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Olive leaf components apigenin 7-glucoside and luteolin 7-glucoside direct human hematopoietic stem cell differentiation towards erythroid lineage

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ABSTRACT

The generation of blood cellular components from hematopoietic stem cells is important for the therapy of a broad spectrum of hematological disorders. In recent years, several lines of evidence suggested that certain nutrients, vitamins and flavonoids may have important roles in controlling the stem cell fate decision by maintaining their self-renewal or stimulating the lineage-specific differentiation. In this study, main olive leaf phytochemicals oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G) were investigated for their potential effects on hematopoietic stem cell differentiation using both phenotypic and molecular analysis. Oleuropein and the combination of the three compounds enhanced the differentiation of CD34+ cells into myelomonocytic cells and lymphocytes progenitors and inhibited the commitment to megakaryocytic and erythroid lineages. Treatment with Lut7G stimulated both the erythroid and the myeloid differentiation, while treatment with Api7G specifically induced the differentiation of CD34+ cells towards the erythroid lineage and inhibited the myeloid differentiation. Erythroid differentiation induced by Api7G and Lut7G treatments was confirmed by the increase in hemoglobin genes expressions (*α-hemoglobin*, *β-hemoglobin* and *γ-hemoglobin)* and erythroid transcription factor *GATA1* expression. As revealed by microarray analysis, the mechanisms underlying the erythroid differentiation-inducing effect of Api7G on hematopoietic stem cells involves the activation of JAK/STAT signaling pathway. These findings prove the differentiation-inducing effects of olive leaf compounds on hematopoietic stem cells and highlight their potential use in the ex vivo generation of blood cells.

1. Introduction

Hematopoiesis is a tightly regulated process maintained by a small pool of hematopoietic stem cells (HSCs) capable of undergoing self-renewal and generating mature progeny of all the hematopoietic cell lineages (Abdelhay et al., 2012). The two defining features of HSCs, self-renewal and multi-lineage differentiation, make these cells an attractive source for stem cell-based therapies. HSC transplantation and the infusion of the ex vivo expanded progenitors of a specific lineage can be a life-saving procedure in the treatment of a broad spectrum of disorders, including hematologic, immune, and genetic diseases (Walasek et al., 2012; Hino et al., 2000; Lippi et al., 2011; Siddiqui et al., 2011). Thus, strategies to expand either hematopoietic progenitors or the differentiated sub-populations are an area of active research. Characterizing and identifying regulators of stem cell fate decision may also help to design approaches that stimulate endogenous stem cells to promote healing and regenerative medicine as an alternative to stem cell transplantation (Bickford et al., 2006; Shytle et al., 2010). The decision of symmetric or asymmetric division of HSC depends on both intrinsic and extrinsic factors. The former include the specific hematopoietic lineage to which the cell belongs and its stage of maturation. In this regard, multiple proteins have been identified as regulators of HSC fate including transcription factors, epigenetic modifiers and cell cycle regulators (Walasek et al., 2012; Kubota and Kimura, 2012). On the other hand, the extrinsic factors include multiple cytokines, adhesion molecules and other signals produced by

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stromal cells occupying HSC microenvironment and likely other cells in the body. Multiple cytokines, including SCF, TPO, EPO, Flt-3L, IL-11, IL-3, IL-6, and GM-CSF, and combinations of these, have been studied in in vitro HSC expansion and differentiation protocols of mouse and human cells (Walasek et al., 2012; Sauvageau et al., 2004). In recent years, a growing body of evidence suggests that in addition to cytokines/factors, chemical and natural compounds can also have potent effects on hematopoietic cell expansion protocols. A number of compounds such as retinoic acids, valporic acid, 5-aza-2-deoxycytidine methyltransferase (5azaD), and the lipid mediator prostaglandin E_2 (PGE_2) have been identified for their ability to control stem cell fates in culture, where the effects ranged from the enhancement of stem cell expansion to the stimulation of lineage-specific differentiation (Walasek et al., 2012; Purton et al., 1999; De Felice et al., 2005; Hoggatt et al., 2009; Milhem et al., 2004; Bickford et al., 2006). Shytle et al. (2007) showed that a particular combination of blueberry extract, green tea extract, carnosine, and vitamin D3, a proprietary nutraceuticals formulation known as NT-020, demonstrated synergistic activity in promoting proliferation of hematopoietic stem cells. Therefore, it appears that it is possible to use certain natural products, either alone or in combination, in order to direct the stem cell culture to maintain the self-renewal or to stimulate the differentiation of the committed progenitors (Bickford et al., 2006; Shytle et al., 2010, 2007).

