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Antiproliferative, proapoptotic and morphogenic effects of the flavonoid rutin on human glioblastoma cells

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ABSTRACT

In this study, we investigated the effects of the flavonoid rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside) on glioma cells, using the highly proliferative human cell line GL-15 as a model. We observed that rutin (50–100 μM) reduced proliferation and viability of GL-15 cells, leading to decreased levels of ERK1/2 phosphorylation (P-ERK1/2) and accumulation of cells in the G2 phase of the cell cycle. On the other hand, 87.4% of GL-15 cells exposed to 100 μM rutin entered apoptosis, as revealed by flow cytometry after AnnexinV/PI staining. Nuclear condensation and DNA fragmentation were also observed, further confirming that apoptosis had occurred. Moreover, the remaining cells that were treated with 50 μM rutin presented a morphological pattern of astroglial differentiation in culture, characterised by a condensed cell body and thin processes with overexpression of GFAP. Because of its capacity to induce differentiation and apoptosis in cultured human glioblastoma cells, rutin could be considered as a potential candidate for malignant gliomas treatment.

1. Introduction

Glioblastomas are very invasive and proliferative tumours that occur in cerebral hemispheres (Louis et al., 2007). The current treatment for this type of cancer consists of surgical resection followed by radiotherapy and/or chemotherapy, providing patients with a median survival of less than one year (Brandes, Pasetto, & Monfardini, 2000). New compounds such as tamoxifen and retinoids have been tested as alternative drugs, but pre-clinical treatments remain inconclusive (Costa et al., 2001). Therefore, new compounds with antitumor potential are needed as an alternative to the classical cytotoxic treatments.

Plants are a good source of putative antitumour drugs, and a large number of plant-derived compounds are being tested for antineoplastic activity. *Dimorphandra mollis Bent* (Caesalpinoidae) is a perennial shrub indigenous to Brazil that has been traditionally used to treat a wide range of pathologies, such as ulcerative lesions of skin or diarrhoea and haemorrhages (Robbers, Tyler, & Speedie, 1997). This plant is a robust source of rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside), a well-known flavonoid that has been extensively studied and possesses various pharmacological properties. Among the pharmacological properties attributed to flavonoids isolated from various medicinal plants around the world, an antitumour activity of flavonoids against mammary cancer has been described (Fresco, Borges, Diniz, & Marques, 2006; Lamartiniere et al., 2002; Ramos, 2007). Rutin and its aglycone quercetin form have been shown to have chemopreventive activity in both a variety of colonic cancer cell lines and in animal models (Deschner, Ruperto, Wong, & Newmark, 1991; Lipkin, 1999; Yang et al., 2000). Fernandez and colleagues (2006) have reported a depressant action for rutin and others available flavonoid glycosides on the central nervous system (CNS), suggesting that these molecules cross the blood–brain barrier. However, little is known about any neurotoxic and antitumour activities in the CNS. An inhibitory effect was demonstrated in rat C6 and human U138MG glioma cell lines treated with the aglycone quercetin (Braganhol et al., 2006; Chen, Jeng, Lin, Wu, & Chen, 2006). Moreover, we recently observed that rutin

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affects astrocytes and microglial cells in primary culture, inducing them to secrete neurotrophic factors such as NO and TNFα (Silva et al., 2008). Understanding the role of such natural molecules on the growth and viability of human glioma cells may help to elucidate the antitumour activity of these drugs and generate new potential candidates for glioma treatment. In this study, we investigated the effects of rutin on the highly proliferative human glioblastoma cell line GL-15 (Bocchini, Casalone, Collini, Rebel, & Lo Curto, 1991) with regards to cell growth, viability and phenotypic modification.

2. Methods

2.1. Cell line and culture

GL-15 (Bocchini et al., 1991) stock cultures (passages 83–105) were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C in medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal 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 per week. Stock cultures were subcultured into new plates every 3–4 days and cultures for experiments were seeded into polystyrene culture plates as needed. After 24 h the culture medium was changed and the various analytical procedures were performed on cultures.

