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Effects of cadmium on cell death and cell proliferation in chick embryos

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Abstract

The aim of this study was to examine cell death and cell proliferation in chick embryos destined to have ventral body wall defects as a result of cadmium (Cd) treatment. Embryos in shell-less culture were treated with 50 μL Cd acetate (8.9×10^{-5} M Cd^{2+}) at Hamilton–Hamburger (H.–H.) stage 16–17, or with equimolar sodium acetate. TdT-Mediated dUTP nick end labelling (TUNEL) showed the mode of cell death to be apoptosis commencing 4 h after treatment in somites and neural tube. Desquamation also occurred in the peridermal layer of the ectoderm. Cd caused no changes in the S-phase population of any tissue except ectoderm. The peridermal layer of the latter had a 40% reduction in labeling index (LI) 5.25 h after treatment but increased thereafter, being 30% greater than control values at 25.25 h. The occurrence of gross malformation was strongly correlated with the degree of apoptosis and in turn with the extent of peridermal desquamation. Pre-treatment with zinc acetate (10× the dose of Cd) prevented gross malformation, apoptosis and the effect of Cd on peridermal proliferation. We hypothesize that the ventral body wall defect resulting from Cd treatment in chick embryos is the result of changes in the somites perhaps following interruption of a signalling pathway originating in ectoderm.

Keywords: Cadmium; Gastroschisis; Zinc; Apoptosis; Periderm; Somite; TUNEL; Cell proliferation

1. Introduction

Cadmium (Cd) is a known teratogen in avian [1,2] rodent [3–5] and xenopus [6] embryos, causing facial, eye and ear defects, limb abnormalities, body wall defects, neural tube defects, heart, lung and kidney anomalies, developmental delay, and death. It also has a multiplicity of toxic effects on widespread animal species [7]. An important environmental teratogen, Cd production has risen significantly up to the last quarter of the 20th century.

In a previous study [2], we showed that treatment of chick embryos in shell-less culture at 60 h of incubation (Hamilton–Hamburger (H.–H.) stages 16–17) resulted in a defect consisting of failure of closure of the anterior body wall in the lower limb region of the embryo. Histological examination showed abnormal growth of the lateral plate mesoderm in a dorsal direction in association with this defect. The lower limbs were also carried dorsally by the aberrant mesoderm so that they projected backwards. In addition, there was a distinct bend in the notochord in the region of the gastroschisis. These defects, which occurred in about 47% of treated embryos, could be completely prevented by pre-treatment with zinc (Zn). Histological studies also revealed that 4 h after the administration of Cd, the peridermal cells of the ectoderm began to detach from each other and desquamate. At the same time, the basal layer of the ectoderm, normally consisting of a single row of elongated cells, became thicker and consisted of smaller more rounded cells. In addition, abnormal cell death occurred in ectoderm, somites and neural tube beginning at 4 h after treatment and appearing to increase in extent thereafter. Again, all these cellular changes could be prevented by Zn pre-treatment. In view of the results of experiments by Prozialeck and Niewenhuis [8] on epithelial cells in vitro, it seemed a likely hypothesis that the earliest effect of Cd was to interfere with cadherin binding between peridermal cells. These authors used trans-epithelial electrical resistance and electron microscopy to demonstrate that Cd has a damaging effect on intercellular junctions in LLC-PK1 cells. Later work by the same authors [9] and by their associates [10] showed that exposure to sublethal concentrations of Cd decreased the amount of E-cadherin associated with cell–cell contacts, and decreased trans-epithelial resistance within 4 h of exposure. If Cd interferes with cadherin binding between peridermal cells, the consequent

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disruption of periderm and the rest of the ectoderm could then have further effects on mesodermal tissues, perhaps through disruption of a signalling cascade. Alternatively, the occurrence of cell death or alterations in cell proliferation kinetics in various tissues could lead to the observed malformations.

Therefore, we thought it worthwhile to investigate further the early cell and tissue changes induced by Cd. In particular, we wanted to establish the exact mode of cell death and to quantify its occurrence. In addition we wanted to see if there were any changes in cell proliferation in the tissues that participate in the development of the abnormality since there is evidence that Cd can cause mitotic arrest in certain circumstances [11,12] and promotion of cell proliferation in others [13]. Finally, we wanted to see if the initial peridermal cell desquamation could be correlated with the degree of abnormal cell death and with abnormal growth of the lateral plate mesoderm.

2. Materials and methods

2.1. Embryos and treatments

Fertilised unincubated eggs of the Cobb strain were obtained from a local commercial hatchery (Manor Farm, Cellbridge, Co. Kildare). The eggs were placed in a forced draught incubator at 38 °C and 65–75% relative humidity until they reached the required stage for explantation and treatment. After 60 h of incubation, embryos which had reached stages 16–17 of Hamburger and Hamilton [14] were explanted into shell-less culture [2,15] and then treated after a further hour in the incubator to ensure that all embryos were at physiological temperature at the moment of dosing. All doses were given in sterile water at a volume of 50 μL delivered with a precision micropipette. The following compounds and concentrations were used. Cd^{2+} was given as the acetate at a dose of 8.9×10^{-5} M, as this is the concentration that results in 47% body wall defect without excessive embryo mortality [2]. Controls received an equimolar amount of Na^{2+} as the acetate. A third group were given Zn^{2+} as the acetate, at a concentration 10 times that of the Cd^{2+} dose followed by Cd 15 min later. This dose of Zn had previously been found to confer 100% protection against the effects of Cd. All chemicals were analytical grade.

