Cytochrome c Release from Mitochondria of Early Postimplantation Murine Embryos Exposed to 4-Hydroperoxycyclophosphamide, Heat Shock, and Staurosporine

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Cell death is an early and common event in the pathogenesis associated with the abnormal development induced by a variety of teratogens. Previously, we showed that the cell death induced in day 9 mouse embryos by three teratogens, hyperthermia (HS), 4-hydroperoxycyclophosphamide (4-CP), and sodium arsenite (As), is apoptotic in nature involving the activation of caspase-3, cleavage of poly(ADP-ribose) polymerase (PARP), and DNA fragmentation. We now show that HS, 4-CP, and staurosporine (ST) induce the release of cytochrome c from mitochondria with kinetics suggesting a causal relationship with the activation of caspase-3 and caspase-2. This causal relationship is supported by data showing that procaspase-3 and -2 can be activated *in vitro* by the addition of cytochrome c to a S-100 fraction prepared from control day 9 embryos. Together, these data support the notion that these three teratogens induce changes in embryonic mitochondria resulting in the release of cytochrome c and the subsequent activation of caspase-9, the upstream activator of caspase-3. Previously, we also showed that cells within the day 9 mouse embryo are differentially sensitive/resistant to the cell death-inducing potential of HS, 4-CP, and As. The most dramatic example of this differential sensitivity is the complete resistance of heart cells, characterized by the lack of caspase-3 activation, PARP cleavage, and DNA fragmentation. We now show that this block in the terminal phase of the apoptotic pathway in heart cells is associated with a lack of teratogen-induced release of cytochrome c. Together, our data indicate that mitochondria play a pivotal role in cell death during the early phases of teratogenesis. © 2000 **Academic Press**

Key Words: Cytochrome c; caspase-3; mitochondria; apoptosis; murine postimplantation embryo; *in vitro* embryo culture; hyperthermia; 4-hydroperoxycyclophosphamide; cyclophophamide; staurosporine.

Programmed cell death (PCD) plays an integral role in the normal development of most, if not all, embryonic organs (Glucksman, 1951; Jacobson *et al.,* 1997). Likewise, inappro-priate cell death plays an important role in abnormal development induced by a variety of teratogens (Scott, 1977; Knudsen, 1997). In addition, teratogens often induce cell death in areas of normal PCD (Menkes *et al.,* 1970; Milaire and Rooze, 1983; Sulik *et al.,* 1988), suggesting a mechanistic link between PCD and teratogen-induced cell death. Recent work indicates that this mechanistic link is apoptosis, a cell death pathway involving an ever increasing list of factors that 1) receive the apoptotic stimulus, 2) transduce the signal intracellularly, 3) regulate the decision to live or die, and 4) execute the cell death sentence.

Key to the execution phase of the apoptotic pathway is the activation of cysteinyl aspartate-specific proteases, i.e., caspases, that are constitutively expressed in cells as inactive proenzymes. To date there are 10 known human caspases (caspase-1 to -10), all related to the *Caenorhabditis elegans* homologue, ced-3, that carries out the execution phase of PCD in this roundworm (Porter *et al.,* 1997). Once activated, a subset of these caspases proteolytically cleave a variety of target substrates (Cryns and Yuan, 1998). Presumably, the destruction of these caspase targets culminates in the orderly demise of the cell, thereby preventing inflammation and the unnecessary destruction of surrounding cells. One of the key executioner caspases is caspase-3, an enzyme activated by a variety of apoptotic stimuli (Faleiro *et al.,* 1997) and essential for PCD that normally occurs during brain development (Kuida *et al.,* 1996).

Because caspase-3 has proven to be such a key enzyme in both PCD and toxicant-induced cell death, the regulation of its activation has been a focus of recent research. Initial work indicated that caspase-3 could be activated by other caspases, including caspase-1, -8, and -10 (Tewari *et al.,* 1995; Boldin *et al.,* 1995; Fernandes-Alnemri *et al.,* 1996). Although caspase-8 is known to activate caspase-3 in Fas-mediated cell death (Stennicke *et al.,* 1998), other work has implicated a key role for the mitochondria in the activation of caspase-3 by other apoptotic stimuli (Reed, 1997).

An interest in the role of mitochondria in apoptosis was triggered by a report by Liu *et al.* (1996) showing that the induction of apoptosis in cell free extracts, i.e., activation of

caspase-3, cleavage of poly(ADP-ribose) polymerase (PARP), and induction of DNA fragmentation, required cytochrome c. These authors also showed that, in cells undergoing apoptosis, cytochrome c is released from the mitochondria and accumulates in the cytoplasm. On the basis of these results, the authors suggested that mitochondria participate in the execution phase of apoptosis by releasing cytochrome c, which in turn activates caspase-3. More recent work has revealed some of the mechanistic details concerning the activation of caspase-3 by cytochrome c. Using a cell fractionation approach, Wang and coworkers have shown that cytochrome c initially binds to a protein called Apaf-1, the mamalian homologue of *C. elegans* Ced-4 (Zou *et al.,* 1997). This binding requires dATP. The cytochrome c/Apaf-1 complex then forms a ternary complex with procaspase-9. The formation of this cytochrome c/Apaf-1/procaspase-9 ternary complex facilitates the activation of procaspase-9, culminating in the caspase-9-mediated activation of caspase-3 (Li *et al.,* 1997).

Subsequent to the initial report showing that cytochrome c is released from mitochondria in cells induced to undergo apoptosis by exposure to staurosporine (ST) (Liu *et al.,* 1996), a variety of apoptotic stimuli have been shown to induce cytochrome c release in cultured cells. These apoptotic stimuli include ionizing radiation, *cis* platinum, methylmethansulfonate (Kharbanda *et al.,* 1997), Ara-C (Kim *et al.,* 1997), etoposide (Yang *et al.,* 1997), UVB, actinomycin D, hydrogen peroxide (Kluck *et al.,* 1997), 1-methyl-4-phenylpyridinium (Du *et al.,* 1997), campothecin, bleomycin, VP-16, TNF-α (Tang *et al.,* 1998), tributyltin (Stridh *et al.,* 1998), arsenite (Chen *et al.,* 1998), ter-butylhydroperoxide (Zamzami *et al.,* 1998), and growth factor withdrawal (Neame *et al.,* 1998; Vander Heiden *et al.,* 1997). In addition, other studies have shown that Bax, a proapoptotic factor, directly induces cytochrome c release from isolated mitochondria (Jurgensmeier *et al.,* 1998) whereas Bcl-2 and Bcl-X, two antiapoptotic factors, block cytochrome c release (Yang *et al.,* 1997; Kluck *et al.,* 1997; Kharbanda *et al.,* 1997; Kim *et al.,* 1997; VanderHeiden *et al.,* 1997). Taken together, available data indicate that the efflux of cytochrome c from mitochondria plays a critical role in the terminal phase of apoptosis induced by a variety of apoptotic agents.