2.2. Flavonoid and treatment

The flavonoid rutin was extracted from *D. mollis* seeds by recrystallisation in tetrahydrofuran (THF), according to the Merck S.A. procedures in LAPEMM-FFar-UFBA. The methanol extract obtained from broadbeans of *D. mollis* was fractionated into n-BuOH and aqueous layers through solvent fractionation. Repeated silica gel column chromatography of the n-BuOH layer and recrystallisation from THF produced the flavonol glycoside rutin (99% purity). For experiments it was dissolved in dimethyl sulfoxide (DMSO, Sigma, St Louis, MO) at a concentration of 20 mM and stored in the dark at −20 °C. When applied to cells, rutin was dissolved in the medium at final concentrations of 1, 10, 50 and 100 μM and incubated for 24–72 h. Control cells were treated with the same volume of DMSO that was used as a vehicle for rutin. DMSO (0.5%) did not show any significant effect on the analysed parameters when compared to cultures that were not exposed to this solvent.

2.3. Cell viability and proliferation

2.3.1. LDH activity

Membrane integrity was evaluated by measuring lactate dehydrogenase (LDH) activity in culture medium from control and treated cells, and evaluated as an index of cell death. Cells were grown in 40 mm diameter plates (1.5×10^5 cells/plate) treated with 1–100 μM rutin or 0.5% DMSO (control) for 24–72 h. The culture medium was then removed and LDH activity (IU/l) was measured according to the manufacturer's protocol (Doles, Goiás, Brazil). Three independent experiments were carried out for each experimental point. Experimental results were expressed in terms of LDH activity (IU/ml of culture medium) in at least three replicate experiments.

2.3.2. Trypan blue staining

Growth curves and membrane integrity were obtained after Trypan blue staining of control or treated cells seeded on 40 mm polystyrene culture dishes (1.5×10^5 cells/plate). Both adherent and floating cells were obtained after trypsinisation 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% (v/v). Three replicate experiments were performed for each analysis; the number of viable and non-viable cells/μl was determined after 24–72 h exposure to rutin (1–100 μM) by counting four 10 μl samples of cell suspension for each experiment in a Burker chamber (Boehringer Mannheim).

2.3.3. Thymidine incorporation

The effect of rutin on cellular proliferation was studied by measuring 2-(methyl-^{3}H) thymidine incorporation (1 μCi/ml, Amersham). In brief, cells were cultured in 96 well plates (TPP Switzerland; 1×10^4 cells/well) and incubated in four replicates with 1–100 μM rutin, or 0.5% DMSO (control) for 24 and 72 h. Cells were pulse-labelled for the final 5 h by adding 1 μCi/200 μl of [^{3}H]-thymidine to the culture medium. After this incubation, cells were collected in a glass fibre filter (Packard) using a cell harvester (Filtermate 196, Packard, Meriden, CT, USA) and allowed to dry for 24 h. Incorporation of radioactive thymidine was determined by gas scintillation in a direct beta counter (Packard). Cell counts were performed for four independent experiments and results (mean ± S.D.) were expressed as percentage of control incorporation (considered as 100%).

2.3.4. Flow Cytometry

Flow cytometry analysis of DNA content was performed to assess the cell cycle phase distribution after rutin (50 μM) was added to logarithmically growing GL-15 cells. After exposure, GL-15 cells were harvested by trypsinisation, fixed with cold ethanol and stained with propidium iodide (Biosource, Camarillo, CA). Flow cytometry was performed on a BD FACS Calibur. The software BD Cell Quest™ Pro was used to generate histograms and determine the cell cycle phase distribution. Experiments were performed at least three times.

2.3.5. Western blot analysis for MAPK signalling

To investigate the regulation of MAPK signalling by rutin in glioblastoma cells, we determined the expression of phosphorylated and unphosphorylated forms of ERK1 (44 kDa) and ERK2 (42 kDa). Total protein content was determined in control or treated cells seeded in 40 mm polystyrene culture dishes (1.5×10^5 cells/plate). After 24–72 h exposure, cells were rinsed twice with PBS, lysed and harvested in a 2% (w/v) SDS, 2 mM EGTA, 4 M urea, 0.5% (v/v) Triton X-100, 62.5 mM Tris–HCl buffer (pH 6.8) supplemented with 1 μl/ml protease inhibitor cocktail (Sigma, St Louis, MO). Protein content was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using a protein assay reagent kit (Bio-Rad, Hercules, CA). Twenty micrograms of total protein extract were loaded onto a discontinuous 4% stacking and 12% running SDS polyacrylamide gel (SDS–PAGE). Electrophoresis was performed at 200 V for 45 min. Proteins were then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Equal protein loading was confirmed by staining membranes with Ponceau Red (Sigma). Membranes were then blocked for 1 h at room temperature in 20 mM TBS (pH 7.5) containing 0.05% Tween 20 (TBS-T) and 5% powdered skim milk. Subsequently, membranes were incubated with rabbit anti-ERK1/2 (1:1000, Santa Cruz) or mouse anti-phospho-MAP kinase 42/44 (1:1000, Santa Cruz) diluted in TBS-T containing 1% powdered skim milk for 1 h. Conjugated alkaline phosphatase goat anti-rabbit IgG or goat anti-mouse IgG (1:5000 in TBS-T, Bio-Rad, Hercules, CA) was used as a secondary antibody. Immunoreactive bands were visualised using an AP-conjugated substrate kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Quantification was performed by scanning densitometry (ScanJet 4C – HP) of three independent experiments

