Genotoxic Stress Response Gene Expression in the Mid-Organogenesis Rat Conceptus

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The ability of the conceptus to respond to genotoxic stress may be critical for normal development, particularly after exposure to genotoxic teratogens. Members of the phosphatidylinositol 3-kinase (PI3K) superfamily are involved in controlling cell cycle activity and maintaining genomic stability. The expression of PI3K family members ATM, ATR, and DNA-PKcs, and downstream genes p53, GADD45, and p21, was examined in the mid organogenesis rat conceptus in vivo on gestational days (GD) 10 through 12 and in vitro following exposure to genotoxic stress. ATM was the most highly expressed PI3K family member in both yolk sac and embryo proper, with transcript levels increasing ~fourfold in the embryo from GD 10 to 12. Transcript concentrations for ATR, DNA-PKcs, and downstream genes were low in both tissues; all genes had increased transcript levels exclusively in the GD 12 embryo. Transient oxidative stress, induced by short-term, in vitro embryo culture, had no effect on transcript levels in either tissue. Culture for 24 or 44 h significantly decreased ATM transcript levels in both embryo and yolk sac, but downstream genes were unaffected compared to GD-11 and -12 in vivo levels, respectively. Exposure to 4-hydroperoxycyclophosphamide (4-OOHCPA), an activated form of the nitrogen mustard cyclophosphamide (CPA), had no effect on transcript levels for any of the genes examined. Therefore, while transcripts for genotoxic stress-response genes are present in the mid organogenesis rat conceptus, their expression is not regulated by exposure in culture to either transient oxidative stress or a genotoxic alkylating agent. The inability of the conceptus to upregulate transcripts in response to insult may contribute to an increased susceptibility to stressors during organogenesis.

Key Words: DNA damage; oxidative stress; cyclophosphamide; phosphatidylinositol 3-kinase; gene expression; cell cycle checkpoint.

The ability to sense and signal within the cell that DNA damage has occurred is one of the most important cellular defenses against genotoxic agents. DNA-damage sensors activate downstream cellular targets that delay the cell cycle, leading to checkpoint arrest (Lowndes and Murguia, 2000), modify DNA repair activity, and activate the apoptotic cascade (Shiloh, 2001). Members of the phosphatidylinositol 3-kinase (PI3K) superfamily are serine/threonine protein kinases that act as genotoxic stress sensors. This family includes ataxia-telangiectasia mutated (ATM; Savitsky et al., 1995), ATM- and Rad3-related (ATR; Cimprich et al., 1996), and the DNA-dependent protein kinase (DNA-PK; Lees-Miller et al., 1990). ATM is activated following ionizing radiation (IR; Canman and Lim, 1998) and oxidative stress (Rotman and Shiloh, 1997), ATR following UV radiation (UVR; Tibbetts et al., 1999) and exposure to alkylating agents such as cisplatin (Damia et al., 1998), and DNA-PK in response to double-strand breaks (DSBs; Smith and Jackson, 1999). Activation of ATM and ATR leads to the activation of p53 (Canman and Lim, 1998); activation of p53 transcriptionally upregulates p21 and the growth arrest and DNA damage-inducible gene 45 (GADD45; Wang et al., 1999). GADD45 triggers G1/M checkpoint arrest (Wang et al., 1999), while activation of p21 leads to G1/S checkpoint arrest (Sherr and Roberts, 1999), and can activate the apoptotic machinery (Dotto, 2000). DNA-PK, composed of a catalytic subunit, DNA-PKcs and the Ku70/80 heterodimer (Smith and Jackson, 1999), binds to DNA DSBs and activates the apoptotic machinery (Wang et al., 2000).