2.2. Histological study of cell death

At 2, 4, 8 and 24 h after treatments, embryos were removed and fixed in 2% formaldehyde (prepared by depolymerising paraformaldehyde), 2% glutaraldehyde in 0.1 M Sorensen's buffer at pH 7.2 at room temperature. After dehydration in graded ethanol they were embedded in araldite and 1 μm sections were cut with a diamond knife on an ultramicrotome. Sections were mounted on gelatinised slides and stained with 1.5% toluidine blue.

The neural tube, somites and lateral plate mesoderm were examined blindly and scored for the extent of cell death by two observers whose scores were then averaged. Two slides, each with eight sections, were surveyed from each of six embryos per treatment group. Criteria used for the presence of cell death were the observation of pyknotic cells or of darkly staining phagocytosed debris within other cells. The extent of cell death was scored on a scale from 0 to 5. A score of 0 was given when no apoptotic debris was seen in sections from an embryo. The presence of up to three cells with debris was scored as 1 and three to six as 2. A score of 3 indicated that up to 30% of cells contained debris, 4 indicated between 30 and 50% of cells with debris and 5 indicated more than 50%. Scores were averaged for each embryo and the means compared by the Mann–Whitney test. A p-value less than 0.05 was taken to indicate statistical significance.

2.3. Characterisation of cell death by TUNEL

To further assess the extent of cell death and to see if cells were dying by apoptosis, tissues were examined by TdT-Mediated dUTP nick end labelling (TUNEL) using the Apoptosis Detection System, Fluorescein purchased from Promega (UK). In this method, fluorescein-tagged 12-dUTP is incorporated onto the 3′-OH ends of cleaved DNA, catalysed by the enzyme Terminal deoxynucleotidyl Transferase (TdT). Hence, fluorescein-labelled cleavage regions of DNA can be visualised in wax-embedded sections using confocal microscopy. Embryos were harvested as before, fixed in 4% formaldehyde in PBS, embedded in paraffin wax and sectioned at 7 μm. Sections were placed on silanised slides, dewaxed in xylene and rehydrated with descending concentrations of ethanol. After washing in PBS (pH 7.4), treatment with proteinase-K, and refixation in methanol-free 4% formaldehyde, each section was covered with 50 μL of a solution consisting of equilibration buffer, nucleotide mix and TdT enzyme, all supplied with the kit. Sections for negative control were treated with the above minus TdT enzyme. After incubation for one hour at 37 °C, the reaction was terminated by immersing the slides in 2×SSC for 15 min. Finally, sections were counterstained with propidium iodide (1 μg/ml) for 15 min in the dark and mounted in 50% glycerol under glass coverslips which were sealed with nail varnish. Sections were examined and recorded with a BIORAD MRC 1024 confocal laser-scanning microscope using a standard filter set at 585 nm for flourescein and at 620 nm for propidium iodide.

2.4. Studies with Lyso Tracker Red

Because TUNEL uses wax sections, it has a relatively low sampling potential. Therefore, to get a clearer picture of the extent of cell death, whole mounted embryos were studied after LysoTracker (LT) Red staining. LT Red is a weakly basic amine that selectively accumulates in cellular compartments with a low pH within. Lysosomes, due to an ATP-dependent proton pump, have a low internal pH. Once inside, the dye becomes protonated and thus becomes trapped within these organelles. Death by apoptosis results in the formation of apoptotic bodies, which are small vesicles consisting of highly cross-linked protein envelopes containing low molecular weight DNA fragments [16]. These bodies are phagocytosed quickly after formation and degraded by lysosomes. LT Red can be used to stain apoptotic bodies (Molecular Probes, 2001). This combination of methods was thought to be suitable as DNA fragmentation with concomitant preservation of cellular membranes and organelles provide reliable markers of apoptosis [17,18].

Embryos were recovered at 2, 4 and 8 h after treatment and were incubated for 30 min at 38 °C in a solution consisting of 50 μL LT red (Molecular Probes) and 50 μL dimethyl sulphoxide (Sigma) in PBS to achieve a final concentration of LT of 5 μM. After rinsing with PBS, embryos were immersed in 4% formaldehyde and fixation was continued for 24 h at room temperature. After rinsing again in PBS, embryos were dehydrated in ascending concentrations of methanol and cleared in methanol:BABB (Sigma). The latter consists of 40% methanol, 40% benzyl benzoate and 20% benzyl alcohol. After a further 30 min in BABB, the embryos were whole-mounted in 1.5 mm depression glass slides, cover slipped and sealed with nail varnish. Serial optical sections were recorded in 8 μm z-plane steps from the lumbar regions with the confocal microscope using a 585 nm filter.

2.5. Autoradiography with tritiated thymidine (^{3}H-TdR)

To study the effects of treatments on cell proliferation, embryos which had been treated with Cd, Na or Cd after zinc pre-treatment received 15 μCi ^{3}H-TdR (specific activity, 25 Ci/mmol, Amersham) 1.25 h before harvesting at 2, 4, 8 and 24 h after treatment. Six embryos were recovered for each time and treatment point. After embedding in resin as before 1 μm sections were mounted on gelatinised slides and dipped in LM nuclear track emulsion (Amersham) diluted 1:1 with distilled water and exposed in light-proof boxes at 4 °C for 7 days. After photographic processing with D-170 developer and fixation, the sections were stained with 0.5% azure II. The total number of cells and the number of labelled cells (cells in S-phase) was counted in the periderm, basal ectoderm, lateral plate mesoderm, sclerotome, dermomyotome and endoderm. From these counts the labeling index (percentage of labelled cells; LI) was calculated. Groups were compared using ANOVA and Tukey–Kramer post-test for multiple comparisons between means.