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Intra-laboratory validated human cell-based *in vitro* vasculogenesis/angiogenesis test with serum-free medium

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ABSTRACT

Vasculogenesis and angiogenesis are the processes by which new blood vessels are formed. We have developed a serum-free human adipose stromal cell and umbilical cord vein endothelial cell based vasculogenesis/angiogenesis test. In this study, the test was validated in our GLP laboratory following the OECD Guidance Document 34 [1] using erlotinib, acetylic salicylic acid, levamisole, 2-methoxyestradiol, anti-VEGF, methimazole, and D-mannitol to show its reproducibility, repeatability, and predictivity for humans. The results were obtained from immunostained tubule structures and cytotoxicity assessment.

The performance of the test was evaluated using 26 suspected teratogens and non-teratogens. The positive predictive value was 71.4% and the negative predictive value was 50.0%, indicating that inhibition of vasculogenesis is a significant mechanism behind teratogenesis. In conclusion, this test has great potential to be a screening test for prioritization purposes of chemicals and to be a test in a battery to predict developmental hazards in a regulatory context.

1. Introduction

Blood vessels are constructed by two distinct processes, vasculogenesis (initiated from endothelial progenitor cells) [2–4] and angiogenesis (new vessels sprout from pre-existing vessels) [5,6]. The importance of properly formed vasculature is highlighted by the fact that the cardiovascular system is the first functional organ to develop in the embryo [7]. Disrupted vasculogenesis may lead to impaired vasculature formation during embryonic development, which often causes embryonic malformations at different severity levels (depending on the timing of the exposure and the dose of the chemical) [8]. Vascular disruption, especially during the first trimester of pregnancy, has been proposed to be the mechanism for a large spectrum of malformations, such as scalp anomalies, abdominal wall defects, limb reduction and restriction of growth, or death of the embryo or fetus [9,10].

On the other hand, angiogenesis may be the intended target of drug treatment. Especially in cancer treatments, the primary mechanism of many drugs is the prevention of blood vessel formation [11], but unintentional loss of blood vessels or impaired angiogenesis can also cause pathological conditions, such as diabetic ulcers [12,13] and pre-eclampsia [14,15]. The reverse situation – excessive angiogenesis – is a worsening factor in several diseases, such as macular degeneration [16,17] and endometriosis [18,19]. The anti-angiogenic or pro-angiogenic properties of chemicals used in everyday life (including environmental chemicals) is a growing safety concern [20]. The adverse outcome pathways (AOP) for embryonic vascular disruption and developmental defects is included in the Organisation for Economic Co-operation and Development (OECD) AOP list (OECD Project 1.6).

Animal tests are presently used to predict vascular disruption [21], although their relevance in predicting effects in humans have been questioned [22–25]. Furthermore, there are *in vitro* tests available for studying vascular distruptors, but none are yet accepted for regulatory use. Thus, easy-to-use and human biology-based test models are urgently needed. Our model is solely based on human umbilical cord vein endothelial cells (HUVEC) and adipose stromal cells (hASC) with a minimal amount of xenologous agents [26,27]. The advantage is that both vasculogenesis and angiogenesis are captured. Vasculogenesis is initiated from endothelial precursor

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and stem cells in the hASC population [28,29], while the angiogenesis process is led by the HUVEC population [30,31]. Serum-free medium is used in the test [32].

The objective of this study was to demonstrate that the developed vasculogenesis/angiogenesis test is suitable and reliable for its intended use, which is to detect potential vascular disrupters (*e.g.* industrial chemicals, biocides, and pharmaceuticals) of vasculogenesis and angiogenesis in the human cellular system. The test was validated using seven reference (profiency) chemicals; five well-known inhibitors of blood vessel formation – levamisole, acetyl salicylic acid, erlotinib, anti-VEGF, and 2-methoxyestradiol – and two non-inhibitors – D-mannitol and methimazole. The performance and relevance of the test was further evaluated by using a larger set of chemicals and comparing the results to actual *in vivo* concentrations found in patients.

2. Materials and methods

2.1. Materials

The materials used were EGM-2 medium (EBM-2 Basal medium, #CC-3156 and EGM-2 SingleQuots supplements #CC-4176, both Lonza), DMEM/F12 Dulbecco's modified Eagle medium: nutrient mixture F-12 (Gibco Invitrogen # 21331), HS Human serum (PAA #C15-021), L-glutamine (Gibco Invitrogen #25030), Penicillin/streptomycin (Gibco Invitrogen #15070), Recombinant human FGF basic (R&D Systems #233-FB), Recombinant human VEGF (R&D Systems #293-VE), Sodium pyruvate 100 x (Gibco Invitrogen #11360), BSA Bovine Serum Albumin Fraction V (Roche Diagnostics #10735086001), L-ascorbic acid (Sigma #4544), Sodium hydroxide solution 0.1 mM (Fluka #38210), Hydrocortisone (Sigma #H0888), 0.2% Heparin solution (Stemcell Technologies #07980), ITS premix [lyophilized powder containing 25 mg insulin, 25 mg transferrin, 25 μg Selenious Acid] (BD Biosciences #354351), T3 3,3′,5-Triiodo-L-thyronine (Sigma #T6397), Anti-von Willebrand Factor, rabbit primary antibody (Sigma Aldrich #F-3520), Anti-collagen IV, mouse primary antibody (Sigma Aldrich #C1926), Polyclonal Antibody to Rabbit IgG TRITC (Sigma Aldrich #T6778), Polyclonal Antibody to Mouse IgG FITC (Sigma Aldrich #F4143), Triton X-100 (BDH Prolabo #437002), DPBS Phosphate Buffered Saline with Ca++/Mg++ (Lonza # BE17-513F), PBS Phosphate Buffered Saline (Lonza # BE17-516F), TrypLE Express (Gibco Invitrogen #12604), ddH_2O (Gibco Invitrogen #15230), and Cell proliferation Reagent WST-1 (Roche #11644807001). The test chemicals used are shown in Table 1.