Both oxidative stress (Wells et al., 1997) and alkylating agents (Glantz, 1994) are teratogenic, inducing specific malformations during susceptible stages of development. The embryo culture system has been utilized as a model system to study these teratogens. Transient oxidative stress is induced within 30 min following the initiation of culture (Ozolinš and Hales, 1997). Exposure of the conceptus to active metabolites of cyclophosphamide, a nitrogen mustard, is used to model the action of alkylating agents as teratogens (Slott and Hales, 1988). The rat conceptus is most sensitive to the effects of genotoxic teratogens during mid organogenesis, gestational day (GD) 10–12 (Jirakulsomchok and Yielding, 1984; Little and Mirkes, 1987; Platzek et al., 1982). Impaired responses to DNA damage may enhance the consequences of teratogen exposure and lead to specific birth defects. Previous studies have examined the gene expression profiles of the major DNA repair pathways in the mid organogenesis conceptus (Vinson et al., 1999). The ability of the conceptus to respond to genotoxic stress may be critical for normal development, particularly after exposure to genotoxic teratogens. Members of the phosphatidylinositol 3-kinase (PI3K) superfamily are involved in controlling cell cycle activity and maintaining genomic stability. The expression of PI3K family members ATM, ATR, and DNA-PKcs, and downstream genes p53, GADD45, and p21, was examined in the mid organogenesis rat conceptus in vivo on gestational days (GD) 10 through 12 and in vitro following exposure to genotoxic stress. ATM was the most highly expressed PI3K family member in both yolk sac and embryo proper, with transcript levels increasing ~fourfold in the embryo from GD 10 to 12. Transcript concentrations for ATR, DNA-PKcs, and downstream genes were low in both tissues; all genes had increased transcript levels exclusively in the GD 12 embryo. Transient oxidative stress, induced by short-term, in vitro embryo culture, had no effect on transcript levels in either tissue. Culture for 24 or 44 h significantly decreased ATM transcript levels in both embryo and yolk sac, but downstream genes were unaffected compared to GD-11 and -12 in vivo levels, respectively. Exposure to 4-hydroperoxycyclophosphamide (4-OOHCPA), an activated form of the nitrogen mustard cyclophosphamide (CPA), had no effect on transcript levels for any of the genes examined. Therefore, while transcripts for genotoxic stress-response genes are present in the mid organogenesis rat conceptus, their expression is not regulated by exposure in culture to either transient oxidative stress or a genotoxic alkylating agent. The inability of the conceptus to upregulate transcripts in response to insult may contribute to an increased susceptibility to stressors during organogenesis.

Key Words: DNA damage; oxidative stress; cyclophosphamide; phosphatidylinositol 3-kinase; gene expression; cell cycle checkpoint.

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and Hales, 2001a,b). Little is known about regulation of the expression of DNA damage-sensor genes during development. The present study was undertaken to elucidate the expression profiles of the PI3K superfamily genes during mid organogenesis in the rat conceptus, and to determine whether exposure to transient oxidative stress or to DNA damage with 4-hydroperoxycyclophosphamide (4-OOHCPA) alters the expression of these genes.

**MATERIALS AND METHODS**

**Tissue preparation.** Timed-pregnant virgin female Sprague-Dawley rats (225–250 g) were obtained from Charles River Canada (St. Constant, Québec, Canada) and housed in the McIntyre Animal Resource Centre (McGill University). Rat chow (Purina Rat Chow 5012, St. Louis, MO) and water were provided ad libitum, and animals were exposed to a 14-h light: 10-h dark cycle. All treatments were in accordance with a protocol approved by the Animal Care Committee of McGill University. Gestational-day zero was defined as the morning following mating. On GD 10, 11, and 12, uteri were removed and dissected into embryo and yolk sac tissues, and stored at –80°C.