Due to its established beneficial effects on health, olive leaf has gained the interest of the scientific and industrial community, and thus, has emerged as commercially valuable nutraceuticals. Olive leaves contain many bioactive compounds that have antioxidant, anti-microbial, antihypertensive, anti-viral, anti-inflammatory, hypoglycemic, neuroprotective, and anti-cancer properties (Kontogianni and Gerothanassis, 2012; Pereira et al., 2007; Somova et al., 2003; Micol et al., 2005; Wainstein et al., 2012; Seddik et al., 2011; Bouallagui et al., 2011). Olive leaf extract and compounds, such as apigenin 7-glucoside, showed anti-leukemia effects by inducing the differentiation of different leukemia cell lines (Abaza et al., 2007; Samet et al., 2014a; Tsolmon et al., 2011). While myeloid leukemia cell lines are frequently used to study differentiation of myeloid cells, these cells have a highly abnormal karyotype and often display functional differences from their normal myeloid counterparts (Choi et al., 2009). We have previously demonstrated that olive leaf components have the potential to enhance the differentiation of hematopoietic stem cells rather than stimulating their self-renewal (Samet et al., 2014b). In this study, we identify the lineage-differentiation effects of each compound on the human hematopoietic stem cells and give insight into the underlying mechanism.

2. Materials and methods

2.1. Sample preparation and cell culture

Stock solutions of 100 mM of oleuropein (Olp), apigenin 7-glucoside (Api7G) and luteolin 7-glucoside (Lut7G), (Sigma Aldrich), were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C until use. Cryopreserved CD34+ hematopoietic stem/progenitor cells (HSPCs) were purchased from Life Technologies™ (GIBCO, Life Technologies™) and cultured in StemPro®-34 serum-free medium supplemented with StemPro®-34 Nutrient Supplement. Cytokines, SCF, IL-3 and GM-CSF (GIBCO, Life Technologies™) were used at final concentrations of 100 ng/mL, 50 ng/mL and 25 ng/mL respectively. For all experiments, cells were seeded at 2×10^4 cells/mL in 6-well plates and incubated for 24 h before the addition of olive leaf phytochemicals at the desired final concentrations.

The individual effect of each compound was assessed by treatment with Olp, Api7G or Lut7G at a final concentration of 50 μM based on a preliminary study of the morphology and the viability of CD34+ cells cultured in the presence of different concentrations of each compound (Samet et al., 2014b). DMSO (vehicle) at 0.05% was added to control cells. In order to evaluate the effect of the combination of these three compounds, cells were also treated with combination (comb) of Olp, Api7G and Lut7G at 55 μM, 5 μM, and 5 μM, respectively. The concentrations of the compounds in the mixture were determined based on the HPLC analysis of the ethanol extract of olive leaf and the compounds' effective concentration for the induction of differentiation of human chronic myelogenous leukemia K562 cells in our previous study (Samet et al., 2014a).

2.2. Cell viability and cell number

The viability and number of CD34+ cells were determined using flow cytometry on the 3rd, 6th and 9th days of culture with each compound (Olp, Api7G or Lut7G) or their combination (Comb). DMSO-treated CD34+ cells served as control cells. After incubation for the indicated time, treated cells were harvested, suspended in Guava ViaCount reagent (Guava Technologies) and allowed to be stained for at least 5 min in darkness. The Guava ViaCount reagent contains two DNA-binding dyes. The nuclear dye stains only nucleated cells, while the viability dye brightly stains dying cells. The differential permeability of these two dyes enables the Guava ViaCount assay to distinguish between viable and non-viable cells. The cell number and viability were automatically measured using the Guava ViaCount application in Guava PCA flow cytometry (Guava Technologies). Morphological changes were detected by observation under a phase contrast microscope (Leica Microsystem).

2.3. Cell cycle analysis

HSPCs were cultured for 3, 6 or 9 days, harvested, washed with 1 × PBS, fixed with 70% ethanol, and incubated at 4 °C for more than 12 h. The fixed cells were then centrifuged and washed with 1 × PBS twice. Cell cycle reagent (Guava Technologies) was added, and the cells were incubated in the dark for 30 min at room temperature. The population of cells in each cell cycle phase was determined using the cell cycle software of Guava PCA.

2.4. Differentiation marker expression

Phenotypic analysis of the expanded cells were carried out on the 3rd, 6th and 9th days by analyzing the expression of surface markers such as CD34, CD38, CD33, CD10, CD14, CD41 and GlycophorinA (Abcam). The expanded control and treated cells were collected, the number adjusted to 1×10^5 cells and washed with 1 × PBS. After the addition of antibodies, the cells were incubated for 40 min on ice. Excess antibody was removed by washing with 1 × PBS before analysis using Guava PCA. The expression level of each surface antigen in treated cells was normalized to its expression level in control cells.

2.5. RNA isolation and real-time PCR analysis

Total RNA extraction of treated CD34+ cells, as well as control cells, was conducted on the 2nd, 5th and 8th day of culture using Isogen reagent (Nippon Gene Co., Ltd) following the manufacturer's instructions. The isolated RNA was quantified using Nanodrop 2000 (Thermo Scientific). First strand cDNA synthesis was carried out from about 1 μg of total RNA using Superscript III (Invitrogen) and oligo(dT) primers according to manufacturer's protocol. Real-time PCR was performed using TaqMan master mix and specific gene primers from Applied Biosystems, specific for the 7500/7500 Fast RT-PCR system. Primers for *α-hemoglobin*, *β-hemoglobin*, *γ-hemoglobin*, *GATA1*, and the internal control *β-actin*, with the assay IDs listed in Supplementary table (Table S1), were all purchased from Applied Biosystems.