Previously, we reported that three selected teratogens, cyclophosphamide, heat shock, and sodium arsenite, induced an apoptotic form of cell death in early postimplantation mouse embryos characterized by the rapid activation of caspase-3, cleavage of PARP, and induction of DNA fragmentation (Mirkes and Little, 1998). In addition, we showed that these characteristic changes occurred in the embryo proper, where morphological cell death was evident later, but not in the heart, an organ completely resistant to cell death induced by these three teratogens. These latter results support a key role for caspase-3 in teratogen-induced cell death. The present studies were undertaken to determine 1) whether selected teratogens induce the release of cytochrome c, 2) if so, the temporal relationship between cytochrome c release and caspase-3 acti-

vation, and 3) whether there is a relationship between cytochrome c release and heart cell resistance.

MATERIALS AND METHODS

In vitro embryo culture. Primigravida Swiss–Webster mice were obtained from a local supplier. The morning following copulation was designated day 0 of gestation. On day 8.5 of gestation, conceptuses from multiple litters were explanted using the whole-rodent embryo culture system established by New (1978) with the following modifications. Embryos from all litters were equally distributed among the different treatment groups. For each treatment group, 10 to 12 embryos were cultured in 12 ml of media containing 80% heat inactivated rat serum/20% Hanks' Buffered saline (HBSS)/50 U/ml penicillin/50 μg/ml streptomycin, gassed with a mixture of 5% O_2/5% CO_2/90% N_2, and incubated overnight on a roller apparatus at 37°C. The following morning, embryo cultures were regassed with 20% O_2/5% CO_2/75% N_2 and continued in culture for 1 h prior to treatment.

Exposure conditions. Treatment of the embryos was initiated by direct addition of freshly prepared 250× solution of 4-hydroperoxycyclophosphamide (4-CP), a preactivated analogue of cyclophosphamide (a gift of Michael Colvin, Johns Hopkins University), or 2000× stock solution of ST (Calbiochem, San Diego, CA) in DMSO to the culture medium, resulting in a final concentration of 20 μM 4-CP or 0.5 μM ST. Embryos for heat shock were exposed to 43°C for 15 min and then returned to 37°C (Mirkes, 1985). Embryos were continued in culture with drug or following heat shock for up to 10 h. At indicated times, treated and control embryos were removed from culture, dissected free of associated membranes, and rinsed in cold HBSS. For experiments using embryo parts, the embryo was further dissected into head, heart, and trunk. Exposure to teratogens and subsequent analyses of cytochrome c accumulation and caspase-3 and caspase-2 activation were repeated at least three times.

Sample preparation. The embryos or embryo parts were placed in a small volume of Buffer A (20 mM Hepes–KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml Aprotinin, and 5 μg/ml PepstatinA) on ice for 10 min with occasional agitation to disaggregate the tissue. The cells were then disrupted by gently expelling the sample five times through a 25-gauge needle, placed on ice for 5 min, and again expelled through the 25-gauge needle. The homogenate (lysate) was centrifuged 10 min at 12,000 rpm at 4°C, resulting in a pellet (P-12) containing the nuclei and mitochondria. The supernatant was again centrifuged at $100,000g$ for 30 min to assure removal of all mitochondria. This supernatant is designated the S-100. The P-12 pellet was lysed by sonication in RIPA buffer (Mirkes and Little, 1998). After aliquots of the S-100 and the P-12 preparations were taken for protein quantification by BCA (Pierce, Rockford, IL), the samples were stored at −80°C until ready for analysis.

Western blot analysis. Aliquots of lysed sample containing equivalent amounts of protein were added to equal volumes of 2× Laemmli buffer, boiled 5 min, applied to 12.5% PAGE (Laemmli, 1970), and transferred to PVDF membranes. Immunoblot analysis was carried out as previously described (Mirkes and Little, 1998) using 3% nonfat milk in Tris-buffered saline (TBS)/0.5% Tween (Tw) for blocking and antibody dilutions. The primary antibodies used were monoclonal anti-cytochrome c at 1:1000 (Pharmingen 7H8. C12 San Diego, CA), monoclonal anti-cytochrome oxidase IV at 1:1000 (Molecular Probes A6431 Eugene, OR), rabbit polyclonal anti-caspase-3 at 1:5000 (Merck Frosst MF R393, Quebec, Canada), rabbit polyclonal anti-caspase-2 at 1:500 (Santa Cruz sc-626, Santa Cruz, CA), monoclonal anti-actin at 1:5000 (Sigma AC-15, St. Louis, MO), rabbit polyclonal anti-caspase-9 at 1:3000 (Cal Biochem 218779, LaJolla, CA), and rabbit polyclonal anti-Apaf-1 at 1:5000 (Cayman Chem. 160780, Ann Arbor, MI). Primary antibodies were incubated overnight (except Actin for 2 h) and washed 4× with TBS/Tw. HRP-linked anti-mouse or anti-rabbit secondary antibodies (Amersham Life Sciences, Arlington Heights, IL) were used at 1:3000 for 2 h and membranes were

washed 2× with TBS/Tw and 3× TBS. Antigen-antibody complexes were visualized by development with ECL Plus (Amersham Life Science, Arlington Heights, IL) and autoradiography. Western blots presented are representative of results obtained in at least three independent experiments.

Caspase-3 enzyme assay. Caspase-3 enzyme activity in lysates (50 mM HEPES, 1 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, pH 7.4) prepared from day 9 mouse embryo heads, hearts, and trunks was assayed using the Caspase-3 Cellular Activity Assay Kit (CalBiochem, San Diego, CA) following manufacturer's instructions. Enzyme activity was determined by the rate of cleavage of a caspase-3 colorimetric substrate (DEVD-pNA) normalized to embryo protein content (pmol/min/μg protein).