and analysed with ImageJ 1.33u (Wayne Rasband, National Institute of Health, USA). Antibody specificity and linearity of the densitometric analysis system was assessed by serial dilutions of total protein from cells in control conditions within a range of 5–20 μg of protein per lane.

2.4. Morphological changes and differentiation

2.4.1. Rosenfeld's staining and immunocytochemistry

Morphological changes were primarily assessed by analysing Rosenfeld's staining. All control and treated cells seeded on 40 mm polystyrene culture dishes (1.5×10^5 cells/plate) were rinsed three times with PBS (without Ca^{2+} and Mg^{2+}) and fixed for 10 min with methanol at −20 °C. Fixed cells were stained with Rosenfeld's reagent (1 ml) for 20 min at room temperature. Plates were then rinsed with water, air-dried, analysed in an optic phase microscope (Nikon TS-100) and photographed using a digital camera (Nikon E-4300).

Morphological changes and astroglial differentiation were also studied by immunocytochemistry for the cytoskeletal proteins vimentin, a marker of immature glial cells, and GFAP (glial fibrillary acidic protein), an astrocyte marker. All control and treated cells seeded on 40 mm polystyrene culture plates (1.5×10^5 cells/plate) were rinsed three times with PBS and fixed with cold methanol at −20ºC for 10 min. Non-specific binding of antibody reagents was blocked by preincubating the plates with 3% bovine serum albumin (BSA) in PBS. Cells were incubated with mouse monoclonal antibodies against anti-vimentin (clone 3B4, 1/250, Biomakor, Israel) or rabbit polyclonal antibodies specific for GFAP (1/250, DAKO, Denmark) diluted in PBS, for 12 h at 4 °C under slow agitation. After three washes with PBS, cells were incubated with rhodamine-conjugated antibodies specific for mouse or rabbit IgG (1/500, Boehringer Mannheim) diluted in PBS, for 2 h at room temperature. Thereafter, cells were analysed using an epifluorescence microscope (Olympus BX-2) and photographed. Ten randomised fields were analysed.

2.4.2. Protein assay and western blotting

GFAP and vimentin expression was also investigated by western immunoblotting. Total protein content was determined in control or treated cells seeded on 40 mm polystyrene culture dishes (1.5×10^5 cells/plate). After 24–72 h exposure, cells were rinsed twice with PBS, lysed and harvested in a 2% (w/v) SDS, 2 mM EGTA, 4 M urea, 0.5% (v/v) Triton X-100, 62.5 mM Tris–HCl buffer (pH 6.8) supplemented with 1 μl/ml protease inhibitor cocktail (Sigma, St Louis, MO). Protein content was determined by the method of Lowry et al. (1951) using a protein assay reagent kit (Bio-Rad, Hercules, CA). For western immunoblot analysis, 20 μg proteins was loaded onto a discontinuous 4% stacking and 12% running SDS polyacrylamide gel (SDS–PAGE). Electrophoresis was performed at 200 V for 45 min. Proteins were then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Equal protein loading was confirmed by staining membranes with Ponceau Red (Sigma). Thereafter, membranes were blocked for 1 h at room temperature in 20 mM TBS (pH 7.5) containing 0.05% Tween 20 (TBS-T) and 5% powdered skim milk. Subsequently, membranes were incubated with rabbit anti-GFAP (1:1000, SIGNET, Dedham, MA) or mouse anti-vimentin (1:1000, clone 3B4, OXFORD Biotechnology, Oxford, United Kingdom) monoclonal antibody diluted in TBS-T containing 1% powdered skim milk for 1 h. Conjugated alkaline phosphatase goat anti-rabbit IgG or goat anti-mouse IgG (1:5000 in TBS-T, Bio-Rad, Hercules, CA) was used as a secondary antibody. Immunoreactive bands were visualised using AP-conjugated substrate Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Quantification was obtained by scanning densitometry (ScanJet 4C – HP) of three independent experiments and analysed with ImageJ 1.33u. Antibody specificity and linearity of the densitometric analysis system was assessed by serial dilutions of total protein from cells in control conditions within a range of 5–20 μg of protein per lane.

