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# Cytotoxicity of 5-fluorouracil: Effect on endothelial differentiation via cell cycle inhibition in mouse embryonic stem cells

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ABSTRACT

Embryonic stem cells (ESCs) are known to characteristics for pluripotency and self-renewal, but the precise mechanisms of ES-derived cells to specific toxicants have not been determined. Here, we evaluated the cytotoxicity of 5-fluorouracil (5-FU) and see its effect on cell viability, proliferation, and differentiation in mouse ESC-derived endothelial differentiation. Mouse ESCs were exposed to 5-FU (10 μM) and combined with probucol (50 μM) for 24 h, which is an antagonist of 5-FU. Changes in gene expression as a result of 5-FU exposure in mouse ESC-derived endothelial precursor cells (ES-EPCs) were assessed using an oligonucleotide microarray (AB1700). The expression of Oct-4 was decreased during the differentiation of mouse ESCs into endothelial cells; otherwise, the expression of PECAM was increased. Mouse ES-EPCs were shown to have a decrease in viability (49.8%) and PECAM expression, and induce G1/S phase (31.1%/60.6%) when compared with/without treatment of 5-FU. Expression of cell cycle-related proteins was increased in endothelial precursor cells exposed to 5-FU without probucol treatment. From theses results suggest that 5-FU inhibit endothelial differentiation as well as inducing the G1/S phase arrest. We propose that mouse ES-EPCs might be a useful tool for screening the cytotoxicity of compounds in endothelial cells.

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## 1. Introduction

Embryotoxicity tests using animals are a traditional strategy to identify potentially hazardous chemicals. They can also be used to confirm the absence of toxic properties in the development of potentially useful new substances (Brown et al., 1995). These *in vivo* tests have several limitations, such as validation of animal models, enormous cost, high labour intensity, time required to generate meaningful results, and ethics for animal experiments (Knight, 2007; Bremer and Hartung, 2004). Therefore, there is a need for alternative methods to evaluate the potential reproductive toxicity of chemical substances, by *in vitro* systems. To develop a new alternative screening test, many scientists have tried to use cell lines, primary cell cultures of dissociated cells from mice or rat embryo limb buds, midbrains for micromass tests, or whole embryos from rat (Steele et al., 1983).

Mouse embryonic stem cells (mouse ESCs) are able to differentiate into various cell types, including three germ layers as pluripotent cells derived from the inner cell mass of blastocysts. They can also undergo unlimited self-renewal (Evans and Kaufman, 1981; Martin, 1981; Ramalho-Santos et al., 2002). Therefore, an embryonic stem cell test (EST), which mirrors growth and differentiation, is an *in vitro* test system well-suited for the evaluation of the embryotoxic potential of substances (Spielmann et al., 1997).

Endothelial, endothelial-like cells, and endothelial precursor cells (EPCs) derived from stem cells have been explored to establish a toxicity screening system for endothelial-specific toxicants (Kim and von Recum, 2008). The feasibility of these screening systems depends on the differentiation processes of the ESCs used; guided differentiation into target cell types and accurate investigation of the mechanisms of endothelial toxicity are necessary. Recently, we reported that endothelial-like and endothelial cells derived from mouse ESCs using EGM medium and optimal protocols are more sensitive to 5-FU toxicity than undifferentiated endothelial cells as well as a mouse endothelial cell line (Kim et al., 2008).

*Abbreviations:* Mouse ESCs, mouse embryonic stem cells; EBs, embryoid bodies; EGM-2, endothelial growth medium-2; LIF, leukaemia inhibitory factor; MEF, mouse embryonic fibroblast; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5,-diphenyl tetrazolium bromide; 5-FU, 5-fluorouracil; PECAM, platelet endothelial cell adhesion molecule; Probucol, 4,4′-(isopropylidenedithio) bis (2,6-di-t-butylophenol); CDK, Cyclin-dependent kinase; CDKI, Cyclin-dependent kinase inhibitor; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde-3 phosphate dehydrogenase.

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5-Fluorouracil (5-FU) is one of the most widely used as an anti-cancer or anti-angiogenesis agents for advanced carcinoma and works through G1/S cell cycle arrest and the induction of apoptotic death of the cancer cells (Lewin et al., 1987; Dimery and Hong, 1993). Also, 5-FU induces a direct toxic effect on the endothelium (Kinhult et al., 2003). However, the exact molecular biological role of 5-FU on cell cycle regulation in the endothelial differentiation of mouse ESCs has not been fully explained yet.

The objectives of this study were to investigate the specific action of 5-FU on the endothelial differentiation of cells derived from mouse ESCs. Secondly, to investigate the correlation between cell cycle regulation and endothelial differentiation in mouse ESCs exposed by 5-FU.

## 2. Materials and methods

### *2.1. Cell culture conditions and endothelial cell differentiation*

Mouse $D_3$ ESCs (ATCC Cat. No. CRL-1934, Rockville, MD, USA) were co-cultured with mitomycin C-treated mouse embryonic fibroblast (MEF) cells in high glucose DMEM (Gibco-BRL, Invitrogen, Carlsbad, CA) containing 15% fetal bovine serum (FBS; Hyclone, Ogden, UT), 1000 U/ml LIF/ESGRO (Chemicon, Temecula, CA), and basic ES medium components [50 U/ml penicillin and 50 μg/ml streptomycin (Gibco-BRL, Invitrogen, Carlsbad, CA), 1% non-essential amino acids (Gibco-BRL, Invitrogen, Carlsbad, CA) and 0.1 mM β-mercaptoethanol (Gibco-BRL, Invitrogen, Carlsbad, CA)]. The hanging drops method (20 μl per drop; $1 \times 10^5$ cells $ml^{-1}$) was used to induce differentiation as described by Heuer and theirs colleagues (Heuer et al., 1993) with minor modifications. After incubation for 3 days, embryoid bodies (EBs) were transferred to gelatine-coated wells of chamber slides (Nunc, Denmark) or 60 mm dishes to allow attachment. To promote endothelial cell differentiation, 3-day-old EBs were placed in medium consisting of EBM-2, 5% FBS, and growth factor cocktail (EGM2-MV Bullet Kit; Clonetics/BioWhittaker, Walkersville, MD).