In vitro caspase activation. A 6-μg aliquot of S-100 preparation from control embryos, containing endogenous procaspases, was incubated with +/−2 mM dATP and +/−0.02 μg/μl bovine heart cytochrome c (Sigma) for 2 h at 37°C in a final volume of 6 μl. Reaction was terminated by the addition of 2× Laemmli and boiling for 5 min. Samples were then analyzed by Western blot as described above.

RESULTS

Previously (Mirkes and Little, 1998), we showed that caspase-3 is activated in early postimplantation mouse embryos exposed to selected teratogens. In the present studies, day 9.0 mouse embryos were exposed *in vitro* to three different teratogens, i.e., 43°C for 15 min, 4-hydroperoxycyclophosphamide(4-(P)) (20 μM), or 0.5 μm staurosporine (ST). At 7.5 h after initiation of exposures, a time when teratogen-induced cell death in sensitive tissues of the embryo is morphologically obvious, groups of embryos were removed from culture and used to determine whether these exposures induce the release of cytochrome c from mitochondria. Cytochrome c release from mitochondria was assessed by determining whether cytochrome c was present in the S-100 fraction (mitochondria-free) prepared from embryo homogenates compared to its normal localization in the mitochondrial fraction (P-12). Because we also wanted to correlate the release of cytochrome c with the activation of caspase-2 and caspase-3, we initially compared the distribution of cytochrome c, cytochrome oxidase (a mitochondrial inner membrane protein), caspase-3, and caspase-2 in the S-100 and P-12 fractions prepared from control and teratogen-treated day 9 mouse embryos. Results presented in Fig. 1A show that, as expected, cytochrome c is present in the P-12 fraction regardless of whether embryos were unexposed or exposed to HS, 4-CP, or ST; whereas, the S-100 fractions from control embryos contain a barely detectable level of cytochrome c. In contrast, S-100 fractions from teratogen-treated embryos contain clearly elevated levels of cytochrome c compared to S-100 fractions from control embryos. These results indicate that cytochrome c is released from mitochondria of embryos exposed to HS, 4-CP, and ST. Results presented in Fig. 1B confirm that the S-100 fraction is devoid of mitochondria (absence of cytochrome oxidase), thus ruling out the possibility that the cytochrome c observed in the S-100 fractions from treated embryos results from contaminating mitochondria. Figure 1C confirms our previous observations that caspase-3 is activated by teratogens and further
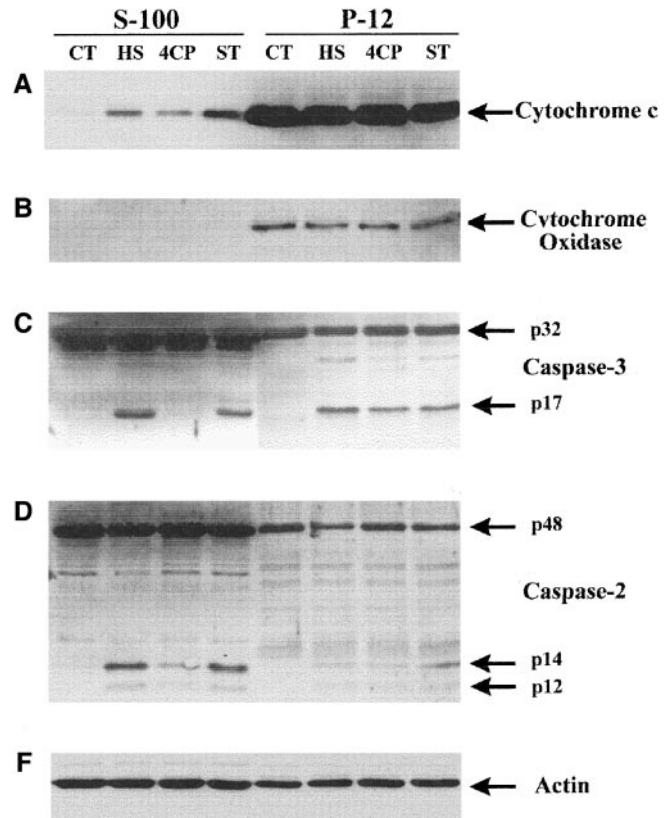

FIG. 1. Cytochrome c release and caspase-2 and -3 activation in teratogen-treated embryos. Western blot analyses of cultured day 9 mouse embryos at 7.5 h after receiving either no treatment (CT), a heatshock of 43°C for 15 min (HS), or continuous exposure to 20 μM 4-hydroperoxycyclophosphamide (4-CP) or 0.5 μM staurosporine (ST). Homogenates of the embryos were fractionated into S-100 (cytosol) and P-12 (mitochondria and nuclei) preparations. Equivalent amounts of protein (6 μg) were applied to 12.5% SDS gels and transferred to PVDF membranes. Membranes were probed with appropriate primary antibodies, cytochrome c (A), cytochrome oxidase (B), caspase-3 (C), caspase-2 (D), and actin (E), followed by HRP-labeled secondary antibodies and visualized by development with ECL-plus and autoradiography. Caspase activation was determined by the presence of the cleaved subunits of the active caspase (p-17 for caspase-3 and p-14 and p-12 for caspase-2). Analysis of cytochrome oxidase (B), a mitochondrial marker protein, was used to detect any mitochondrial contamination in the S-100 fractions. Actin levels (E) were also assessed to verify equivalent protein loading. Western blot data presented are representative of those obtained in at least three separate embryo culture experiments.

shows that the p17 subunit of activated caspase-3 is present in both the S-100 and P-12 fractions. For unknown reasons, the relative amount of the p17 subunit of caspase-3 is much reduced in the S-100 fraction prepared form embryos exposed to 4-CP compared to the P-12 fraction; therefore, in all subsequent experiments, caspase-3 activation is assessed by Western blot analysis using samples from P-12 fractions. Finally, Fig. 1D shows that caspase-2 is also activated by these three teratogens and that the p14 and p12 subunits of activated caspase-2 are present primarily in the S-100 and to a lesser extent in P12 fractions prepared from treated embryos. Thus,

S-100 fractions were used in all subsequent analyses of caspase-2 activation.