2.5. Apoptosis detection

The present study was designed to investigate whether rutin exerts antiproliferative effects on glioblastoma cells by inducing apoptosis or necrosis. Thus, the 100 μM higher concentration of rutin was tested.

2.5.1. Annexin V analysis

Apoptosis was detected by monitoring phosphatidylserine externalisation using an Annexin V-FITC/Propidium iodide staining kit (US-BD Biosciences Clontech). Untreated and treated cells were cultured for 24 h in 40 mm diameter plates (1.5×10^5 cells/plate). Approximately 1.5×10^4 cells were then resuspended in 100 μl of binding buffer containing Annexin V-FITC (0.125 μg/ml), propidium iodide (5 μg/ml), ddH_2O and annexin buffer, following the kit recommendations. After 15 min incubation at room temperature in the dark, cells were analysed by flow cytometry using a FACSort BD machine (US-BD Biosciences Clontech). At least 10,000 events were recorded, and annexin-V-positive cells (apoptotic) and propidium iodide-positive cells (necrotic) were presented as percentages of labelled cells related to the total number of cells counted.

2.5.2. Chromatin staining

Apoptosis of GL-15 cells was also determined by Hoechst 33258 (Sigma–Aldrich, St. Louis, USA) staining, which allows us to detect and quantify cells with fragmented and condensed chromatin. After washing with PBS, cells cultured on 40 mm plates (1.5×10^5 cells/plate) were fixed for 10 min with methanol at −20 °C for 20 min. Subsequently, fixed cells were stained with Hoechst 33258 at a final concentration of 5 μg/ml in PBS, for 10 min at room temperature in a dark chamber. Thereafter, the cells were washed with PBS and the plates were analysed by fluorescence microscopy (Olympus AX70) and photographed. The apoptotic index represents the percentage of fragmented nuclei and was determined in a microscopic field of at least 500-cells/experimental point.

2.5.3. Single cell electrophoresis assay of DNA

DNA integrity and single-strand breaks were monitored using single cell gel electrophoresis (comet assay) performed under alkaline conditions, essentially following the procedure described by Ribeiro (2006) with some modifications. Briefly, all control and treated cells seeded on 40 mm polystyrene culture dishes (1.5×10^5 cells/plate) were incubated for 24 or 72 h in the presence of rutin, using 0.5% DMSO as a negative control or 1 h direct exposure to UV light as a positive control. After treatment the cells were scraped at 4 °C and centrifuged at 1000*g* for 10 min. The pellet was diluted in 150 μl of PBS, and 30 μl of cell suspension was mixed with 300 μl of 1% (w/v) low-melting-point agarose (Sigma–Aldrich), and applied on the surface of a normal-melting-point agarose-precoated slide to form a microgel and allowed to set at −20 °C for 5 min. Microgels were submersed in cell lysis buffer (14.61% NaCl, 3.72% EDTA, 0.12% Tris pH 10, 1% Triton X-100 and 10% DMSO) for 1 h at 4 °C with protection from light. Following cell lysis, slides were washed with PBS for 10 min to remove salt and detergent from the microgel. Slides were placed in a horizontal electrophoresis unit and were allowed to equilibrate for 20 min with electrophoresis buffer (0.034% EDTA, 0.3 N NaOH, pH 13). Electrophoresis was performed at 0.13 A and 25 V for 25 min. Slides were rinsed with neutralisation solution (12.12% Tris HCl, pH 7.5) three times for 5 min each, fixed with 100% ethanol, air-died and stored protected from light until analysis. For analysis and scoring,

DNA comet slides were stained with 0.1% ethidium bromide (25 μl) for 5 min and coverslips were applied before image analysis. Comet assay samples were analysed at 200× magnification using an epifluorescence microscope (Olympus BX-2) with a rhodamine filter. Three replicate experiments were performed with two slides per experiment and UV irradiation point. The electrophoresed DNA appears as a comet, with undamaged DNA in the head and fragmented DNA migrating to form a tail. Comet images were recorded using a Variocam digital camera (PCO, Germany). Two hundred randomly selected cells were scored from each slide (two slides per dose), and the percentage of comet cell (comet rate) was calculated.