2.2. Cells

The human adipose tissue samples and human umbilical cords were received from Tampere University Hospital, Tampere, Finland. The use of human adipose tissue and human umbilical cords were approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland with permit numbers of R03058 and R08028, respectively. All donors gave written informed consent. This study conforms to the principles outlined in the Declaration of Helsinki. hASC and HUVEC were isolated as described in Sarkanen et al. [26].

2.3. Cell culture

hASC and HUVEC were expanded separately before seeding for exposure. The hASC cells were propagated for 7–14 days in hASC cell culture medium (DMEM/F12 supplemented with 10% HS and 1% L-glutamine) and the HUVEC cells were propagated for 3–4 days in EGM-2 medium. The co-culture for the test (hASC, 20,000 cells/cm^2; HUVEC, 4000 cells/cm^2) was established on 48 well plates one day before the exposure to chemicals.

Individual differences between cell batches inevitably exist when the cells are isolated directly from primary human tissue. To overcome the batch-to-batch variation, we included pre-set quality acceptance criteria for the cell batches. For HUVEC, the ability of cell batches to adequately form tubules is scored before the batch is accepted for use. hASC are evaluated with a cytometer for the expression of stem cells markers CD73, CD90, and CD105 [33,34].

2.4. Chemical testing

Chemical stocks for the exposures were freshly prepared on the days of use. The exact purity of the chemicals was used in the concentration calculations. Twelve different concentrations of each chemical with two replicates were used. The dilutions were made in serum-free stimulation medium (SFSM) as described in Huttala et al., 2015 (DMEM/F12, 2.56 mM L-glutamine, 0.1 nM 3,3′,5-Triiodo-L-thyronine sodium salt, ITS^{TM} Premix: 1.15 μM: 6.65 μg/ml insulin, 6.65 μg/ml Transferrin, 6.65 ng/ml selenious acid, 1% Bovine serum albumin, 2.8 mM Sodium pyruvate, 200 μg/ml Ascorbic acid, 0.5 μg/ml Heparin, 2 μg/ml Hydrocortisone, 10 ng/ml VEGF, and 1 ng/ml FGF-β) [27]. When the chemical was dissolved in dimethyl sulfoxide (DMSO), the dilution series was also prepared in DMSO. Then, the dilutions were further diluted in SFSM in order to reduce the DMSO concentration to 0.5%, thus the amount of solvent was constant in all test cultures and the only variable was the test chemical. Twenty-four hours after starting the hASC and HUVEC co-culture (*i.e.* on day 0 of the test), the HUVEC cell culture medium was removed and the test chemicals, positive controls (*i.e.* SFSM), the negative controls (SFSM without ascorbic acid, hydrocortisone, heparin, VEGF and FGF-β), and the vehicle controls were added to the co-culture wells (Exposure 1). On the third day after exposure, the exposure was repeated (Exposure 2). The total exposure time was 6 days, after which a WST-1 cytotoxicity assay was conducted by adding 50 μl of WST-1 reagent per well and incubating the plates at 37 °C in 5% CO_2 for 1.5 h. The resultant absorbance was measured at 450 nm with a Varioskan Flash Multimode Reader. After the WST-1 assay, the cells were fixed with 70% ethanol for 30 min, permeabilized for 15 min with Triton X-100, blocked for 30 min with 10% BSA, and then the primary antibodies against von Willebrand factor (dilution 1:100) and collagen IV (dilution 1:500) were applied overnight. The next day, secondary antibodies were applied for 45 min; with anti-rabbit TRITC for von Willebrand factor and anti-mouse FITC for collagen IV. The stained fluorescent tubular structures were evaluated using the automated microscope image analysis system Cell-IQ (CM Technologies, Tampere, Finland). In the analysis, the tubule area and the number of branching points were determined. The result was weighted 0.75 * for tubule pixels and 0.25* for number of branches to obtain tubule formation score. The tubule formation score was normalized to positive tubule formation control (the highest level of tubule formation induced with stimulatory factors = 100%) to obtain the percentage of control value (% of control) and depicted as mean ± SD. EC_{50} values were calculated using GraphPad Prism 6.05 software using a sigmoidal Hill's logistic equation (when applicable to the data).

2.5. Intra-laboratory validation

The validation of the vasculogenesis/angiogenesis test was performed in a Good laboratory practice (GLP) laboratory in accordance with OECD Guidance Document 34 [1]. In the validation, two technicians performed the test with seven reference (profiency) chemicals (shown in Table 1) and three separate testing times using three different HUVEC cell batches from different donors, but the