On the basis of these results, we next determined whether teratogen-induced cytochrome c release precedes or is coincident with the activation of caspase-3, a terminal caspase prominently activated by a variety of apoptotic stimuli, and caspase-2, a long prodomain caspase that is expressed at relatively high levels in various embryonic tissues exhibiting high levels of programmed cell death during development (Kumar et al., 1994; Raff et al., 1993). Results presented in Fig. 2A show that the amount of cytochrome c present in a S-100 fraction (cytosol) is low at early times after heat shock (1 and 2.5 h), and at these times there is no detectable difference between heat-shocked and control embryos. However, at 5 h after heat shock, the level of cytochrome c in the S-100 fraction is clearly elevated in exposed embryos compared to controls and is detectable at even higher levels at later times (7.5 and 10 h). Using P-12 and S-100 fractions from the same samples in which cytochrome c release was assessed, we also monitored the activation of caspase-3 and caspase-2, respectively. Results presented in Fig. 2B show that a detectable activation of caspase-3 (appearance of p17 subunit) can first be seen 5 h after embryos are heat shocked. In addition, we also determined whether caspase-2 is activated in embryos exposed to
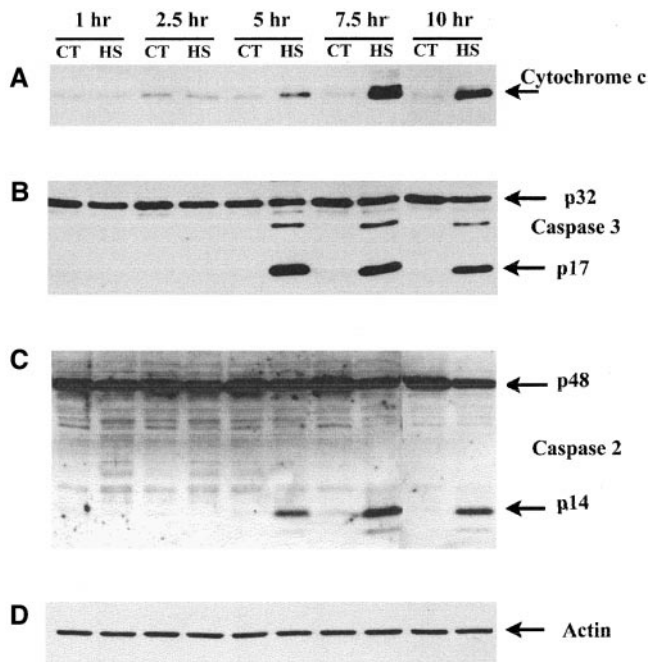

FIG. 2. Time course of cytochrome c release (A) and caspase-3 (B) and -2 (C) activation in heat-shocked mouse embryos. Western analyses of mouse embryos at 1, 2.5, 5, 7.5, and 10 h following a heat shock of 43°C for 15 min (HS) or no treatment (CT). S-100 preparations (4 μg protein) were used for all analyses except for caspase-3, which used the P-12 preparation (8 μg protein). Details are the same as for Fig. 1. Actin levels (D) were also assessed to verify equivalent protein loading. Western blot data presented are representative of those obtained in at least three separate embryo culture experiments.
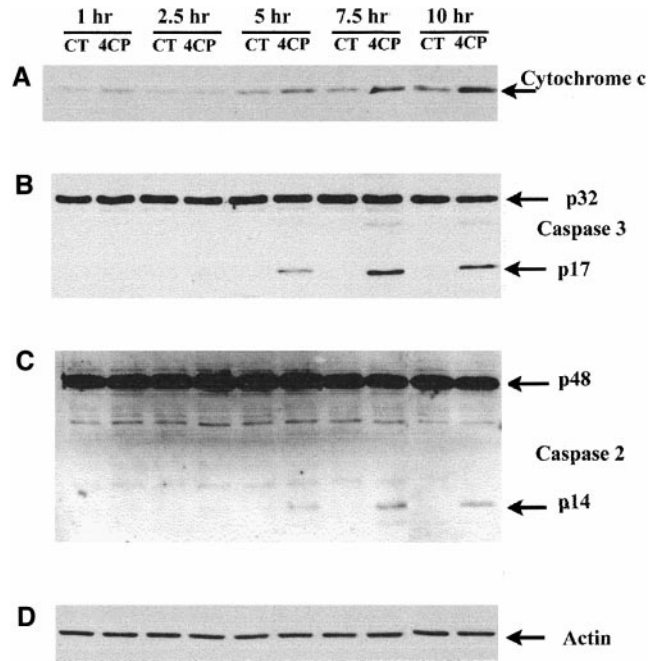

FIG. 3. Time course of cytochrome c release (A) and caspase-3 (B) and -2 (C) activation in 4-OOH cyclophosphamide-treated mouse embryos. Western analyses of mouse embryos at 1, 2.5, 5, 7.5, and 10 h of continuous exposure to 20 μM 4-OOH cyclophosphamide (4-CP) or no treatment (CT). Details are the same as for Fig. 2. Actin levels (D) were also assessed to verify equivalent protein loading. Western blot data presented are representative of those obtained in at least three separate embryo culture experiments.

hyperthermia. Results presented in Fig. 2C show that caspase-2, like caspase-3, is activated (appearance of p14 subunit) 5 h after embryos were exposed to hyperthermia. Thus, heat shock-induced redistribution of cytochrome c is coincident with the activation of caspase-3 and caspase-2.

