Repression of induced apoptosis in the 2-cell bovine embryo involves DNA methylation and histone deacetylation

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ABSTRACT

Apoptosis in the bovine embryo cannot be induced by activators of the extrinsic apoptosis pathway until the 8–16-cell stage. Depolarization of mitochondria with the decoupling agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP) can activate caspase-3 in 2-cell embryos but DNA fragmentation does not occur. Here we hypothesized that the repression of apoptosis is caused by methylation of DNA and deacetylation of histones. To test this hypothesis, we evaluated whether reducing DNA methylation by 5-aza-2'-deoxycytidine (AZA) or inhibition of histone deacetylation by trichostatin-A (TSA) would make 2-cell embryos susceptible to DNA fragmentation caused by CCCP. The percent of blastomeres positive for TUNEL was affected by a treatment × CCCP interaction (P < 0.0001). CCCP did not cause a large increase in the percent of cells positive for TUNEL in embryos treated with vehicle but did increase the percent of cells that were TUNEL positive if embryos were pretreated with AZA or TSA. Immunostaining using an antibody against 5-methyl-cytosine antibody revealed that AZA and TSA reduced DNA methylation. In conclusion, disruption of DNA methylation and histone deacetylation removes the block to apoptosis in bovine 2-cell embryos.

Introduction

During preimplantation development, the mammalian embryo goes through a period where it is resistant to proapoptotic signals. In the best studied example, the bovine, this period lasts from the 2-cell stage through the 8–16-cell stage [1–5]. Inhibition of the extrinsic pathway for apoptosis at the 2-cell stage is caused in part by resistance of the mitochondria to depolarization [4,5]. In addition, a second block exists that is revealed when the mitochondrial membrane is artificially depolarized by carbonyl cyanide 3-chlorophenylhydrazone (CCCP). In this case, caspase-9 and caspase-3 activation takes place but DNA fragmentation does not occur [4]. Thus, DNA is resistant to caspase-3 mediated events such as activation of caspase-activated DNase (CAD).

One possible explanation for DNA resistance to CAD may reside with the structure of DNA in the early preimplantation embryo. At the 2-cell stage, little transcription takes place [6–7] and DNA is highly methylated [8]. DNA demethylation occurs over the next several cleavage divisions [9]. Thus, the stage of development at which susceptibility to apoptosis is acquired (the 8–16-cell stage) is also a time of when DNA methylation is reduced [9] and transcription is activated [6].

DNA methylation can reduce the accessibility of DNases to DNA as shown for DNase I [10]. Here we hypothesize that the repression of apoptosis responses in response to mitochondrial depolarization in the 2-cell embryo is caused by DNA methylation that makes internucleosomal DNA inaccessible to activated CAD. Moreover, we hypothesize that repression requires deacetylated histones.

Materials and methods

Reagents. Materials for in vitro maturation of oocytes, in vitro fertilization, and embryo culture were obtained as described previously [11]. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was purchased from Sigma (St. Louis, MO) and was maintained in 100 mM stocks in dimethyl sulfoxide (DMSO) at –20 °C in the dark. The CCCP stock solution was diluted in embryo culture medium (called KSOM-BE2, see Ref. [12] for recipe) to 100 μM in 0.1% DMSO on the day of use. 5-Aza-2'-deoxycytidine (AZA) and trichostatin-A (TSA) were obtained from Sigma and used at a final concentration of 100 μM and 100 nM, respectively. The In Situ Cell Death Detection Kit (TMR red) was from Roche Diagnostics Corporation (Indianapolis, IN), Hoechst 33342 was from Sigma, polyvinylpyrrolidone (PVP) was from Eastman Kodak (Rochester, NY). Anti-5-methylcytosine (mouse IgG1; clone 162 33 D3) was purchased from Biolegend (San Diego, CA). The Zenon Alexa Fluor 488 mouse IgG1 labeling kit 488 and Prolong® Antifade Kit were obtained from Invitrogen.
Molecular Probes (Eugene, OR). All other reagents were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

**Experiment 1—Effects of cytosine demethylation and inhibition of histone deacetylation on induction of apoptosis by CCCP in 2-cell embryos.** Procedures for production of embryos in vitro were performed as previously described [12]. After fertilization of matured oocytes for 8 h at 38.5 °C in an atmosphere of 5% (v/v) CO2 in humidified air, putative zygotes were cultured in groups of 30 in 50-μl microdrops of KSOM-BE2 overlaid with mineral oil at 38.5 °C in a humidified atmosphere of 5% (v/v) CO2 and 5% (v/v) O2 with the balance N2. At 18 h post insemination (hpi), embryos were harvested and placed in groups of 30 in fresh 50-μl microdrops of KSOM-BE2 containing either 0.1% DMSO (vehicle), 100 μM AZA or 100 nM TSA. At 28–30 hpi, 2-cell embryos were harvested and placed in groups of 10–20 in 50-μl microdrops of KSOM-BE2 containing the same treatment as previously (vehicle, AZA or TSA) and either vehicle (0.1% DMSO, v/v) or 100 μM CCCP. Embryos were cultured for 24 h, harvested and then analyzed for TUNEL labeling.

