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Flavonoids suppress human glioblastoma cell growth by inhibiting cell metabolism, migration, and by regulating extracellular matrix proteins and metalloproteinases expression

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ABSTRACT

The malignant gliomas are very common primary brain tumors with poor prognosis, which require more effective therapies than the current used, such as with chemotherapy drugs. In this work, we investigated the effects of several polyhydroxylated flavonoids namely, rutin, quercetin (F7), apigenin (F32), chrysin (F11), kaempferol (F12), and 3′,4′-dihydroxyflavone (F2) in human GL-15 glioblastoma cells. We observed that all flavonoids decreased the number of viable cells and the mitochondrial metabolism. Furthermore, they damaged mitochondria and rough endoplasmic reticulum, inducing apoptosis. Flavonoids also induced a delay in cell migration, related to a reduction in filopodia-like structures on the cell surface, reduction on metalloproteinase (MMP-2) expression and activity, as well as an increase in intra- and extracellular expression of fibronectin, and intracellular expression of laminin. Morphological changes were also evident in adherent cells characterized by the presence of a condensed cell body with thin and long cellular processes, expressing glial fibrillary acidic protein (GFAP). Therefore, these flavonoids should be tested as potential antitumor agents in vitro and in vivo in other malignant glioma models.

1. Introduction

The malignant gliomas are very common primary brain tumors responsible for about 40% of all primary tumors and 78% of all malignant tumors of the central nervous system [16]. Over 80% of these tumors are considered high-grade (grades III and IV) when diagnosed according to the current classification of the World Health Organization (WHO). Glioblastoma (GB) is the most aggressive form of gliomas that affect the brain, is highly infiltrative, and is morphologically very heterogeneous. Currently, the protocol adopted for the treatment of patients with glioblastoma is based on surgery followed by radiation therapy and chemotherapy with temozolomide (TMZ) [29]. However, despite advances in treatment, the average life expectancy of patients with GB is approximately 14 months, and only a small number of patients can survive for up to 5 years after diagnosis.

The excessive proliferation, diffuse ability to infiltrate the surrounding brain tissue and the suppression of anti-tumor immune response are crucial biological aspects that contribute to the malignant phenotype of glioblastomas and limit the success of current treatment protocols. The tumor invasion is a complex process, in which the neoplastic cells initiate the migration on the primary tumor site, adhere to the extracellular matrix (ECM), degrade its components through proteolytic enzyme activities, and invade the normal tissue. Therefore, the crucial elements for the invasion of tumor include adhesion and migration that involve interactions

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between tumor cells and the ECM components surrounding the tumor [8,28].

The ECM is an organized and complex network of molecules that are typically composed of large glycoproteins, including fibronectins, collagens, laminins and proteoglycans, which assemble fibers or other macromolecular arrangements. The ECM acts as a reservoir of growth factors and fluids, and plays an important role in the organization of tissues, cellular microenvironments and niches of stem cells. It is tissue specific and adapts to changes in the development, age and disease [28].

The enzyme matrix metalloproteinases (MMPs) are a family of endopeptidases, which selectively degrade extracellular matrix components (EMC). MMPs are involved in the migration and invasion of tumor cells, because they contribute for proteolytic degradation of basement membrane. Among MMPs, MMP2 (Gelatinase A, EC 3.4.24.24) has been widely studied because it is involved in the malignancy of tumor cells [19].

Flavonoids constitute a group of polyphenolic components originated from different plant species that have a low molecular weight, and present in a variety of fruits, vegetables, cereals, tea, wine, and fruit juices [9]. They exhibit a variety of biological activities such as anti-inflammatory, antioxidant, antiviral and antitumor actions [10]. Inhibition of the growth of cancer cells by flavonoids in vitro and in vivo has been reported in several studies. Flavonoids such as quercetin and rutin, commonly found in the diet, have shown antiproliferative and apoptotic effects in glioma cells in vitro [5,20,25]. Quercetin and other flavonoids have also been associated with inhibition of migration and invasion of some types of tumors [15,27,34]. Despite this, there are few studies evaluating the mechanisms by which different flavonoids inhibit tumor growth and migration of central nervous systems tumors. Understanding the role of flavonoids on the growth, viability and the processes of migration and invasion of gliomas may help to elucidate the antitumor activity of these natural products and contribute to generate new potential candidates for therapy. Therefore, in this study, we investigated the effects of polyhydroxylated flavonoids on cell viability, phenotypic changes, migratory and invasive capacity in human glioblastoma cells of the GL-15 lineage [4].