To determine whether hyperthermia-induced cytochrome c release and caspase activation represent a general response of embryos exposed to teratogens that induce apoptosis, we used a similar Western blot approach to monitor cytochrome c release in embryos exposed to 4-CP and ST, two other teratogens known to induce cell death as part of their developmental toxicity (Fujinaga et al., 1994; Mirkes and Little, 1998). In these experiments, embryos were cultured continuously in the presence of either 4-CP or ST. Results presented in Fig. 3A show that, like hyperthermia, an increase in cytochrome c in the S-100 is first observed 5 h after embryos are exposed to 4-CP. Likewise, the activation of caspase-3 and caspase-2 are coincident with the release of cytochrome c (Figs. 3B and 3C). Results presented in Fig. 4 show that the release of cytochrome c and the activation of caspase-3 and caspase-2 are also coincident, occurring 5 h after the initiation of treatment, in embryos exposed in vitro to staurosporine. Although limited to three teratogens, these results indicate that the release of cytochrome c into the cytoplasm may be a common event in
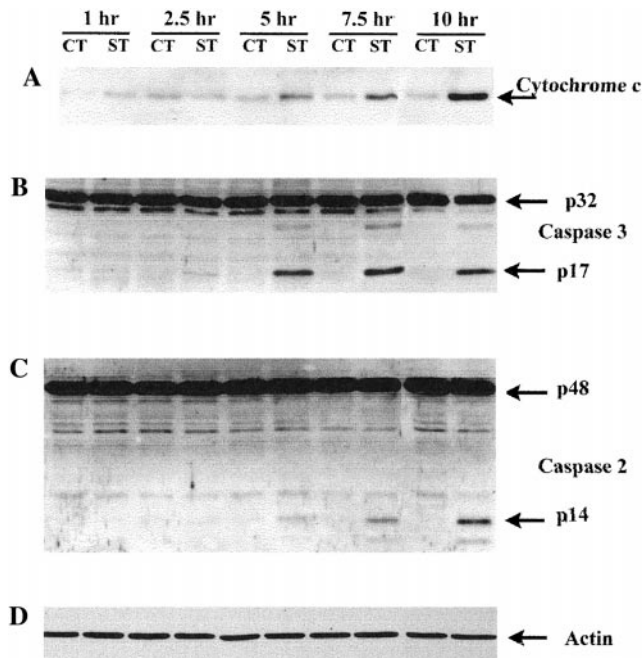
FIG. 4. Time course of cytochrome c release (A) and caspase-3 (B) and -2 (C) activation in staurosporine-treated mouse embryos. Western analyses of mouse embryos at 1, 2.5, 5, 7.5, and 10 h of continuous exposure to 0.5 μM staurosporine (ST) or no treatment (CT). Details are the same as for Fig. 2. Actin levels (D) were also assessed to verify equivalent protein loading. Western blot data presented are representative of those obtained in at least three separate embryo culture experiments.

teratogen-induced apoptosis in postimplantation murine embryos.

To determine whether there is a functional link between the accumulation of cytoplasmic cytochrome c observed in Figs. 1–4 and the activation of caspase-3 and caspase-2 in embryos

exposed to teratogens, we turned to an *in vitro* system to ask whether the addition of exogenous cytochrome c to a S-100 fraction prepared from control embryos would result in the activation of caspase-3. Results presented in Fig. 5A show that exogenous cytochrome c (beef heart) can activate caspase-3 (cleavage of the p32 procaspase-3 to yield p17 and p19 subunits). This cytochrome c-mediated activation of caspase-3 is slightly enhanced by the addition of dATP. Results presented in Fig. 5B show that exogenous cytochrome c also activates caspase-2 (cleavage of the p48 procaspase-2 to yield the p14 subunit). Again, cytochrome c-mediated activation of caspase-2 is enhanced by the addition of dATP.

The above results indicate that three known teratogens induce the release of cytochrome c into the cytoplasm and the concomitant activation of caspase-3 and caspase-2. In addition, these teratogen-induced changes in these known apoptotic factors occur in a time frame consistent with a causal role in teratogen-induced cell death. Because cells of embryonic heart are resistant to teratogen-induced cell death, we next asked whether this resistance was correlated with a failure to release cytochrome c from mitochondria and to activate caspase-3 and caspase-2. To accomplish this, hearts were dissected from control and hyperthermia-treated day 9 embryos and used to assess cytochrome c release and the activation of caspase-3 and caspase-2. For comparison, the remainder of the embryo was dissected into two pieces, i.e., head and trunk, in which significant teratogen-induced cell death occurs. It is important to point out that all three parts of the embryo are heterogeneous, containing multiple tissues and cell types, and that this heterogeneity is lost when samples are disrupted prior to Western blot analysis. Thus, the results presented in subsequent Western blots represent an "average" for each embryo part. Results presented in Fig. 6A show that a clearly demonstrable accu-
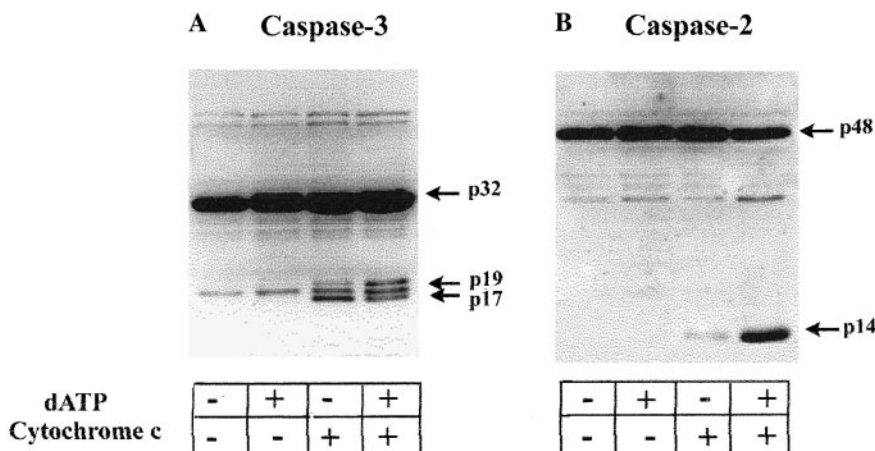

