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The differentiation of cardiomyocytes from mouse embryonic stem cells is altered by dioxin

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-para-dioxin (TCDD) causes abnormalities during heart development. Cardiomyocytes derived from embryonic stem (ES) cells are a robust model for the study of early cardiomyogenesis. Here, we evaluated the effects of TCDD at key stages during the differentiation of mouse ES cells into cardiomyocytes analysing: (i) the transcription of lineage differentiation (*Brachyury*, *Nkx-2.5*, *Actc-1*), cardiac-specific (*Alpk3*, *cTnT*, *cTnI*, *cTnC*) and detoxification phase I (*Cyp1A1*, *Cyp1A2* and *Cyp1B1*) and phase II (*Nqo1*, *Gsta1* and *Ugt1a6*) genes; (ii) the global gene expression; (iii) the ultrastructure of ES-derived cardiomyocytes; (iv) level of ATP production and (v) the immunolocalisation of sarcomeric α-actinin, β-myosin heavy chain and cTnT proteins. We show that TCDD affects the differentiation of ES cells into cardiomyocytes at several levels: (1) induces the expression of phase I genes; (2) down-regulates a group of heart-specific genes, some involved in the oxidative phosphorylation pathway; (3) reduces the efficiency of differentiation; (4) alters the arrangement of mitochondria, that show twisted and disrupted cristae, and of some sarcomeres, with misalignement or disarrangement of the myofibrillar organisation and (5) reduces ATP production. This study provides novel evidences that TCDD impairs cardiomyocyte differentiation. Sarcomeres and mitochondria could be a target for dioxin toxicity, their disruption representing a possible mechanism developing cardiac injury.

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1. Introduction

The heart is considered one of the target organs of dioxin (2,3,7,8-tetrachlorodibenzo-para-dioxin or TCDD) toxicity. Dioxin is one of the most dangerous and diffused man-made xenobiotics. In the environment, dioxins tend to accumulate in the food chain and humans, being at the top of it, accumulate the highest quantities. Dioxins are of concern to the public, as short-term exposure to high levels may result in skin lesions, such as chloracne, patchy darkening of the skin and altered liver function. Long-term exposure induces impairment of the immune system, liver damage, cancer, disruption of hormone signalling pathways, reproductive and developmental defects (Mandal, 2005), including altered heart formation (Yeager et al., 2006). Like the liver and thymus, the heart expresses the AhR-Arnt-DRE system. The AhR (aryl hydrocarbon receptor) is a ligand-activated transcription factor belonging to the basic helix-loop-helix/Per-AhR-Arnt-Sim (bHLH/PAS) superfamily of proteins. The mechanism of induction of gene transcription by TCDD involves ligand recognition and binding by the AhR, nuclear translocation, and dimerization with the AhR nuclear translocator (Arnt). The TCDD-AhR-Arnt heterodimer binds to Dioxin-Responsive Elements present in dioxin-inducible genes and modulates their expression (Nebert et al., 2000; Kelling et al., 1987; Schmidt et al., 1996; Xie et al., 2006). Chronic exposure of rats to TCDD determines a dose-dependent increase in the incidence of degenerative cardiovascular lesions and ultimately cardiomyopathy (Jokinen et al., 2003). Adult mice exposed subchronically to TCDD developed high blood pressure and cardiac enlargement

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(Kopf et al., 2008), whilst exposure to an acute high dose of TCDD determined an increase in triglyceride levels (Dalton et al., 2001). Some of these changes may be ascribed to altered gene expression, inflammation and oxidative stress (Lund et al., 2005; Arzuaga et al., 2007), whereas others are related to direct effects on calcium signalling in cardiomyocytes (Xie et al., 2006).

During development, TCDD has been shown to cause heart and vascular abnormalities in a variety of animal models (Jokinen et al., 2003; Kopf et al., 2008; Dalton et al., 2001; Lind et al., 2004). These studies have demonstrated dioxin-related decrease in cardiomyocyte proliferation very early during development in chick and zebrafish embryos and in the murine fetus (Ivnitski et al., 2001; Antkiewicz et al., 2005; Thackaberry et al., 2005a; Goldstone and Stegeman, 2006). Thackaberry et al. (2005b), by microarrays analysis of gene expression, have shown global changes in cardiac gene transcription following TCDD exposure during *in vivo* development of the fetal murine heart, indicating possible alterations during cardiac cell differentiation.