2. Methods

2.1. Flavonoids and treatment

The flavonoids 3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside (rutin) and 3,3′,4′,5,6-pentahydroxyflavone (quercetin or F7) were extracted and characterized from *Dimorphandra mollis*, and 4′,5,7-trihydroxyflavone (apigenin or F32) was extracted and characterized from *Croton betulaster* in the Laboratory of Organic Chemistry and Natural Products (Institute of Chemistry, UFBa) and in the Laboratory of Research in Materia Medica (Faculty of Pharmacy, UFBa). 5,7-dihydroxyflavone (chrysin or F11; Aldrich, St Louis, MO), 3,4′,5,7-tetrahydroxyflavone (kaempferol or F12; Sigma, St Louis, MO), and 3′,4′-dihydroxyflavone or F2 (Extra synthese-R&D Chemicals) were kindly provided by Prof. *Guy* G. *Chabot* from Laboratoire de Pharmacologie Chimique et Génétique, *Université Paris* Descartes. Structures of these flavonoids are shown in Table 1. All flavonoids were dissolved in dimethylsulfoxide (DMSO, Sigma, St Louis, MO) at a concentration of 20 mM and stored in the dark at −20 °C. When applied to cells, flavonoids were dissolved in the medium at a final concentration of 50 μM and incubated for 24 or 48 h. Control cells were treated with the same volume of DMSO (0.5%) that was used as a vehicle for flavonoids, and it did not show any significant effect on the analyzed parameters when compared to cultures that were not exposed to this solvent.

2.2. Cell line and cultures

GL-15 [4] and U251 cells (kindly provided by Vivaldo Moura-Neto) derived from human glioblastomas were used between 20 passages, maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY), a nutrient mixture (7 mM glucose, 2 mM glutamine, 0.011 g/l pyruvate) and antibiotics (100 IU/ml penicillin G, 100 μg/ml streptomycin). Cells were grown in 100 mm diameter tissue culture plates (TTP, Switzerland) containing 10 ml medium, which was replaced three times a week. Stock cultures were subcultured into new plates every 3–4 days and cells for experiments were seeded into polystyrene culture plates as needed. After 24 h, cells were trypsinized and subcultured in other plates (96-, 24- or 6-wells) according to the analytical procedures to be performed. Dissociated sphere-derived TG-1 cells (Patru et al. [21]; kindly provided by Hervé Chneiweiss) were cultured in 75 cm^2 tissue culture flasks plated at 2500–5000 cells/cm^2 in DMEM: F-12 medium (1:1) containing the N2, G5 (containing FGF and EGF) and B27 supplements (all from Invitrogen, France). They were dissociated in single-cell suspension each week with a renewal of two third of their culture medium.

2.3. Cell viability and proliferation tests

2.3.1. Trypan blue staining

Membrane integrity and cell viability were evaluated after Trypan blue staining in the control group and treated cells seeded on 40 mm polystyrene culture dishes (1.6×10^4 cells/cm^2). Both adherent and floating cells were obtained after trypsinization and were centrifuged for 10 min at 1300× *g*. Cells were then suspended in 200 μl DMEM without supplements and stained with Trypan blue at a final concentration of 0.1% (p/v). Three replicate experiments were performed for each analysis; the number of viable and non-viable cells/μl was determined after 48 h exposure to flavonoids by counting four 10 μl samples of cell suspension for each experiment in a Burker chamber (Boehringer Mannheim).

2.3.2. MTT test

Flavonoids were tested for its cytotoxicity towards GL-15, U251 and dissociated TG-1 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO) test in 96 well plates (TPP Switzerland) after cells had become confluent (95%). Cells were seeded at a density of 5.0×10^4 cells/cm^2. The cells were exposed to flavonoids for 48 h and 2 h before the end of exposure time, the culture medium was replaced by a solution of MTT (1 mg/ml) diluted in DMEM and then the plate was incubated for 2 h. Thereafter, cells were lysed with 20% (w/v) SDS, 50% (v/v) DMF, pH 4.7 adjusted with a solution of 80% (v/v) acetic acid, 2.5% (v/v) 1 M HCl, and plates were kept overnight at 37 °C in order to dissolve formazan crystals. The cell cytotoxicity was quantified based on the conversion of yellow MTT to purple MTT formazan by mitochondrial dehydrogenases of living cells. The optical density of each sample was measured at 580 nm using a BIO-RAD 550 PLUS spectrophotometer. Eight replicate wells were used for each analysis.

2.3.3. BrdU cell proliferation assay

Proliferation was evaluated using the BrdU cell proliferation assay (Sigma–Aldrich, Inc). After 48 h of exposure to flavonoids, BrdU (10 μM) was added to wells of the plate. Cells were incubated for 2 h in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were fixed and DNA was denatured by treatment with denaturing solution (2 N HCl) for 20 min at room temperature. Mouse