FIG. 5. *In vitro* activation of mouse procaspases by cytochrome c. Mitochondrial free S-100 fractions prepared from untreated day 9 mouse embryos were incubated with or without 2 mM dATP and 0.02 μg/μl cytochrome c for 2 h at 35°C. Activation of procaspase-3 (A) and procaspase-2 (B) were shown by the presence of expected subunits on Western immunoblots. Data presented are representative of those obtained using S-100 fractions prepared from at least three different groups of untreated day 9 mouse embryos.
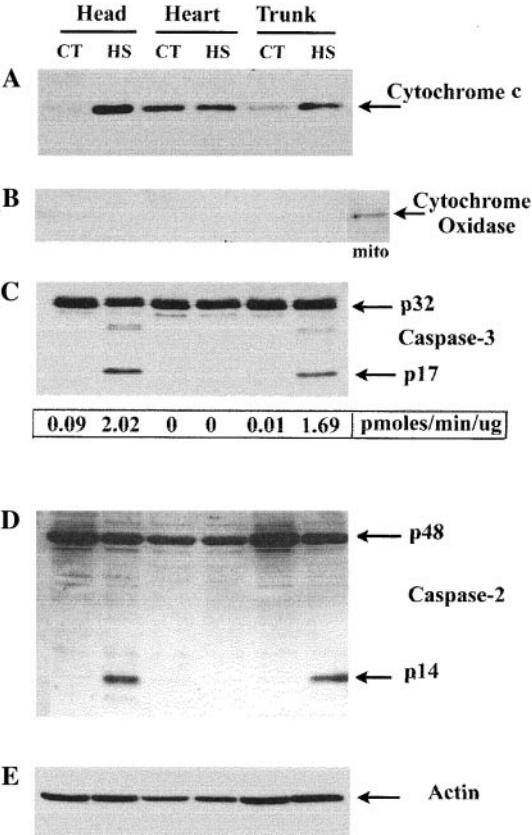
FIG. 6. Cytochrome c release (A) and caspase-3 (C) and -2 (D) activation in heads, hearts, and trunks of heat-shocked mouse embryos. At 7.5 h after receiving a heat shock of 43°C for 15 min (HS) or no treatment (CT), day 9 mouse embryos were dissected and isolated heads, hearts, and trunks were homogenized and fractionated into S-100 (cytosol) and P-12 (nuclei and mitochondria). Equivalent amounts of protein were applied to 12.5% SDS gels and analyzed by Western immunoblot using appropriate antibodies as described in Fig. 1. Bottom of C shows caspase-3 enzyme activities (pmol/min/μg protein) for each treatment group. Analysis of cytochrome oxidase (B), a mitochondrial marker protein, was used to detect any mitochondrial contamination in the S-100 fractions. As a positive control for the presence of cytochrome oxidase, an aliquot of a p12 preparation containing mitochondria (mito) was analyzed (B). Actin levels (E) were also assessed to verify equivalent protein loading. Data presented are representative of those obtained using S-100 or P-12 fractions prepared from at least three different groups of untreated day 9 mouse embryo heads, hearts, and trunks.

mulation of cytochrome c occurs in S-100 fractions prepared from the head and the trunk of treated embryos compared to controls. In both the control head and trunk, little cytochrome c is detected; however, in the control heart, the level of cytochrome c is significantly higher. Nonetheless, the level of cytochrome c present in the S-100 fraction prepared from treated hearts is not increased compared to control. In fact, the level of cytochrome c may be diminished in treated hearts compared to control. Results in Fig. 6B show that the cytochrome c present in the S-100 fractions is not the result of mitochondrial contamination. Results in Fig. 6C confirm previously published data showing that caspase-3 is activated in

the heads and trunks from hyperthermia-treated embryos but not in the heart. Using a caspase-3 enzyme assay, we confirm these Western blot results showing that heat shock induces caspase-3 activity in the head and trunk but not in heart extracts. In addition, we now show that caspase-2 is activated in the heads and trunks of hyperthermia-treated embryos but again not in the heart (Fig. 6D). These results indicate that, although relatively high levels of cytochrome c are present in S-100 fractions prepared from control hearts, hyperthermia does not induce any further accumulation of cytochrome c in the cytoplasm. In addition, the apparent failure of heart mitochondria to release cytochrome c after exposure to hyperthermia is positively correlated with the lack of activation of caspase-3 and caspase-2 and ultimately with the absence of cell death.

On the basis of these results, we next asked whether heart caspase-3 could be activated *in vitro* by the addition of exogenous cytochrome c. Results presented in Fig. 7A show that, whereas caspase-3 can be activated in S-100 fractions prepared from the head and trunk of control embryos, particularly from the head, much less activation is observed in S-100 fractions prepared from control hearts. These latter results are more dramatically highlighted when S-100 fractions from hearts of heat-shocked embryos are incubated with cytochrome c (Fig. 7B). Addition of exogenous cytochrome c to heat shock-activated S-100 fractions from hearts fails to activate caspase-3 to levels observed in heads and trunks and does not dramatically increase caspase-3 activation above what is induced in control heart S-100 fractions.

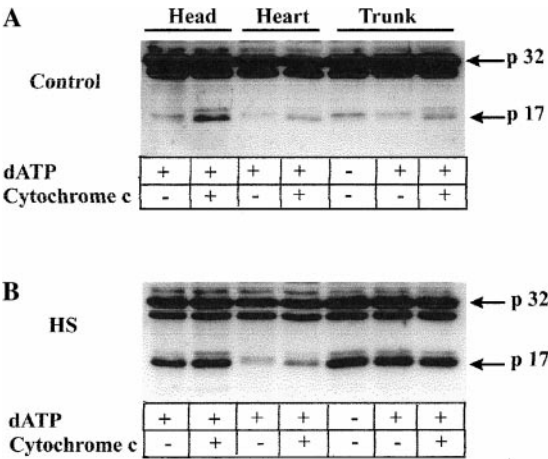Our data showing that teratogens do not induce the release of