**Embryo culture.** Rat conceptuses were explanted from timed-pregnant dams on GD 10 and cultured using established techniques (New, 1978). The whole-embryo culture model removes any confounding maternal effects that may occur following drug exposure. For short-term oxidative stress studies, conceptuses with an intact yolk sac and ectoplacental cone were cultured for 0.5, 1.5, 3, and 6 h at 37°C in 90% heat-inactivated rat serum supplemented with penicillin and streptomycin. For 4-hydroperoxycyclophosphamide (4-OOHCPA) studies, conceptuses were cultured as above in the presence of vehicle (sterile water) or 10 μM 4-OOHCPA (a gift from M. Colvin) for either 24 or 44 h. In culture, 4-OOHCPA breaks down spontaneously in solution to its active metabolites (Slott and Hales, 1988), phosphoramide mustard and acrolein. Following culture, embryos were removed and dissected as described above.

**Antisense RNA (aRNA) technique.** The aRNA technique was used to examine the DNA-repair gene-expression profile on a per embryo basis, as previously described (Vinson and Hales, 2001a,b). This technique is essentially a “reverse Northern blot,” allowing for the simultaneous examination of multiple gene transcripts from a single tissue sample. Individual embryos and yolk sacs were sonicated on ice in lysis buffer (1 mg/ml digitonin, 5 mM DTT, 0.5, 1.5, 3, and 6 ha t 37°C in 90% heat-inactivated rat serum supplemented with penicillin and streptomycin. For 4-hydroperoxycyclophosphamide (4-OOHCPA) studies, conceptuses were cultured as above in the presence of vehicle (sterile water) or 10 μM 4-OOHCPA (a gift from M. Colvin) for either 24 or 44 h. In culture, 4-OOHCPA breaks down spontaneously in solution to its active metabolites (Slott and Hales, 1988), phosphoramide mustard and acrolein. Following culture, embryos were removed and dissected as described above.

**Gene array membranes and hybridization.** To examine the expression of multiple genes using a single tissue sample, gene-expression arrays were created. In particular, the expression of six ATM pathway and family-member genes was examined. These were: ATM, ataxia-telangiectasia-related (ATR), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), growth arrest and DNA-damage-inducible gene 45 (GADD45), p21, and p53. To create these arrays, nylon membranes (Zeta-Probe GT, Bio-Rad, Mississauga, Ontario) were slot-blotted (Bio-Dot SF, Bio-Rad) with equimolar amounts of cDNAs, as per the manufacturer’s protocol. Wherever possible, nonrodent cDNA probes were used. Nonrodent probes were chosen based on their high homology to rodent cDNAs for the genes of interest. Nonrodent probes were used for DNA-PKcs, p21, and ATR, all of which were human in origin. Gene expression arrays were prehybridized for 30 min in hybridization buffer (7% SDS, 0.12 M Na2HPO4, pH 7.2, 0.25 M NaCl, 50% formamide). Heat denatured, radiolabeled aRNA probes were hybridized overnight to arrays at 42°C.

Following hybridization, the arrays were washed in solutions of decreasing stringency (from 2× SSC/0.1% SDS to 0.1× SSC/0.1% SDS) at 42°C for 20 min each and exposed to phosphorimager plates overnight. Arrays were stripped of probe by boiling in 0.1× SSC/0.5% SDS twice for 20 min each. Stripping efficiency was determined by exposing membranes to phosphorimager plates overnight. Arrays were reused a maximum of five times, at which point no appreciable degradation in signal was observed (data not shown). Blots were initially randomly assigned to each sample group being studied; stripped blots were not reused for other individual samples from the same sample group. In addition, each sample group was tested on blots with, on average, the same number of stripping cycles, to minimize any chance of expression level bias owing to the stripping procedure.