2.6. Statistical analysis

Results are expressed as mean ± standard deviation. One-way ANOVA followed by the Student-Newmann-Keuls test was used to determine the statistical differences among groups differing in only one parameter. Student's *t* test was used to compare two groups. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Cell viability and growth

The effect of rutin on the proliferation of GL-15 human glioblastoma cells over a 72 h period was investigated using both trypan blue staining and thymidine incorporation assays (Fig. 1A, B). Growth curves of viable cells obtained from the trypan blue staining showed that the number of viable GL-15 cells/μl was not changed significantly after 24 h exposure to 1–10 μM rutin. However, treatment with 50–100 μM rutin for 24 h reduced significantly ($p < 0.05$) the number of viable cells (data not shown). After 72 h exposure, growth inhibition was more significant and was also observed in the presence of the lowest rutin concentrations used (1 and 10 μM; Fig. 1A). A significant ($p < 0.05$) growth inhibitory effect of rutin on GL-15 cells was also evidenced by ^{3}H-thymidine incorporation. When compared to cells in control conditions (0.5% DMSO), the level of ^{3}H-thymidine incorporation by GL-15 cells exposed to 10 μM rutin was 72.01 ± 8.36% and 39.72 ± 6.73% after 24 h and 72 h exposure, respectively (Fig. 1B). On the other hand, rutin had a stronger inhibitory effect on growth of GL-15 cells when used at higher concentrations. A single application of 50 μM rutin caused a reduction in ^{3}H-thymidine incorporation, namely to 12.90 ± 3.88% after 24 h and 26.65 ± 4.53% after 72 h exposure. At the highest rutin concentration (100 μM) growth inhibition of around 90% was achieved and ^{3}H-thymidine incorporation was only of 7.82 ± 2.24% and 10.26 ± 1.74% after 24 h and 72 h exposure, respectively.

Because rutin displayed an inhibitory effect on GL-15 cell growth, we analysed the cell cycle distribution after treatment by flow cytometry. In control conditions, the proportion of GL-15 cells in the G1 phase increased with time with a concomitant

