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Monolayer cultivation of osteoprogenitors shortens duration of the embryonic stem cell test while reliably predicting developmental osteotoxicity

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ABSTRACT

Osteotoxic compounds administered during pregnancy can initiate skeletal congenital anomalies in the embryo. *In vitro*, developmental osteotoxicity of a compound can be predicted with the embryonic stem cell test (EST), the only *in vitro* embryotoxicity model identified to date that entirely abrogates the use of animals. Although the previously identified endpoint osteocalcin mRNA expression robustly predicts developmental osteotoxicity, it can only be assayed after 5 weeks of *in vitro* culture with existing embryoid body (EB)-based differentiation protocols. Therefore, the goal of this study was to characterize novel earlier endpoints of developmental osteotoxicity for the EST.

The currently used EB-based differentiation protocol was modified so that a monolayer culture of pre-differentiated cells was inoculated. The expression profile of five bone-specific mRNAs, including osteocalcin, over the course of 30 differentiation days suggested an acceleration of pre-osteoblast specification in the monolayer over the EB-based protocol. Similarly, calcification was already visible after 14 days of culture in monolayer cultures. Employing image and absorption-based techniques to measure the degree of mineralization in these cells after compound treatment, the three compounds Penicillin G, 5-fluorouracil (5-FU) and all-trans retinoic acid (RA) were then tested after 14 days in monolayer cultures and compared to embryoid body-based differentiations at day 30. By modifying the culture the three test substances were classified correctly into non- or strong osteotoxic. Moreover, we were successful in shortening the assay duration from 30 to 14 days.

1. Introduction

Toxicology programs are designed to identify toxicities that may potentially be encountered in humans, helping to ensure that the initial human studies are conducted safely and ethically. In this context, embryotoxicity studies provide supportive information to augment the interpretation of toxicology findings. Embryotoxicity tests are currently performed according to OECD guidelines *in vivo* in animal models. To reduce the amount of animal testing, a variety of *in vitro* assays have been proposed, such as the Micromass-Test, whole embryo culture or FETAX test. Ultimately, these tests have been routinely used by industry although their predictive value lies only between 70% and 80% (Genschow et al., 2002). However, they are very labor-intensive and require the killing of pregnant animals.

Embryonic stem cells (ESCs) are a powerful and valuable tool to examine general embryotoxic potentials of compounds, a circumstance that has been proven by the German Ministry for Risk Assessment (BfR) with an EU-wide evaluation study of the classic Embryonic Stem Cell Test (EST) (Genschow et al., 2000; Spielmann et al., 2001). The EST compares important aspects of prenatal toxicity: (a) the inhibition of differentiation of embryonic stem cells (ESCs) into contracting cardiomyocytes caused by compound treatment, and (b) the difference in sensitivity of ESCs compared to adult fibroblasts. However, the semi-quantification of either present or absent contracting cardiomyocytes has been difficult to standardize and requires experienced personnel.

While the EST has traditionally evaluated developmental toxicity in the endpoint cardiogenesis, assessment of skeletal differentiation has been introduced as a novel endpoint in the next step of assay refinement (zur Nieden et al., 2004). This was primarily based on the rational that toxicological studies in animal models have previously shown that the heart is *not* the primary target organ for most embryotoxic substances. In the past decade, protocols have been established that allow ESC differentiation into bone and its characteristic cell type, the osteoblast, which actively

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secretes the bone matrix (Phillips et al., 2001; Buttery et al., 2001; zur Nieden et al., 2003). The use of quantitative mRNA expression analysis of the bone-specific osteocalcin (OCN) gene in the improved EST model has allowed for the correct classification of a test set of six substances according to their known *in vivo* osteotoxic potential (zur Nieden et al., 2004). Although predictive and reliable, OCN expression has not gained significant attention as an endpoint after it had been introduced in 2004 (zur Nieden et al., 2004), simply because less costly means of measuring differentiation outcome are preferred to keep costs low. Assaying for the degree of mineralization in the osteogenic cultures has more recently been shown to successfully discriminate strong developmental osteotoxicants from non-developmental osteotoxicants (zur Nieden et al., 2010), providing a more inexpensive and affordable endpoint read-out.