**Quantification and analysis of aRNA data.** Images of the gene expression arrays were obtained using a STORM phosphorimager (Molecular Dynamics, Sunnyvale, CA), and gene expression quantified using ImageQuant 5.0 software for Windows NT (Molecular Dynamics). Gene expression intensity values on each membrane were normalized relative to the expression of vimentin, a structural protein; vimentin was chosen because its expression remained constant between the different time points and tissues. The intensity value from pUC18 plasmid DNA blotted onto each array (nonspecific hybridization) was also subtracted from each gene intensity value prior to normalization to vimentin. In order to ensure the consistency and reliability of the data, several replicates for each tissue and treatment were performed; each embryo or yolk sac sample was extracted from tissues from separate litters. Data are from four separate GD 10–12 yolk sacs and embryo samples for each in vivo time point, from three to seven separate samples for culture-alone data per culture time point, and from three samples for culture with 4-OOHCPA data per culture time point, each obtained from separate litters.

**Statistical analysis.** Statistical analysis was done on an individual-gene basis using one-way ANOVA, followed by either the Tukey post-hoc test or the Student’s t-test with SigmaStat version 2.03 software (SPSS, Chicago, IL).

**RESULTS**

**Expression of PI3K Superfamily and Downstream Stress-Response Genes during Organogenesis in Vivo**

The expression patterns of PI3K family members, ATM, ATR, and DNA-PKcs in rat yolk sac and embryo-proper on GD 10, 11, and 12 are shown in Figure 1. ATM was the most highly expressed PI3K family gene examined in both tissues at all time points (Fig. 1A). ATM expression was similar in both tissues on GD 10 and 11; a dramatic fourfold increase in the steady-state concentrations of ATM transcripts occurred between GD 11 and 12, exclusively in the embryo. ATR and DNA-PKcs were expressed at low levels in the yolk sac between GD 10 and 12 (Figs. 1B and 1C). The embryo-proper displayed transcript levels near the limit of detection for ATR and DNA-PKcs on GD 10, with higher transcript levels for both genes observed on GD 11 and 12 (Figs. 1B and 1C). The expression profiles for downstream genes, p53, GADD45, and p21, are shown in Figures 1D–1F. The expression of all three genes was low in both tissues. Transcript levels did not change significantly during organogenesis in the yolk sac; in the embryo, GADD45 and p21 transcript levels mirrored...
the increase seen for \(ATM\), increasing on GD 12 compared to GD 10 (for \(GADD45\); Fig. 1E) or compared to GD 10 and 11 (for \(p21\); Fig. 1F).

**Effect of Embryo Culture on PI3K Superfamily and Downstream Stress-Response Gene Expression**

Embryo culture induces short-term oxidative stress (Ozoliņš and Hales, 1997). To determine if this oxidative stress affects PI3K superfamily gene expression, embryos were cultured for 0.5, 1.5, 3, and 6 h and the expression of PI3K superfamily and downstream genes was determined (Fig. 2). Short-term culture did not affect transcript concentrations of \(ATM\) (Fig. 2A), \(ATR\) (Fig. 2B), or \(DNA-PKcs\) (Fig. 2C), nor did it alter transcript levels for downstream targets \(p53\), \(GADD45\), and \(p21\) (Figs. 2D–2F).

Embryos are cultured frequently for 24 or 44 h, in the presence and absence of putative teratogens, to elucidate the effects of these substances on organogenesis. To compare expression profiles of the ATM family of stress-response genes during organogenesis in vivo with those from embryos cultured in vitro, GD-10 embryos cultured for 24 h were compared to GD-11 embryos in vivo, while GD-10 embryos cultured for 44 h were compared to GD-12 embryos in vivo (Fig. 3). While a slight decrease in \(ATM\) transcript levels was observed in yolk sac following a 24-h culture compared to GD-11 in vivo values (Fig. 3A), a dramatic decrease to 15% of GD-12 levels was observed following 44 h of culture (Fig. 3B). \(p53\) and \(GADD45\) transcript levels were unaffected following either culture period (\(p21\) levels were not examined). In the embryo, \(ATM\) transcript levels decreased to 22% of GD-11 (Fig. 3C), and 35% of GD-12 values (Fig. 3D), after 24- and 44-h culture, respectively. Apart from a decrease in \(GADD45\) transcript levels to undetectable levels following a 44-h culture (Fig. 3D), \(p53\) and \(GADD45\) transcripts were unaffected by long-term culture.