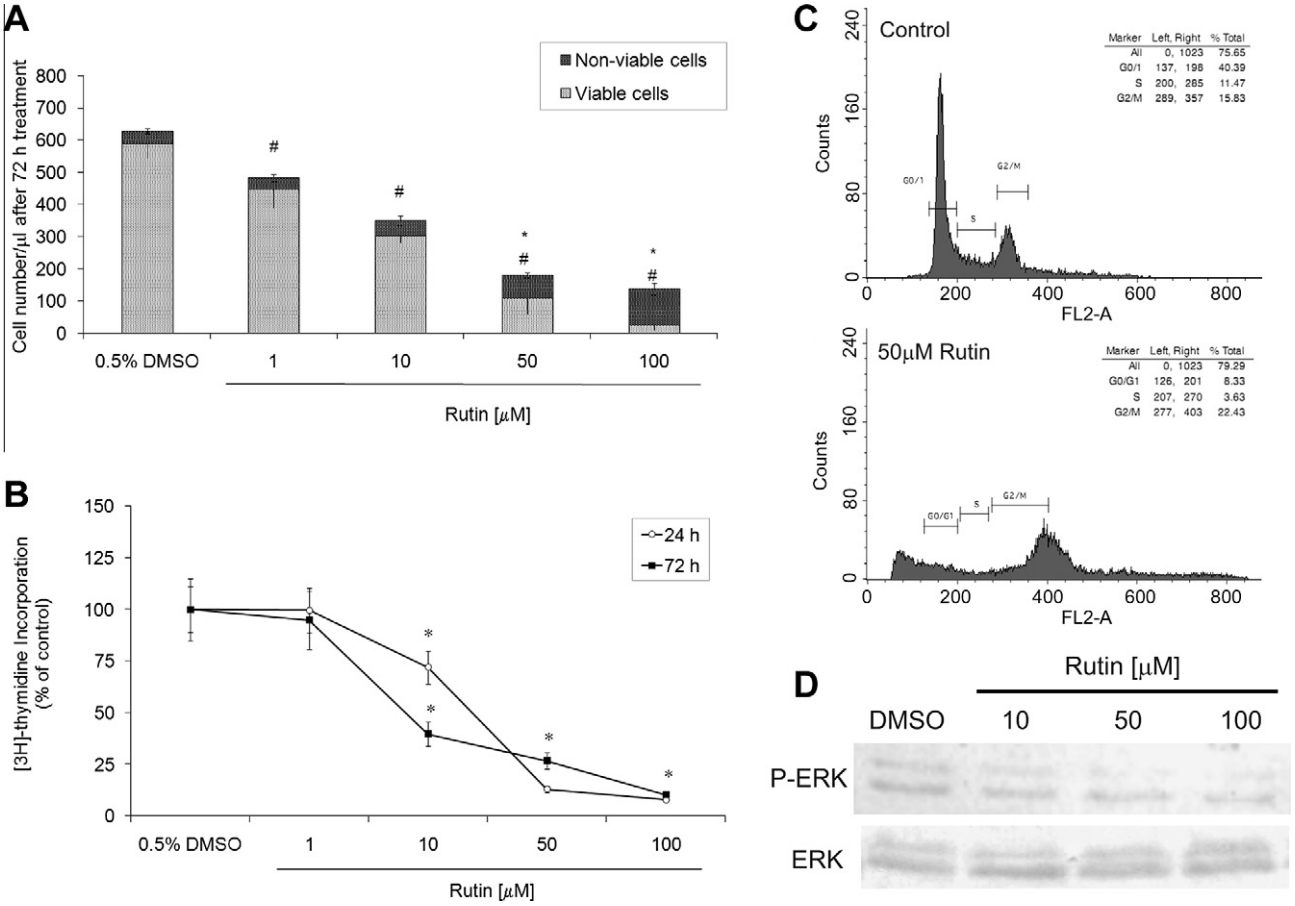

Fig. 1. Effect of rutin on viability and proliferation of GL-15 cells. Cells were incubated in the absence (control, 0.5% DMSO) or presence of rutin at different concentrations (1–100 μM), and cell viability was assessed by Trypan blue staining after 24 h or 72 h treatment. (A) Proportion (%) viable and non-viable (blue) cells after 72 h exposure and Trypan blue staining; (*) = Significant difference from control (0.5% DMSO) values ($p \leqslant 0.05$) referring the number of non-viable cells; (#) = Significant difference from control (0.5% DMSO) values ($p \leqslant 0.05$) referring the number of viable cells. (B) Proliferation assessed by ^{3}H-thymidine incorporation after 24 h or 72 h of the experiment; values for vehicle control samples were set at 100%; (*) = Significant differences from control values ($p \leqslant 0.05$). (C) DNA content analysis by flow cytometry after a 48 h experiment; the DNA content is shown by relative fluorescence and the number of cells in the G0/G1 phase are represented in the first peak, whereas those in the G2/M phase are in the second peak. Cells in S phase are present in the area between the G0/G1 and G2/M peaks. (D) Expression of phosphorylated and non-phosphorylated ERK proteins (p44/p42) from GL-15 cells in control conditions and after 24 h exposure to rutin, as determined by western blot. Results are from at least three independent experiments.

decrease in S phase (Fig. 1C). Thus, rutin induced changes in the cell cycle distribution. However, after 48 h exposure to 50 μM rutin, cells accumulated in the G2 phase.

ERK1 (44 kDa) and ERK2 (42 kDa) are members of the MAPK family that are induced in response to growth stimuli. We therefore investigated if growth inhibition by rutin is correlated with

