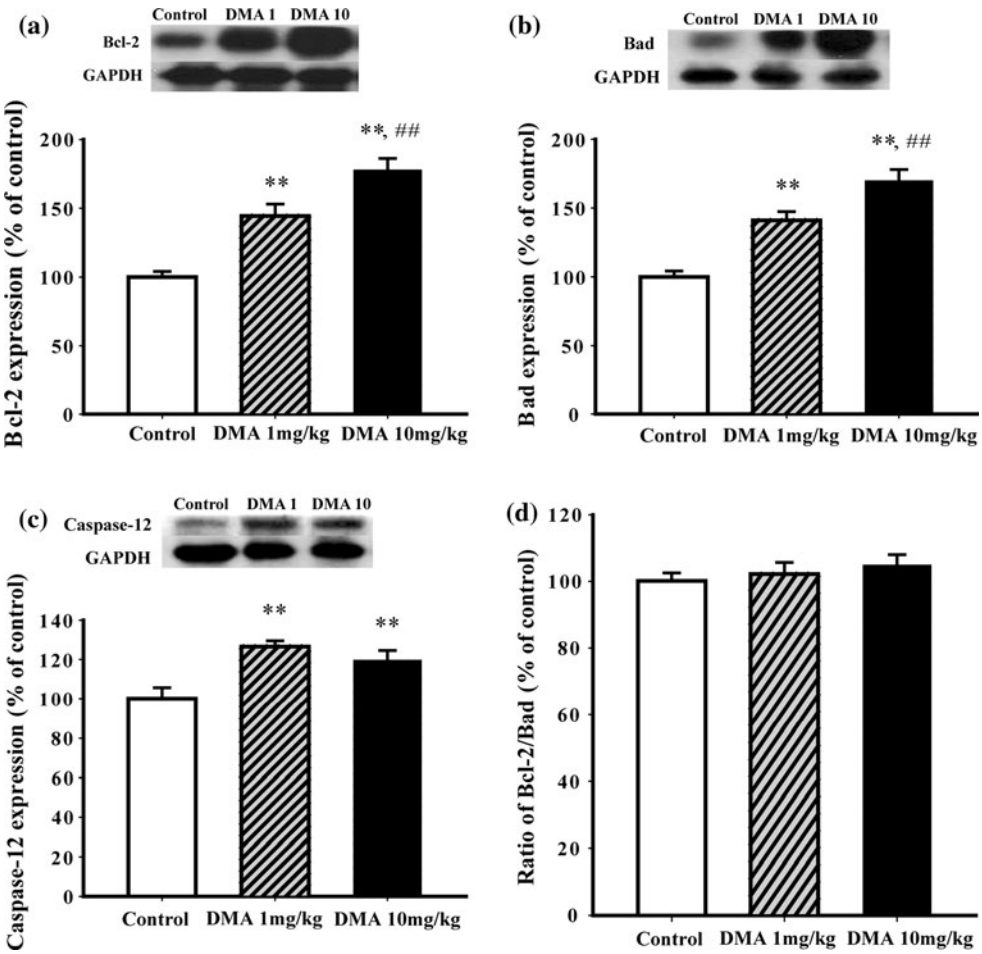

Fig. 2 Relative expression of Bcl-2 (**a**), Bad (**b**), and caspase-12 (**c**) and ratio of Bcl-2/Bad (**d**) in the skin of dams treated with DMA during pregnancy. Example Western blots (*top*) and the results of densitometric analyses of band intensities (*bottom*) are shown. Data represent the mean ± SEM ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group; $^{\#\#}p < 0.01$, compared with the DMA 1 mg/kg group

As exposure is known to alter the expression of a number of genes involved in different important physiological processes, including metabolism genes, stress response genes, damage response genes, cell cycle regulatory genes, genes involved in cell signaling, and apoptosis-related genes (Ghosh et al. 2007). Notably, exposure to As has been shown to reduce DNA repair capacity, resulting in the induction of apoptosis (Andrew et al. 2003; Ghosh et al. 2007).

In the present study, we examined the expression of several apoptosis-related genes (the Bcl-2 family members Bcl-2, -3, Bad, and Bid, and caspases-3, -6, -8, -9, and -12) in mouse skin. We observed increased expression of Bcl-2, Bad, and caspase-12 at both the mRNA and protein levels in skin following topical treatment with DMA during pregnancy. Members of the Bcl-2 family play important roles in the regulation of apoptosis. The archetypal member of this family is Bcl-2, an anti-apoptotic protein located in the outer mitochondrial membrane (Korsmeyer 1999). Bad, a pro-apoptotic member of the Bcl-2 family, binds to and inactivates Bcl-2. Caspase-12, meanwhile, is an ER-associated protein and a key proximal caspase in the caspase activation cascade (Hirai et al. 2011). Activation of caspase-12 ultimately leads to the activation of caspases-3, -6 and -7 and apoptosis (Nakagawa and Yuan 2000). Thus, these results suggest a defect in apoptosis signaling in the skin of DMA-treated mice.