**Effect of 4-OOHCPA on PI3K Superfamily and Downstream Stress-Response Gene Expression**

Consequences of exposing GD-10 embryos to the alkylating agent and teratogen, 4-OOHCPA, on the expression of DNA damage, sensor genes are shown in Figure 4. Conceptuses were exposed to vehicle or drug (10 \(\mu\)M) in culture for 0.5, 1.5, 3, or 6 h (Fig. 4). No significant changes in \(ATM\), \(ATR\), or \(DNA-PKcs\) expression occurred following 4-OOHCPA exposure (Figs. 4A, 4B, and 4C). \(ATR\) and \(DNA-PKcs\) were expressed at or near the limit of detection in the embryo at most
time points (Figs. 4B and 4C). Furthermore, p53, GADD45, and p21 transcript levels were unaffected by 4-OHCPA exposure, in either yolk sac or embryo (Figs. 4D–4F).

DISCUSSION

A key aspect in the response to DNA damage is coordination of the cell cycle to allow DNA repair to take place before DNA synthesis and cell division occur. The importance of PI3K family members for normal growth and development is apparent in the null-mutant animals (Friedberg and Meira, 2000). While some checkpoint functions may overlap between the PI3K family members, loss of more than one genotoxic stress sensor is not compatible with life; deficiencies in both ATM and other members of recombination and/or DNA damage sensor pathways are embryolethal, for example, ATM-null in DNA-PK null-mutant backgrounds (Friedberg and Meira, 2000). Null-mutant mice lacking downstream targets of PI3K family members also exhibit increased tumor susceptibility and checkpoint problems (Hollander et al., 2001; Vogelstein et al., 2000). We undertook analysis of the expression profile of these genes during a susceptible period of development to investigate the ability of the conceptus to respond to genotoxic teratogens.

Expression of PI3K Family Members and Downstream Genes in Vivo

PI3K family members are highly expressed during development in areas undergoing rapid cell division, consistent with a role for ATM in genome maintenance during cell division (Chen and Lee, 1996). ATM was the highest-expressed PI3K family member examined in both tissues. The dramatic increase in ATM transcript levels on GD 12 may be due to high levels of metabolic activity and DNA metabolism, both of which may increase the occurrence of internally generated DNA damage. While most studies have focused on regulation of ATM at the level of kinase activity, ATM transcript or protein levels have been shown as induced in several model systems in which cells are rapidly proliferating and/or differentiating (Clarke et al., 1998; Fukao et al., 1999). Interestingly, there was no increase in kinase activity as seen by Western blot analysis of phospho-(Ser/Thr) residues, which are substrates for both ATM and ATR (Vinson and Hales, unpublished data).
ATM may be regulated either by alterations in the transcription of pre-existing ATM mRNA or by post-transcriptional mechanisms (Savitsky et al., 1997), possibly by specific proliferation-responsive signals within the rat conceptus.

Genes downstream of the ATM superfamily kinases were expressed at very low levels in the conceptus. Earlier studies found that p53 transcript levels are highest during organogenesis in the mouse (Rogel et al., 1985), with tissues undergoing differentiation exhibiting higher levels of p53 transcripts (Schmid et al., 1991). In the rat conceptus, a trend towards higher transcript levels appeared on GD 12, perhaps coinciding with differentiation of p53-dependent tissues. In this study, transcripts for both GADD45 and p21 appear between GD 11 and 12 in the embryo, mirroring ATM and DNA-PKcs transcript profiles, and in parallel to the expression profile for p53.