Procedures for TUNEL were performed as described previously [13]. Slides were examined using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Gottingen, Germany) with Zeiss filter sets 02 (DAPI filter) and 15 (rhodamine filter). Digital images for epifluorescence and for light microscopy using differential interference contrast were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera. Images were merged for presentation. The Hoescht staining was digitally converted to green before merger.

The experiment was replicated six times using a total of 458 embryos.

**Experiment 2—Effects of cytosine demethylation and inhibition of histone deacetylation on DNA methylation.** The experiment was conducted as for Experiment 1 except embryos were examined for DNA methylation at the end of the experiment using immunocytochemistry with an antibody against 5-methylcytosine. Unless otherwise stated, reactions were at room temperature and reagents were diluted in phosphate-buffered saline (PBS; 10 mM KPO4, pH 7.4 containing 0.9% (w/v) NaCl) containing 1 mg/ml polyvinylpyrrolidone (PVP). Embryos were washed in PBS–PVP, fixed in 4% (w/v) paraformaldehyde, washed in PBS–PVP, permeabilized with 0.3% (v/v) Triton X-100 for 30 min, washed extensively in 0.05% Tween 20 and treated with 3 M HCl for 30 min at 37 °C. After neutralization with 100 mM Tris–HCl, pH 8.5 containing 1 mg/ml PVP, embryos were washed in 0.05% (v/v) Tween 20 and non-specific binding sites blocked by incubation in a blocking buffer consisting of PBS–PVP containing 2% (w/v) bovine serum albumin overnight at 4 °C.

The anti-5-methylcytosine antibody used for visualization of DNA methylation was labeled with Fab fragments against mouse IgG conjugated to Alexa Fluor 488 (Zenon™ Mouse Labeling IgG kits, Invitrogen Molecular Probes) as per manufacturer’s instructions. An irrelevant mouse IgG1 was similarly labeled as an isotype control. The labeled complex was diluted in blocking buffer at a final concentration of 5 μg/ml primary antibody and embryos were incubated for 1 h at room temperature in the dark. After several washes in 0.05% (v/v) Tween 20 in PBS–PVP, embryos were placed on slides and coverslips mounted using Prolong® Antifade reagent (Invitrogen). Embryos were examined using a Zeiss Axioplan 2 epifluorescence microscope with Zeiss filter sets 02 (DAPI filter) and 03 (FITC). Intensity of methylation was subjectively scored for each embryo on a scale of 0 (no methylation) to 3. A total of 61 embryos in two replicates were analyzed.

**Statistical analysis.** Data were analyzed by least-squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS for Windows, version 9.2, SAS Institute, Inc., Cary NC). Dependent variables for Experiment 1, calculated on an embryo basis, were total cell number and percent of cells that were apoptotic (i.e., TUNEL positive). Independent variables included pretreatments (vehicle, AZA or TSA), CCCP (yes vs. no) and replicate. The mathematical model included main effects and all interactions. Replicate was considered random and other main effects were considered fixed. F tests were calculated using error terms calculated from expected means squares. Differences between individual means were determined using the pdiff procedure of SAS. The dependent variable for Experiment 3 was methylation score and the independent variable was treatment.

**Results**

**Experiment 1—Effects of cytosine demethylation and inhibition of histone deacetylation on induction of apoptosis by CCCP in 2-cell embryos**

In the first experiment, embryos were treated with either AZA or TSA at the zygote stage to block cytosine methylation or histone deacetylation and then treated with CCCP at the 2-cell stage. Representative images of TUNEL labeling are shown in Fig. 1A–F. least-squares means ± SEM for total cell number are in Fig. 1G and least-squares means ± SEM for the percent of cells that were TUNEL-positive are in Fig. 1H.

Embryo growth, as determined by total cell number at the end of the experiment, was reduced by AZA, and to a lesser extent, TSA (P < 0.05) (Fig. 1G). Regardless of pretreatment, CCCP induced cell-cycle arrest as determined by a reduction in cell number (P < 0.001) (Fig. 1G).

As shown in Fig. 1H, the percent of blastomeres positive for TUNEL was affected by a treatment × CCCP interaction (P < 0.0001). CCCP did not cause a large increase in the percent of cells positive for TUNEL in embryos treated with vehicle (2.0 ± 3.4% vs. 7.7 ± 5.5%; compare Fig. 1A with D) but did cause a large increase in the percent of cells that were positive for TUNEL for embryos pretreated with AZA (5.4 ± 2.9% vs. 42.3 ± 3.2%; compare Fig. 1B, E) or TSA (17.1 ± 2.8% vs. 24.9 ± 4.2%; compare Fig. 1C, F). The magnitude of the TUNEL labeling after CCCP depolarization was less for TSA than AZA (P < 0.01) (Fig. 1H).