While these novel osteotoxic endpoints offer the advantage of getting reproducible results in an inexpensive manner, there are still problems with the assay duration, which is based on the currently available differentiation methodology. Published differentiation protocols for murine ESC osteogenic differentiation call for embryoid body (EB) formation as the initial step of differentiation and subsequent plating of an intact EB onto tissue-culture adhesive substrates (zur Nieden et al., 2003). This technique generates approximately 60% of osteoblasts in each plated EB (zur Nieden et al., 2007). Although this differentiation protocol is very robust and repeatedly gives similar numbers of osteoblasts, one downside is the culture time needed for the cells to mineralize. Such ESC-derived osteoblasts reach a state of full maturity only after 30 days of differentiation *in vitro* as assessed by the expression of osteoblast-specific genes (*i.e.* OCN) (zur Nieden et al., 2003). Taking into consideration that mice gestate 20 days, such a long test duration is not acceptable for a routine screening assay. As results from an *in vivo* study would be accessible earlier, industry has no compelling reason to replace current *in vivo* with *in vitro* tests to reduce overall animal testing. Alternative osteogenic protocols have been suggested in the past, which disperse the EBs before plating on day 5 (Buttery et al., 2001). Those studies imply that OCN expression is detectable in such monolayer cultures after only 21 days of maturation (Buttery et al., 2001). Our goal is therefore to identify superior culture conditions and innovative osteogenic endpoints that would allow for earlier assessment of developmental osteotoxicity, without losing predictive value or robustness of the system. Here, we were able to show that using monolayer cultures of ESC-derived osteoprogenitors combined with IMAGE analysis and assessment of calcification with an absorption-based test, we were able to reduce the length of the osteotoxicity assay in the EST from 30 to 14 days.

2. Materials and methods

2.1. Test compounds

We chose to test Penicillin G (PenG), 5-fluorouracil (5-FU) and all-trans retinoic acid (RA) since a sufficient amount of toxicology has been published for these substances previously. After PenG and 5-FU were originally used for validating the classic EST, they have found wide-spread application as positive and negative control substances for assessing the validity of novel test approaches (Scholz et al., 1998; Spielmann et al., 2001; zur Nieden et al., 2004, 2010). All substances were purchased from Sigma. Since 5-FU and RA are not water soluble, they were dissolved and diluted in DMSO. Final concentrations of DMSO were kept below 0.01%, as this solvent itself has been shown to induce differentiation of ESCs at higher concentrations (Adler et al., 2006). Solvent control cultures containing DMSO only were included for all endpoints. Since sensitivity of ESCs to toxic agents is related to their differentiation state (Laschinski et al., 1991), compound treatment was performed throughout the entire culture time.

2.2. Cytotoxicity assay

The murine embryonic stem cells line D3 was sub-cultured every other day in high LIF (1000 U/ml) medium (zur Nieden et al., 2001) and thus maintained in the undifferentiated state. To determine cytotoxicity of the three test compounds, cultures were started at 3125 cells/cm^2 and cultivated with test compound for 10 days. Cells were then assayed with an MTT test by incubation with MTT solution (Sigma) for 2 h at 37 °C and changes in absorption were measured in a Benchmark Plus spectrophotometer (Biorad) at 570/630 nm (zur Nieden et al., 2001, 2010). Both the test compound and the solvent control were tested in six independent experiments and changes in mitochondrial dehydrogenase activity in compound treated cultures were normalized to solvent control cultures, which were set as 100%. The cytotoxicity of the test substance was determined from a dose–response curve as that concentration, which reduced the growth of the cells by 50% (IC_{50}).