Transient Oxidative Stress and PI3K Pathway Gene Expression

Alteration of ATM expression may be beneficial during organogenesis to protect the embryo against increased oxidative stress as a result of high levels of metabolism or xenobiotic exposure (Wells et al., 1997). Culture elicits transient oxidative stress in the embryo, increasing oxidized glutathione and altering AP-1 transcription factor mRNA levels and DNA-binding activity (Ozoliņš and Hales, 1997). Oxidative stress did not affect ATM transcript levels. Nevertheless, long-term culture, for 24 or 44 h, did result in decreased ATM mRNA levels; this decrease was specific for ATM, as several other DNA repair-pathway genes were unaffected by culture conditions (data not shown). The explanation for this decrease is not clear; embryos cultured for 24 or 44 h have normal oxidized-to-reduced glutathione levels, indicative of a lack of oxidative stress at these time points (Ozoliņš and Hales, 1997). The decrease in ATM transcripts may be due to other stressors in the culture system such as the depletion of specific growth factors and other nutrients, which may lead to dysregulation of cell function. Alteration of ATM expression may be stressor-specific, or the response of the embryo to genotoxic stress may be unique. Limitations in the factors required for proper embryonic growth following extended periods of culture may play a role in the downregulation of stress-response genes.

Effect of 4-OOHCPA on PI3K Family Genes and Downstream Targets

Several DNA-repair pathways repair the damage caused by cyclophosphamide (CPA) (De Silva et al., 2000; McHugh et al., 2000). In particular, the nonhomologous end-joining (NHEJ) pathway is involved in repairing DSBs formed by nitrogen mustards (McHugh et al., 2000); DNA-PK and associated Ku subunits are involved in this recombination repair pathway (Lieber, 1999). Therefore, the expression of these genes may be crucial in repairing the genotoxic damage caused by CPA.

Previous studies have demonstrated a link between genotoxic stress and checkpoint arrest during organogenesis. Embryos cultured with 4-OOHCPA exhibit an accumulation of S-phase cells and G2/M checkpoint arrest, suggesting a relationship between cell cycle arrest and malformations (Little and Mirkes, 1992). CPA doses that are genotoxic but do not lead to malformations have been shown to perturb the cell cycle (Francis et al., 1990), suggesting that the embryo has the ability to repair genotoxic stress and, furthermore, that check-
point arrest is integral in the prevention of teratogenesis. Perturbation of the cell cycle has been shown to occur approximately 5 h after CPA exposure (Little and Mirkes, 1992); it was hypothesized that the G2/M arrest was due to the presence of DNA cross-links that inhibit initiation of mitosis rather than an active arrest process. In this study, no changes in gene expression occurred near this time point for any PI3K family members or their downstream targets following exposure to 4-OOHCPA. If cell cycle arrest in the conceptus requires active transcription of these genes, then perhaps the conceptus is unable to activate these checkpoints, and the arrest seen is indeed due to genotoxic damage-induced structural abnormalities within the genome. The conceptual response may be stress-, tissue-, and developmental stage-specific, because exposure to methylmercury of rodent embryonic neuronal and limb-bud cells, in vitro, induced both cell-cycle arrest and an increase in GADD45 transcripts (Ou et al., 1997).

Apoptosis is a hallmark of the teratogenicity of CPA (Chen et al., 1994; Mirkes and Little, 1998). While CPA can induce DSBs, the role of PI3K family members in the response to these breaks is unclear. It is possible that PI3K family members play a role, both in the normal programmed cell death that occurs during development and in the aberrant cell death following teratogen exposure.

DNA-repair capability and downstream cellular responses to DNA damage are largely unknown during mammalian development. Only when the capacity of the conceptus to sense and respond to genotoxic teratogens is established, will the importance of these responses in the teratogenicity of genotoxic agents be determined. We suggest that one of the determinants of the ability of the conceptus to respond to oxidative stress and other genotoxic agents may be the expression of PI3K family members. The results of this study demonstrate that PI3K family members, as well as their downstream targets, are expressed in a time- and tissue-dependent manner in the rat conceptus during mid organogenesis and that they are not upregulated following genotoxic stress.

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