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Different concentrations of kaempferol distinctly modulate murine embryonic stem cell function

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ABSTRACT

Kaempferol (3,4′,5,7-tetrahydroxyflavone) is a natural flavonoid with several beneficial and protective effects. It has been demonstrated that kaempferol has anticancer properties, particularly due to its effects on proliferation, apoptosis and the cell cycle. However, possible effects on pluripotent embryonic stem cell function have not yet been addressed. Embryonic stem cells have the ability to self-renew and to differentiate into all three germ layers with potential applications in regenerative medicine and *in vitro* toxicology. We show that exposure of murine embryonic stem cells (mESC) to high concentrations of kaempferol (200 μM) leads to decreased cell numbers, although the resulting smaller cell colonies remain pluripotent. However, lower concentrations of this compound (20 μM) increase the expression of pluripotency markers in mESCs. Mitochondrial membrane potential and mitochondrial mass are not affected, but a dose-dependent increase in apoptosis takes place. Moreover, mESC differentiation is impaired by kaempferol, which was not related to apoptosis induction. Our results show that low concentrations of kaempferol can be beneficial for pluripotency, but inhibit proper differentiation of mESCs. Additionally, high concentrations induce apoptosis and increase mitochondrial reactive oxygen species (ROS).

1. Introduction

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are characterized by their unlimited self-renewal potential and pluripotency. ESCs are derived from the inner cell mass (ICM) of the pre-implantation blastocyst (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Mouse embryonic stem cells (mESCs) are derived from the inner cell mass of blastocysts and commonly maintained in cell culture under normoxia conditions with medium supplemented with leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988) and serum containing bone morphogenic proteins (BMPs) (Ying et al., 2003), required to support and maintain cell culture pluripotency. These signaling pathways are able to induce gene expression of the core pluripotency genes Nanog, Oct4 and Sox2, and also contribute for the maintenance of self-renewal (Chen et al., 2008). Another interesting feature of ESCs is their resemblance to some types of cancer cells considering growth capacity, membrane surface markers, activity of some signaling pathways (e.g. Jak/STAT3, PI3K/Akt, WNT/GSK3 (Pereira et al., 2014)) and their metabolism (Folmes et al., 2011; Pereira et al., 2014; Varum et al., 2011). When comparing metabolic characteristics, it is interesting to note that both cancer and stem cells rely on aerobic glycolysis rather than oxidative phosphorylation, which can be related to the production of building blocks required for self-renewal of these highly proliferating cells (Pereira et al., 2014; Varum et al., 2011). Nonetheless, it should be noted that mouse ESCs can switch between glycolysis and oxidative phosphorylation for ATP production purposes upon specific culture conditions and became almost reliant on glycolysis in a primed state for differentiation (epiblast stem cells - EpiSC) (Zhou

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et al., 2012). Human ESC are described as mainly dependent on glycolysis, similarly to mouse EpiSC, but recent reports suggest that they can also be reverted to a more pluripotent state where their metabolism can be similar to mouse ESC (Ware et al., 2014). More recently, iPSCs were generated by reprogramming somatic cells to pluripotency so that they resemble embryonic stem. IPSCs represent a huge promise for regenerative medicine given that in theory patient-derived cells could be obtained, differentiated and used to avoid rejection issues, besides overcoming ethical concerns regarding the use embryonic stem cells (Sousa et al., 2015).

Flavonoids are compounds widely present in plants (Barros et al., 2012), which have a wide range of beneficial properties (Calderon-Montano et al., 2011; Toh et al., 2013; Yao et al., 2004), namely due to their antioxidant capacity (Crespo et al., 2008; Zhang et al., 2014) that can potentiate cardiovascular protection (Toh et al., 2013; Weseler et al., 2011) and be harnessed in an anticancer capacity (Kandaswami et al., 2005; Sak, 2014; Sak and Everaus, 2015), among other potential uses. Kaempferol (3,4′,5,7-tetrahydroxyflavone) is a natural flavonoid widely present in numerous edible plants which are part of a regular diet, such as broccoli, onions, green tea, pumpkin, strawberry and others (Calderon-Montano et al., 2011; Miean and Mohamed, 2001). It has been described that kaempferol can have beneficial and/or protective effects against several diseases (Calderon-Montano et al., 2011; Chen and Chen, 2013), such as cancer, due to its ability to induce apoptosis of cancer cells (Kang et al., 2009; Li et al., 2009; Sharma et al., 2007), which can be correlated with its anti-proliferative, cell cycle arrest, caspase activation and pro-oxidative capacity (Chen and Chen, 2013; Huang et al., 2010; Lee et al., 2014).

The effects of kaempferol in cancer cells have been studied, but there is no information regarding embryonic stem cells. Taking into account the similarities described between cancer and embryonic stem cells (Folmes et al., 2011; Pereira et al., 2014), we aim to evaluate the possible effects of kaempferol on the pluripotency of embryonic stem cells and in their ability to differentiate. In our study we observed that kaempferol potentiates the expression of pluripotent-associated markers in mESC at low concentrations. However, high concentrations of kaempferol induce apoptosis. Additionally, sub-lethal concentrations of kaempferol impaired proper mESC differentiation.