The effects that TCDD exerts on cardiomyocytes whilst they differentiate is still poorly known. To this end, the use of embryonic stem (ES) cells, an established model of *in vitro* cardiomyocyte differentiation (Kehat et al., 2001; Xu et al., 2002) has been exploited for the first time in a recent study, in which TCDD has been found to alter the expression of developmentally regulated genes in a variety of differentiation pathways and in a number of homeotic genes (Wang et al., 2010).

Mouse ES cells differentiate into cardiomyocytes through the formation of embryoid bodies (EBs) that recapitulate, among others, the developmental stages characteristic of mouse fetal heart development (Boheler et al., 2002). ES cell-derived cardiomyocytes express cardiac gene transcripts in a developmentally controlled manner at time-points corresponding to three main developmental stages: (1) early, primary myocardial-like cells, at 4 days of differentiation; (2) intermediate, myofibrillogenic commitment, at 7 days of differentiation and (3) terminal, cardiomyocytes at 12 days of differentiation.

Compared to *in vivo* studies, the use of an *in vitro* model of cardiomyocyte differentiation (cardiomyocytes may be obtained also from human ES cells, Kehat et al., 2001; Schuldiner et al., 2000) has the advantage of allowing a more precise definition of the effects exerted by TCDD on a specific cell type (i.e., cardiomyocytes) compared to the entire heart and permits the identification of primary effects that *in vivo* would be more difficult to distinguish from secondary effects resulting from damages to other tissues and organs (e.g., liver and kidney). Furthermore, this *in vitro* model allows the investigation of the effects of TCDD at different time points during cardiomyocyte differentiation and to study their functionality without quantitative limitations, a difficult task to carry out using animal models.

Whilst some of the molecular changes induced by TCDD during the differentiation of ES cells into cardiomyocytes are beginning to emerge (Wang et al., 2010), its effects on the cellular organisation and on global gene expression have not been investigated yet. Thus, in the presence or absence of TCDD, at specific time points along the process of differentiation of ES cells into cardiomyocytes we have analysed: (1) on day 4, 7 and 12, the pattern of transcription of *Brachyury* (mesoderm marker), *Nkx-2.5* (cardiac mesoderm marker) and *alpha c actin* (*Actc-1*, cardiomyocyte maker) genes; (2) on day 4, 7 and 12, the constitutive expression and TCDD-inducibility of phase I (cytochromes P450 *Cyp1A1*, *Cyp1A2* and *Cyp1B1*) and phase II (*Nqo1*, *Gsta1* and *Ugt1a6*) genes, involved in the detoxification response; (3) on day 12 from EBs formation, the ultrastructural characteristics of ES-derived cardiomyocytes; the immunolocalisation of sarcomeric α-actinin, β-myosin heavy chain (β-myosin) and cardiac troponin T (cTnT) proteins, markers of their differentiated status (Boheler et al., 2002) and by microarrays the transcriptome profile.

2. Materials and methods

2.1. ES cells culture

The mouse low-affinity R1 ES cell line [established from (129X1/SvJ x 129S1) F1 3.5 days post coitum (dpc) blastocyst; kindly provided by Dr. Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada], was cultured as previously described (Neri et al., 2008). Briefly, ES cells were maintained on a mitomycin C-treated STO feeder-layer (ATCC, CRL-2225) in a complete ES medium supplemented with 15% ES Cell Qualified Fetal Bovine Serum (Gibco) and 500 U/ml ESGRO-LIF (Chemicon International). ES cells were routinely passaged every 48 h and maintained in an incubator at 37 °C with 7.5% CO_2 in air. Culture of ES cells in medium containing TCDD (Cambridge Isotope Laboratories, Inc.) was begun after three passages on gelatin, allowing the elimination of the feeder layer of fibroblasts, as confirmed by microscopic observations. TCDD, dissolved in dimethyl sulphoxide (DMSO, used as vehicle, Sigma–Aldrich), was added to the complete ES medium to a final concentration of 100 nmol/l. This concentration was chosen based on the results of our earlier study that showed the highest TCDD-induction of Cyp1A1 expression in undifferentiated mouse ES cells obtained from a TCDD low-affinity strain (Neri et al., 2008). Control samples, cells cultured in the absence (Ctrl) or presence (DMSO) of 0.06% DMSO, were carried out and analysed in parallel.