2.3. Differentiation of ESCs

Osteogenic differentiation of ESCs was induced according to a previously published protocol (zur Nieden et al., 2003, 2004). Briefly, differentiation was initiated in hanging drops from 3.75×10^4 cells/ml. Embryoid body (EB) formation was allowed to take place for 3 days. Subsequently, EBs were grown for an extra 2 days in non-adherent conditions. On day 5 of the culture period, EBs were either plated intact or as a dispersed single cell suspension onto tissue-culture adhesive substrate. The single cell suspension was generated by trypsinizing EBs for 5 min and subsequent titration. Cells were counted with trypan blue exclusion and 50,000 live cells/cm^2 were seeded onto gelatin-coated plastic ware. EBs and monolayer cultures were supplemented with 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 5×10^{-8} M 1α,25OH_2 vitamin D_3 from day 5 onwards (zur Nieden et al., 2003). Differentiation success was microscopically evaluated every second day. Media changes were always performed after microscopy to avoid photodegradation of the compounds and media supplements.

2.4. IMAGE analysis

The degree of calcification of ESC cultures may be quantitatively assessed with morphometric analysis (zur Nieden et al., 2007). This is possible because mineralized osteoblast-specific matrix appears black in phase contrast and brightfield optics (zur Nieden et al., 2003, 2007). The key to this technique is taking grey-scale photographs of cells at the same exposure time and with the same microscopic settings as described (zur Nieden et al., 2010). Pictures were taken on day 30 of the differentiation (EBs) or on day 14, respectively, with the SPOT Advanced Imaging system (Diagnostic Instruments) mounted to an Olympus IX70 inverted microscope and a 2× objective with a consistent gamma adjustment of 1.8. Differences in mean black pixel values were then calculated with the IMAGE J 1.33u software (http://www.rsb.info.nih.gov/ij/) for each compound concentration (three independent experiments with six technical replicates each) and expressed as percent up-regulation above the osteogenic solvent control, which was set as 100%.

2.5. Ca^{2+} determination

The degree of osteogenesis was quantified by measuring the amount of matrix incorporated calcium on day 30 of differentiation (zur Nieden et al., 2005). EBs or cells (5 wells ea concentration, three independent experiments) were washed with 1× PBS to remove any remaining traces of medium. RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% Deoxycholate, 5 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 2 μg/ml leupeptin, 100 μM sodium orthovanadate and 10 mM p-nitrophenylphosphate was used to lyse the cells at 4 °C with gentle rocking. Aliquots of sample lysates were added to Arsenazo III reagent (DCL, Toronto, Canada) alongside a standard curve. The change in absorbance from purple to blue was detected at 650 nm in a Benchmark Plus microplate spectrophotometer (Biorad). The calcium content of each sample was standardized to its total protein content, which was measured from the same lysate with the DC protein assay reagent (Biorad) as previously described (zur Nieden et al., 2010). Values obtained for compound treated cultures were normalized against solvent control cultures.

2.6. Quantitative PCR

Twenty EBs per treatment group or a well of a 24-well plate for monolayer cultures ($n = 3$) were harvested in RNA lysis buffer (Qiagen) after 30 days of osteogenic induction and compound treatment. RNA was isolated with the Qiagen RNeasy Mini Kit as suggested by the manufacturer. RNA concentration was measured fluorometrically upon incorporation of RiboGreen (Invitrogen) and 625 ng of RNA per sample were subjected to cDNA synthesis as described in a total reaction volume of 25 μl (zur Nieden et al., 2001). Expression of genes specific for bone tissue was determined with quantitative real-time PCR in a Biorad iCycler using a SYBR green PCR master mix (Biorad) as described (Cormier et al., 2006). Primer sequences were as previously published (zur Nieden et al., 2003, 2004, 2007, 2010). Post-run melting curves were performed to verify that the measured signal was specific to the desired reaction product. The *n*-fold expression in target samples was calculated with the $\Delta\Delta C_T$ method by standardizing to GAPDH and normalizing to non-treated osteogenic solvent controls for test compounds or to undifferentiated ESCs for time course analyses. Either the non-treated controls or undifferentiated cells were set as 100% to calculate the percent deregulation in target samples.