FIG. 7. *In vitro* activation of mouse head, heart, and trunk procaspase-3 by cytochrome c. Mitochondrial-free S-100 fractions prepared from heads, hearts, and trunks isolated from day 9 mouse embryos at 7.5 h after receiving (A) no treatment (CT) or (B) heat shock of 43°C for 15 min (HS) were incubated with or without 2 mM dATP and 0.02 μg/μl cytochrome c for 2 h at 35°C. Activation of caspase-3 was shown by presence of p-17 subunit on Western immunoblot. Data presented are representative of those obtained using S-100 fractions prepared from at least three different groups of untreated day 9 mouse embryo heads, hearts, and trunks.
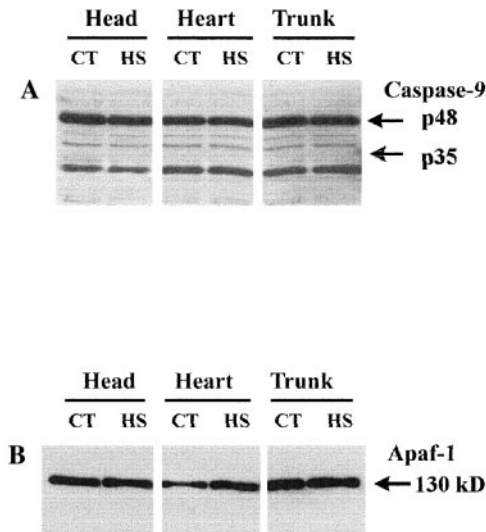
FIG. 8. Caspase-9 and Apaf-1 in embryo heads, hearts, and trunks. Western blot analysis of S-100 fractions prepared from heads, hearts, and trunks of day 9 mouse embryos at 7.5 h after receiving no treatment (CT) or heat shock of 43°C for 15 min (HS). (A) Equivalent amounts of protein are applied to 12.5% SDS gels and probed with a polyclonal antibody to caspase-9. Procaspase-9 (p48) is present in all samples. The cleaved subunit (P-35) of activated caspase-9 was not detectable in any samples. (The band at 28 kDa is unidentified.) (B) Equivalent amounts of protein are applied to 10% SDS PAGE and probed with a polyclonal antibody to Apaf-1. Apaf-1 (130 kD) is present in all samples. Data presented are representative of those obtained using S-100 fractions prepared from at least three different groups of untreated day 9 mouse embryo heads, hearts, and trunks.

cytochrome c from heart mitochondria (Fig. 6A) support the hypothesis that this block to cytochrome c release is responsible for the failure to activate caspase-3 in heart cells. In addition, our *in vitro* data show that, even when heart caspase-3 is presented with an exogenous source of cytochrome c sufficient to activate caspase-3 in heads and trunks, heart caspase-3 is only minimally activated. These results suggest that other factors, in addition to cytochrome c, play a role in controlling the activation of caspase-3 in the heart. Because caspase-3 is thought to be activated by the upstream caspase, caspase-9, and because the activation of procaspase-9 requires the cofactor Apaf-1, we next determined whether procaspase-9 and Apaf-1 are present in heart cells of day 9 mouse embryos.

Figure 8A shows procaspase-9 (48-kDa band) is present in S-100 fractions prepared from control and treated head, heart, and trunk. Recent evidence indicates that activation of procaspase-9 involves Apaf-1-mediated cleavage at Asp 315 to yield a p35 caspase-9 subunit. Subsequently, procaspase-9 can also be cleaved by activated caspase-3 at Asp 330 to yield a p37 subunit (Srinivisula *et al.,* 1998). In mouse embryos exposed to hyperthermia, we could not detect an elevated level of either p35 or p37 (Fig. 8A) in S-100 fractions prepared from heads, hearts, or trunks.

We next determined whether the other cofactor required for procaspase-9 activation, i.e., Apaf-1, was present in heads,

hearts, and trunks of day 9 mouse embryos. Figure 8B is a Western blot of Apaf-1 in control and heat-shocked embryo heads, hearts, and trunks showing that Apaf-1 is present in all three compartments of the day 9 mouse embryo. Although we do not have a functional assay for Apaf-1, these results do show that Apaf-1 is available for interaction with procaspase-9 in the heart, even though procaspase-9 is not activated by HS, 4-CP, and ST, as evidenced by the lack of caspase-3 activation.

DISCUSSION

One of the goals of the present study was to continue our dissection of the apoptotic pathway in early postimplantation murine embryos exposed to selected teratogens known to induce elevated cell death, primarily in the developing central nervous system. Previously we showed that selected teratogens engage the execution phase of the apoptotic pathway characterized by the activation of caspase-3, cleavage of PARP, and fragmentation of DNA. In an effort to elucidate more proximal events in teratogen-induced activation of the apoptotic pathway, we have assessed the role of mitochondria, specifically cytochrome c, in teratogen-induced apoptosis.

The key role of cytochrome c in the apoptotic process was initially identified by fractionating a cytosolic fraction (S-100) prepared from HeLa cells and showing that cytochrome c was one of three proteins (the other two being caspase-9 and Apaf-1) necessary for the *in vitro* activation of caspase-3 (Liu *et al.,* 1996). In this same study, these authors also showed, using a S-100 fraction isolated from staurosporine-treated cells and Western blot analysis, that cytochrome c is released from mitochondria and accumulates in the cytoplasm of cells undergoing apoptosis. Other studies have shown that injection of cytochrome c into cells is sufficient to activate endogenous caspase-3 (Zhivotovsky *et al.,* 1998), further confirming the important role of cytochrome c in the activation of the apoptotic cascade. Since these seminal observations, the release of cytochrome c has now been documented in a number of cell death scenarios using cells exposed to a different apoptotic stimuli, leading to the somewhat paradoxical conclusion that a protein playing a critical role in cellular energy metabolism also plays a key role in cellular suicide (Reed, 1997). In the present study, we have extended these observations derived from cell-based systems to postimplantation murine embryos by showing that three different teratogens, known to induce cell death, also induce the accumulation of cytochrome c in the cytosol prepared from treated embryos. Furthermore, this redistribution of cytochrome c occurs with kinetics coincident with the activation of caspase-2 and caspase-3. These observations are consistent with the idea that cell death induced in early postimplantation mouse embryos by heat shock, cyclophosphamide, and staurosporine is mediated by cytochrome c-induced activation of critical apoptotic caspases such as caspase-2 and -3.

Our results showing the release of cytochrome c from mi-

tochondria together with the activation of caspase-3 also indirectly indicate that these teratogens are activating the mitochondrial apoptotic pathway in which cytochrome c, dATP, and Apaf-1 interact to activate procaspase-9, the upstream activator of caspase-3 (Li *et al.,* 1997). Using an anti-caspase-9 antibody, we have shown that procaspase-9 is expressed in day 9 mouse embryos; however, we have been unable to show activation of caspase-9 on the basis of appearance of caspase-9 subunits. Failure to observe caspase-9 subunits may be related to technical issues, e.g., too little caspase-9 is activated in teratogen-exposed embryos to be detected by Western blot analysis or the available antibody recognizes the proenzyme but not the cleaved subunit. We are currently testing other anti-caspase-9 antibodies to determine whether we can detect the products of procaspase-9 cleavage reported by others. Another possibility is that caspase-9, when complexed with Apaf-1, may not require cleavage to be activated; a possibility supported by studies showing that procaspase-9, mutated in both processing sites and thus unable to form active subunits, is as active in supporting apoptosis as wild-type procaspase-9 (Stennicke *et al.,* 1999).