The main strength of the present study is the application of DMA during pregnancy. Several studies have demonstrated the induction of apoptosis and altered expression of Bcl-2, Bax, Bak, caspase-3, and caspase-9 following treatment with As (Chen et al. 2010; Zhao et al. 2010; Zheng et al. 2010). However, few studies have investigated the expression of apoptosis-related genes in skin following the topical treatment of DMA during pregnancy.

We also evaluated the effect of prenatal exposure to a methylated metabolite of arsenic on the expression of apoptosis-related proteins in the skin of the offspring. To test whether topical exposure to DMA during pregnancy influenced the expression of apoptosis-related proteins in the skin of the offspring, skin expression of Bcl-2, Bad, and caspase-12 protein levels was measured in the offspring of

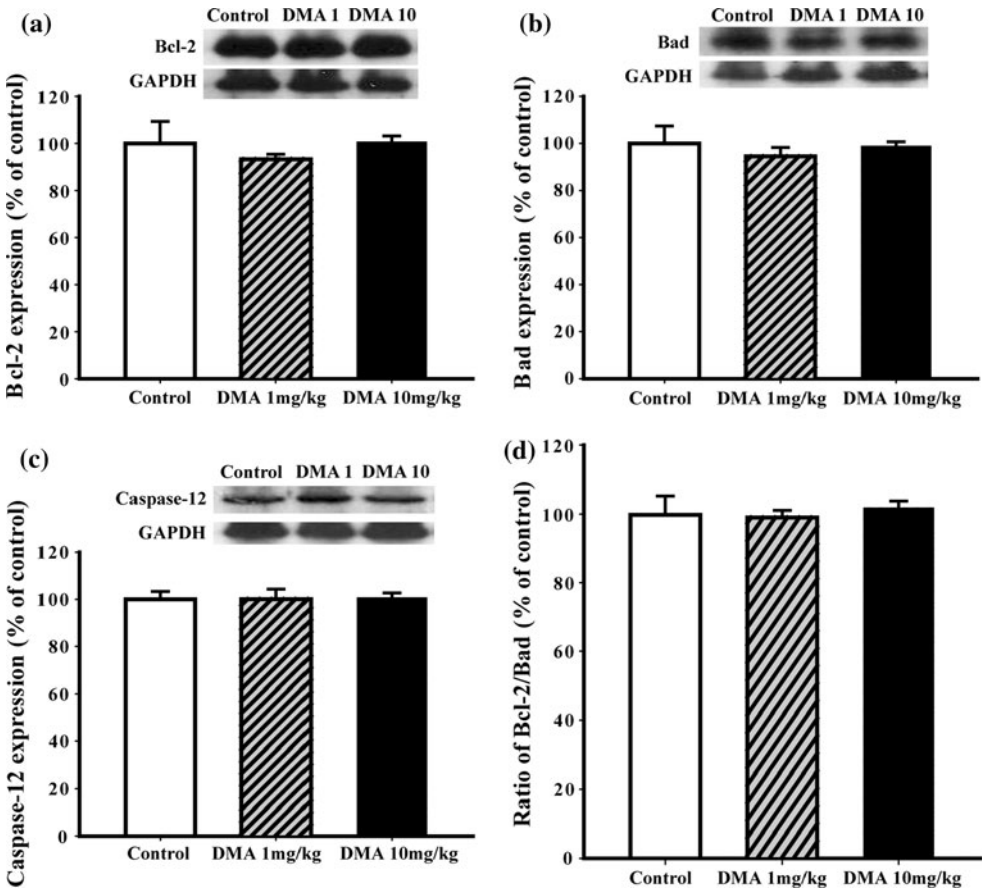

Fig. 3 Relative expression of Bcl-2 (**a**), Bad (**b**), and caspase-12 (**c**) and ratio of Bcl-2/Bad (**d**) in skin of offspring. Example Western blots (*top*) and the results of densitometric analysis of band intensities (*bottom*) are shown. Data represent the mean ± SEM ($n = 5$)

control and DMA-treated dams. Consistent with the mRNA data, Bcl-2 and Bad protein levels in offspring skin were not significantly altered as a result of topical treatment of dams with 1 or 10 mg/kg DMA (Fig. 3). Moreover, treatment of dams with 1 or 10 mg/kg DMA had no significant effect on caspase-12 expression in the skin of their offspring. Even though the mRNA level of caspase-12 was slightly increased with the treatment of DMA, the expressions of other genes and proteins, including the protein expression of caspase-12, were not significantly changed. Although exposure to arsenic during pregnancy is known to negatively affect the offspring (Hopenhayn-Rich et al. 2000), our results suggest that fetal exposure to DMA did not alter the expression of apoptosis-related proteins in the skin. There are a number of possible explanations for this finding. Although the toxicokinetics of dermal As exposure have not been studied, dermal uptake of As is assumed to be very slow. Thus, acute dermal exposure is unlikely to cause systemic health problems, including fetal effects (ATSDR 2007). A second possible explanation is that DMA, the end product in the methylation of inorganic arsenic, was efficiently excreted in the urine, significantly reducing its transplacental passage.

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