2. Methods

2.1. mESC culture and embryoid body (EB) differentiation

Mouse embryonic stem cell line E14Tg2a was kindly provided by Dr. Miguel Ramalho-Santos (University of California, San Francisco, USA). mESC were cultured as described (Pereira et al., 2013; Rodrigues et al., 2015a). Briefly, cells were maintained and propagated in complete medium – KODMEM medium composed of KnockOut-DMEM, 15% KnockOut serum replacement, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin (Life Technologies), 1% non-essential amino acids, 0.1 mM 2-mercaptoethanol (Sigma–Aldrich) and 1.000 U of Leukemia inhibitory factor (LIF) - (Chemicon – Millipore) at 37 °C and 5% CO_2 conditions. All plates were coated with 0.1% gelatin (Sigma) before cell plating.

E14Tg2a mESCs were allowed to grow for 72 h in the complete medium with kaempferol supplementation and LIF. Medium and drug supplementation was renewed every day (Fig. 1a).

mESC differentiation was performed using the embryoid bodies (EBs) suspension protocol as described (Rodrigues et al., 2015b), with minor modifications. Briefly, 10^6 mESCs were plated in a 60 mm non-adherent Petri dish and maintained using KODMEM medium (without LIF supplementation). Cells were cultured at 37 °C in a 5% CO_2 incubator and remained in suspension for three days. Medium was changed every day using the EB sedimentation technique. Briefly, medium-containing EBs was added to a conical 15 mL tube, supernatant medium without cells was removed and fresh medium was added. After three days, EBs were allowed to adhere in a 100 mm tissue-culture Petri dishes in KODMEM medium supplemented with 10% fetal bovine serum (FBS – Life Technologies). EBs were incubated overnight in a 37 °C, 5% CO_2 incubator. From that day on (day 4), EBs were grown with KODMEM medium with no LIF supplementation and kaempferol was added to the medium. Medium was changed every day, with fresh drug supplementation. Pictures were taken from EB cultures at days 6 and 12 of differentiation and total protein was extracted at day 14.

2.2. Cell viability assays: sulforhodamine B and MTT assays

Cell viability was measured by monitoring protein content in a 24 well with Sulforhodamine B (Sigma–Aldrich), an indirect measure of cell proliferation according to the manufacturers instructions. Briefly, after 72 h in culture cells were washed and fixed for 15 min with paraformaldehyde 4% and allowed to dry overnight. A Sulforhodamine B 0.5% solution prepared in 1% acetic acid was added to each well and incubated for 30 min. Unincorporated dye was removed by several washes with 1% acetic acid and samples allowed to dry. Incorporated dye was solubilized in 10 mM Tris and stirring in a gyratory shaker for 10 min. Absorbance was measured at 565 nm. Blank wells without plated cells were used for background correction.

Viability was also measured using the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. Briefly, after 72 h in culture medium was renewed without drug supplementation. MTT (Sigma–Aldrich) was added with a final concentration of 0.5 mg/mL and incubated at 37 °C for 4 h. Dye crystals were then solubilized with acidic isopropanol and absorbance was measured at 570 nm. Wells with medium and without cells were used for background correction.

2.3. Flow cytometry protocols for apoptosis/necrosis, mitochondrial mass, mitochondrial membrane potential, mitochondrial superoxide production and cell cycle analysis

All fluorescent dyes were analyzed by flow cytometry (Becton Dickinson BD FACSCalibur cytometer) and 20,000 gated cells were acquired/analyzed per condition with the Cell Quest Pro Acquisition software (BD Biosciences). GeoMean of graph data were obtained and used for quantification and statistical analysis purposes.

Apoptosis and necrosis were evaluated using annexin V (Immunostep) and propidium iodide (PI; Life Technologies). Cells were harvested, centrifuged and resuspended in Annexin V binding buffer (10 mM Hepes/NaOH (pH 7.4) 140 mM NaCl, 2.5 mM $CaCl_2$) at 10^6 cells/ml. Cells were incubated with propidium iodide (PI; Life Technologies) and Annexin V (5 μL/mL) in the dark for 15 min. Cells were then washed and resuspended in AnnexinV Binding buffer and analyzed by flow cytometry using appropriate settings.

An indirect measure of cell mitochondrial mass was obtained by using MitoTracker® Green FM (Life Technologies), a dye that binds to mitochondria independently of mitochondrial membrane potential. Cells grown in 60 mm dishes were harvested, centrifuged and resuspended at 10^6 cells/mL in PBS1x. 50 nM of dye was added and incubated for 30 min at 37 °C in the dark. Dye was washed, cells resuspended in PBS and analyzed by flow cytometry using appropriate settings.

Mitochondrial membrane potential was measured with TMRM (Tetramethylrhodamine methyl ester; Life Technologies). Briefly, cells grown in 60 mm dishes were detached with accutase,