We have also shown that Apaf-1, a cofactor required for the activation of procaspase-9, is expressed in the day 9 mouse embryo. Thus, early postimplantation mouse embryos contain the necessary factors, i.e., procaspase-9 and Apaf-1, for the activation of caspase-3. The importance of caspase-9 in toxicant-induced cell death is highlighted by the finding that cells derived from caspase-9 null mice are resistant to several apoptotic stimuli (Hakem *et al.,* 1998). More recent studies have shown that Apaf-1 null mice exhibit a phenotype similar to that seen in the caspase-3 and caspase-9 null mice (Yoshida *et al.,* 1998). Like caspase-9 null cells, Apaf-1 null cells are resistant to a variety of apoptotic stimuli. In addition, our studies suggest that the selected teratogens employed all activate the apoptotic pathway, in part at least, by inducing mitochondria to release cytochrome c leading to the activation of Apaf-1 and procaspase-9. Of future interest is an elucidation of teratogen-induced alterations that are upstream of the documented effects on mitochondria. Several possibilities exist, e.g., translocation of Bax from cytosol to mitochondria (Wolter *et al.,* 1997; Gross *et al.,* 1998), cleavage and translocation of BID (Gross *et al.,* 1999), and induction of ceramide (Ghafourifar *et al.,* 1999).

A second goal of these studies was to elucidate the mechanism by which cells of the early postimplantation embryo are differentially sensitive to the cell death-inducing potential of a variety of teratogens. Previously, we showed that hyperthermia, cyclophosphamide, and sodium arsenite induced increased cell death in early postimplantation murine embryos (primarily in the developing CNS); however, cells of the heart were completely resistant (Mirkes and Little, 1998). In addition, we showed that this heart cell resistance is correlated with the absence of teratogen-induced activation of caspase-3, cleavage of PARP, and fragmentation of DNA, indicating that

the terminal aspects of the apoptotic pathway are blocked in heart cells. Results from the present study extend this analysis to more proximal events in the apoptotic pathway and show that the three teratogens used in our present studies do not induce a release of cytochrome c from mitochondria above that present in control heart cells. The failure of heart cell mitochondria to release cytochrome c upon receiving an apoptotic stimulus should, in turn, be correlated with a failure to activate caspase-9; however, we have not been able to confirm this hypothesized lack of caspase-9 activation in the heart. If confirmed, this would indicate that the mechanism of heart cell resistance is related, at least in part, to mitochondrial factors that prevent cytochrome c release. Prime candidates for these factors are members of the Bcl-2 family, particularly Bcl-2, Bax, and Bid, all of which have been shown to play a role in regulating cytochrome c release from mitochondria (Cai *et al.,* 1998).

An unexpected finding in our studies is the observation that the level of cytochrome c in the S-100 fraction prepared from control hearts is considerably higher than the level found in the S-100 fractions from control heads and trunks. Despite the fact that the levels of cytochrome c are high in the S-100 fractions prepared from control and treated hearts, caspase-3 is not activated *in vivo* and only minimally activated *in vitro* after the addition of exogenous cytochrome c. At present, it is not known why heart cell caspase-3 is not activated when apparently sufficient levels of cytosolic cytochrome c are available. One possibility is that the cytochrome c present in control (and treated) hearts is apocytochrome c, a cytoplasmic precursor of holocytochrome known to be unable to activate caspase-3 (Yang *et al.,* 1997). While this remains a possibility, we have been unable to identify this heart cell cytochrome c using available antibodies. Another possibility is that some other factor required for caspase-3 activation is missing or blocked in heart cells, i.e., Apaf-1 and procaspase-9. We have now shown that both procaspase-9 and Apaf-1 are present in day 9 murine embryos and specifically in the heart. What is unknown is whether the Apaf-1/caspase-9/cytochrome c complex (so-called apoptosome) fails to form in heart cells, whether the apoptosome forms but does not result in activated caspase-9, whether caspase-9 is activated but the downstream activation of caspase-3 and -2 is blocked, or whether caspase-3 and -2 are activated but their activity is subsequently blocked. Concerning the latter possibility, a number of factors have been identified and shown to block apoptosis in a variety of contexts. These so-called inhibitors of apoptosis include NAIP, survivin, XIAP, c-IAP-1, and c-IAP-2; the latter three are known to bind to and directly inhibit the terminal caspases-3 and -7 (Deveraux and Reed, 1999). At present, no information is available concerning the expression of these inhibitors of apoptosis in murine embryos. Yet another possibility is suggested by a recent report showing that phosphorylation of serine-196 in procaspase-9 inhibited its protease activity (Cardone *et al.,* 1998). Thus, another possibility is that caspase-9, although present in

the heart, is phosphorylated and thereby inhibited from activating caspase-3.

In summary, we have shown that three different teratogens, presumably exerting their developmental toxicity through different mechanisms, all converge on the mitochondria to induce the release of cytochrome c. Although direct evidence is lacking, this release of cytochrome c together with the documented activation of caspase-3 supports the hypothesis that the three teratogens used in this study induce the formation of the Apaf-1/procaspase-9/cytochrome c complex leading to the activation of caspase-9, a key initiator caspase known to be activated by chemotherapeutic and DNA-damaging agents (Cryns and Yuan, 1998). In addition, we have also presented data showing that cytochrome c release is apparently blocked in embryonic heart cells, leading to the hypothesis that heart cell resistance is related, at least in part, to inhibition of the apoptotic pathway at the level of the mitochondria. Although regulating cytochrome c release may be an important mechanism used by heart cells to inhibit the execution phase of the apoptotic pathway, it is highly likely that redundant inhibitory mechanisms are employed to insure heart cell viability and function. Future work will focus on specific mechanisms regulating mitochondrial release of cytochrome c, whether cytochrome c plays a role in determining which cells in the embryo die during normal development and abnormal development induced by teratogens, and the identification of other factors that play a role in an embryo cell's decision to live or die.

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