### *2.2. Cytotoxicity analysis*

To determine cytotoxic effects of 5-FU on mouse ESCs, the MTT assays were performed in the absence of mLIF as previously described (Spielmann et al., 1997; Scholz et al., 1999). Briefly, 1000 cells were seeded into each well of a 96-well microtitre plate and grown in the presence of a concentration range of 5-FU and probucol. A negative control containing solvent diluted in medium was also included. At day 9, the cells were exposed to 5-FU (10 μM) with/without probucol (50 μM) in a total volume of 200 μl for 24 h. The 5-FU and probucol were dissolved in cell culture medium and ethanol, respectively. The final ethanol concentration in the wells was 0.1%. The controls were incubated with equal volumes of drug solvents to avoid changes that could be due to solvent. About 20 μl of MTT (5 mg/ml) was added to 200 μl culture medium on day 10, followed by incubation at 37 °C for 4 h. After incubation, the MTT solution was carefully removed and 150 μl of DMSO (Sigma, St Louis, MO) was added to each well. The plates were shaken on a plate mixer until all crystals had dissolved. The absorbance of the resulting coloured solution was measured at 570 nm with a Genios luminometer (TECAN, Austria) at a reference wavelength of 630 nm. Cytotoxicity was expressed as a percentage of cells surviving, relative to untreated cultures, and the concentration required to inhibit cell growth by 50% ($IC_{50}$) was calculated. Each experiment was performed using six replicates for each drug concentration and repeated in triplicate.

### *2.3. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)*

Cells, including control and test groups, that had been exposed to 5-FU with/without probucol were directly harvested into tubes containing Trizol (Gibco-BRL, Invitrogen, USA) and mRNA was extracted according to the manufacturer's protocol. The isolated total RNAs were quantified using a spectrophotometer (SmartSpec 3000, Bio-Rad). First-strand cDNA was synthesised from 2 μg of total RNA using an oligo (dT) primer and a SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. First-strand cDNAs were amplified in a final volume of 25 μl containing 0.5 U Ex Taq DNA polymerase (TaKaRa Biotechnology, Korea) and 10 pmol of each target primer (Table 1). PCR conditions were as follows: 5 min at 94 °C, 30 amplification cycles (denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min), followed by a final extension at 72 °C for 5 min. The amplified products were separated on 1.5% agarose gels and visualised with ethidium bromide staining. cDNA samples were adjusted to yield equal GAPDH amplifications.

### *2.4. Immunocytochemistry*

At day 9, the cells were exposed to 5-FU with/without probucol for 24 h. After the 24 h incubation, cells were fixed with freshly prepared MeOH/DMSO (4:1) for overnight incubation at 4 °C. Cells were blocked with blocking solution containing 1% BSA and 0.1% Tween 20 for 30 min, and then incubated with rat anti-mouse PECAM-1 (1:100) (MEC 13.3, Santa Cruze, Biotechnology, Inc.), or rabbit anti-mouse PCNA (1:100) (Santa Cruze, Biotechnology, Inc) at 4 °C for overnight. After washing, cells were incubated with goat anti-rat IgG-FITC (1:100) (Santa Cruz, Biotechnology, Inc) or goat anti-rabbit IgG-TRITC (1:100) (Chemicon, Temecula, CA) for primary antibodies, respectively. After staining, coverslips were mounted in 30% Mowiol (Calbiochem-Novabiochem, Schwalbach, Germany). Images were obtained and analysed using a Bio-Rad confocal microscope (Radiance 2000 FCMP, Bio-Rad, USA).

### *2.5. FACS analysis*

In order to analyse how Oct-4 expression varies in mouse ESCs through different stages of endothelial cell differentiation, endothelial differentiation induced cells for 0, 4, 7 and 10 days were harvested using cell dissociation buffer (Sigma, St. Louis, MO). To analyse PCNA expression, the cells were exposed to 5-FU with/without probucol for 24 h at day 9. After 24 h incubation, control cells and treated cells were harvested using cell dissociation buffer (Sigma, St. Louis, MO). Cells were re-suspended at $10^6$ cells/100 μl in suspension buffer and then incubated with 1 μg/100 μl of rabbit anti-mouse Oct-4 (Santa Cruz, Biotechnology, Inc.), or rabbit anti-mouse PCNA (1:100) (Santa Cruze, Biotechnology, Inc.) for 1 h at 4 °C. Negative controls were incubated for 1 h at 4 °C with fluorochrome labelled irrelevant isotype control antibodies: 1 μg/100 μl goat anti-rabbit FITC-conjugated IgG (Chemicon) or goat anti-rabbit IgG-TRITC (1:100) (Chemicon, Temecula, CA) for primary antibodies, respectively. After staining, cells were analysed without

**Table 1**
Sequences of oligonucleotide primers used for RT-PCR analysis.

| Gene | Primer sequences | Product size (bp) |
|---|---|---|
| PECAM | F 5′-GCCTGGAGAGGTTGTCAGAG-3′<br>R 5′-GGTGCTGAGACCTGCIIII C-3′ | 357 |
| GAPDH | F 5′-TGTTCCTACCCCCAATGTGT-3′ R<br>5′-TGTGAGGGAGATGCTCAGTG-3′ | 396 |

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