The degree of TUNEL labeling in the absence of CCCP was greater for embryos treated with TSA than for control embryos or embryos treated with AZA (P < 0.01). A total of 32% of TSA-treated embryos were >8 cells, a stage when apoptosis is possible. In this subset of TSA-treated embryos, the proportion of cells that were TUNEL-positive was 25.7 ± 4.1%. In control embryos >8 cells, only 1.3 ± 4.6% of cells were TUNEL positive. Thus, some of the TSA-treated embryos underwent apoptosis when developing past the 8-cell stage. None of the AZA-treated embryos were >8 cells. Further analysis of the effect of CCCP on TSA-treated embryos focused on the subset of embryos that were <8 cells (i.e., those that are ordinarily not susceptible to apoptosis). In this subset, which represents 68% of the TSA-treated embryos, there was an increase in the percent of blastomeres that were TUNEL positive after CCCP treatment (10.0 ± 4.2% vs. 24.4 ± 4.5%, P < 0.025).

**Experiment 2—Effects of cytosine demethylation and inhibition of histone deacetylation on DNA methylation**

As determined by reactivity with an antibody to 5-methylcytosine, treatment of putative zygotes with AZA or TSA reduced DNA methylation at 52–54 hpi (Fig. 2). In control embryos treated with vehicle, nuclei reacted strongly with antibody against 5-methylcytosine (Fig. 2A). Immunoreaction product was greatly reduced in embryos treated with AZA (Fig. 2B) and reduced to a lesser extent for embryos treated with TSA (Fig. 2C). The subjective score for degree of DNA methylation was greatest in control embryos...
(2.5 ± 0.1), least in AZA-treated embryos (1.0 ± 0.1), and intermediate in TSA-treated embryos (1.9 ± 0.1). Differences between each mean were significant ($P < 0.0001$).

**Discussion**

The bovine preimplantation embryo undergoes a period from the 2-cell stage to 8–16-cell stage when it is resistant to activators of the extrinsic pathway for induction of apoptosis [1–5]. The block to apoptosis involves resistance of the mitochondria to depolarization and failure of caspase-3 activation to lead to DNA fragmentation [4,5]. Here we show that the resistance of DNA to caspase-3 mediated events is the result of inaccessibility of the DNA caused by a chromatin structure dependent upon DNA methylation and histone acetylation.

5-Aza-2′-deoxycytidine inhibits DNA methylation by incorporation into DNA during replication and subsequent inhibition of DNA methyltransferases (DNMT) [14]. Treatment of embryos with AZA reversed the block to apoptosis so that CCCP treatment caused DNA fragmentation. Experiments with AZA indicate that inhibition of apoptosis caused by mitochondrial depolarization involves DNA methylation preventing accessibility of CAD to DNA. One can visualize two mechanisms by which methylated cytosines could prevent enzymatic cleavage of DNA. Methylated cytosines can repel certain proteins, for example transcription factors [15], and may also repel CAD. Alternatively, methylated cytosines can attract other proteins, such as the Sin3A histone deacetylase complex and a methyl-CpG binding protein called MeCP2 that binds tightly to chromosomes [15].
The fact that embryos treated with TSA were also capable of undergoing DNA fragmentation in response to CCCP suggests that repression of apoptosis involves histone interactions with DNA controlled by histone deacetylation. Results with TSA were more complex to interpret than for AZA because more TSA-treated embryos not exposed to CCCP experienced TUNEL labeling than for AZA-treated embryos. This effect is due to an increase in TUNEL labeling among TSA-treated embryos that were 8 cells or greater. Unlike for AZA, which caused a large reduction in cell number, TSA reduced developmental competence only slightly and many TSA embryos reached the 8–16-cell stage. In these more advanced embryos, TSA caused apoptosis in the absence of CCCP.

Given the role of DNA methylation and histone deacetylation in repressing apoptosis in early stages of development, it is proposed that the acquisition of the capacity for apoptosis at the 8–16-cell stage is dependent upon loss of DNA methylation or changes in histone acetylation. There are large species differences in the pattern of DNA methylation during early development with some species like the mouse experiencing a continual reduction in DNA methylation until the blastocyst stage while other species like the pig and rabbit do not experience large scale demethylation during early development [16]. In the cow, DNA methylation is reduced from the 2-cell to 8-cell stage and then increases by the 16-cell stage [9,17]. There are also changes in histone acetylation that occur during development with Histone H4 K5 and K12 becoming deacetylated at the one and 2-cell stages, followed by reacetylation that reaches a maximum at the 8-cell stage [17].

Given the importance of DNA methylation and histone deacetylation for repressing apoptosis in early cleavage-stage embryos, it is possible that some types of embryonic death result from inadequate DNA methylation or histone deacetylation. Patterns of DNA methylation during early development are clearly important for embryonic development because AZA caused a large reduction in embryo cell number.

In conclusion, repression of apoptosis in the 2-cell embryo involves inaccessibility of caspase-activated DNases to the DNA mediated by a chromatin structure determined by DNA methylation and histone deacetylation. Future work should focus on the particular interactions between methylated cytosines and histones responsible for this inaccessibility as well as the importance of aberrant chromatin structure and premature apoptosis in embryonic death.

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References