2.2. Embryoid bodies formation and cardiomyocytes differentiation

To ensure sample uniformity EBs were formed from 1×10^3 ES cells using the hanging drop method (Wobus et al., 2002) in an ES cell medium without LIF, containing 100 nmol/l TCDD; the medium was changed regularly and used throughout differentiation. After 3 days of culture in drops, EBs were passaged to 100 mm Petri dishes (Corning) for culture in suspension and on day 5, 3 EBs were plated in each 1.9 cm^2 well of a 24 wells plate (Corning). On day 4, 7 and 12 of EBs differentiation, cells were harvested for RT-PCR and immunocytochemistry (on day 12) analyses. Controls (see above) were carried out and analysed in parallel.

2.3. Contraction assay

On day 7 and 12 of differentiation the number of beating EBs was counted (zur Nieden et al., 2004). To evaluate the frequency of contraction, the number of beats *per* min was counted for every ES-derived cardiomyocyte sample in at least 10 beating areas. The experiment was repeated three times. The results are presented as mean ± standard deviation.

2.4. RT-PCR analysis

RT-PCR analyses were performed as previously described (Neri et al., 2008). Briefly, total RNA was isolated using the GenElute Mammalian Total RNA Kit (Sigma–Aldrich), following the manufacturer's instructions. All traces of DNA contamination were eliminated using an on-column DNaseI digestion kit (Sigma–Aldrich). 1 μg of RNA was retrotranscribed and the cycles used for the PCR of all the genes under study were optimised. The optimal number of PCR cycles for each gene was selected within the linear range of the regression curve. The following cycle programme was used for PCR amplification: 94 °C for 5 min, followed by *n* cycles (*Cyp1A1*, *Gsta1* and *Nkx-2.5* *n* = 35; *Cyp1A2*, *n* = 38; *Cyp1B1* and *Brachyury*, *n* = 33; *Nqo1*, *n* = 28; *Actc-1*, *n* = 29; *Ugt1a6*, *n* = 40; *Gapdh*, *n* = 25) at 94 °C for 30 sec, and *Tm* (*Cyp1A* at 61 °C; *Cyp1A2*, *Cyp1B1*, *Gsta1*, *Nqo1* and *Ugt1a6* at 60 °C; *Gapdh* at 55 °C) for 30 s, 72 °C for 30 s, followed by a single step at 72 °C for 7 min. The primer sequences for phase I and phase II genes are those described earlier (Neri et al., 2008). PCR products were sequenced and showed 99% homology with the sequence found in ENSEMBL. 10 μl of PCR product were mixed with 2 μl of loading buffer and were electrophoresed on a 2.5% agarose gel in 0.5× TBE containing 0.1 μg/ml ethidium bromide at 6 V/cm for 45 min. The PCR products were visualised under short-wave length UV and quantified using a Bio-Rad Gel Doc system by comparing, after normalisation on the internal control *Gapdh* product, the known quantity of the low mass ladder marker bands with those obtained from the amplification of the gene sequences.

For quantitative real-time PCR, one twentieth of the cDNA resulting from 1 μg of the retrotranscribed RNA was amplified in triplicate with a Rotorgene 6000 (Corbett Life Science) in 20 μl reaction mixture containing 200 nM of each specific primer and the MESA GREEN qPCR MasterMix Plus for SYBR assay no ROX sample (Eurogentec) at 1× final concentration. The amplification reaction was as follows: 95 °C for 5 min, followed by 30 cycles at 95 °C for 10 s, 60 °C for 15 s, 72 °C for 20 s. The Rotorgene 6000 Series Software 1.7 was used for comparative concentration analysis. β-2-Microglobulin expression was used for sample normalisation. PCR products specificity from each sequence analysed was confirmed using melting curve analysis (from 56 °C to 95 °C) and subsequent agarose gel electrophoresis. Primer sequences were as follows: *Alpk3*, F 5′ AACGGCAGCTTTCTTGTCAC 3′, R 5′ CTCTCCTTGAGGCCTTGGTA 3′; *cTnC*, F 5′ CAGCAAAGGGAAGTCTGAGG 3′, R 5′ TGCAGCATCATCTTCAGCTC 3′; *cTnT*, F 5′ GAAGTTCGACCTGCAGGAAA 3′, R

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