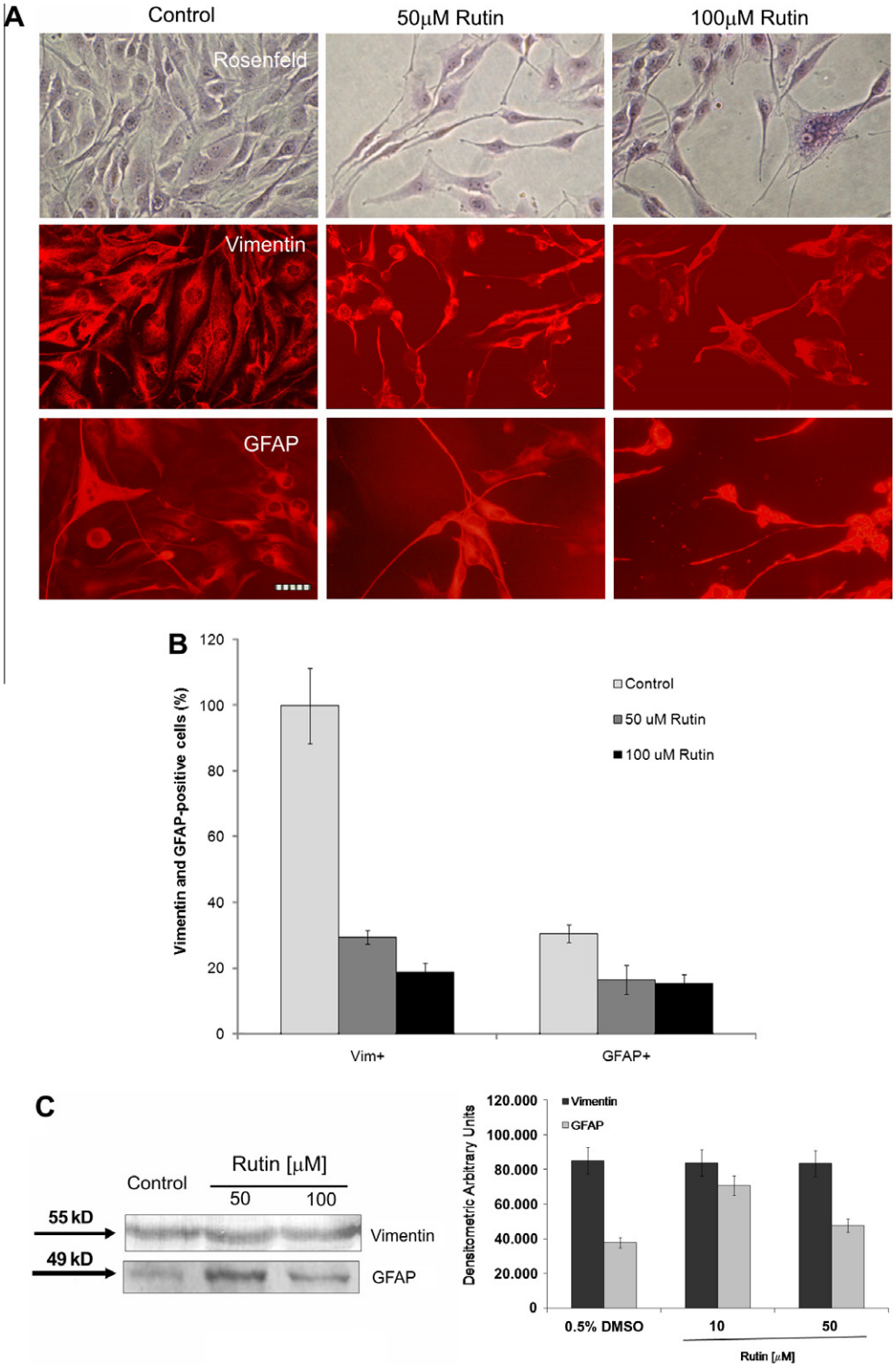

Fig. 2. Effect of rutin on morphology and on vimentin and GFAP expression in GL-15 cells. (A) Photomicrographs after Rosenfeld's staining and immunocytochemistry for vimentin and GFAP proteins in GL-15 cells under control conditions (0.5% DMSO) or treated for 72 h with 50–100 μM rutin. Objective 20 × 0.70, scale bars = 10 μm. This result is representative of three independent experiments. (B) The absolute cell number, vimentin positive cells, and GFAP positive cells were quantified. To normalise the variation between absolute numbers from different experiments, data are represented as proportion (%) for each marker. (C) Western blot analysis of vimentin and GFAP protein expression in GL-15 cells treated with rutin. Samples containing 20 μg of total protein for control conditions (0.5% DMSO) or treated for 72 h with rutin (10–100 μM) were electrophoretically separated through 12% polyacrylamide gel containing 0.1% SDS in running buffer. The immunoreactive bands are representative of three independent experiments and the histogram represents the relative expression of vimentin and GFAP.

ERK1/2 expression and phosphorylation. Fig. 1D shows expression of ERK proteins (p44/p42) in GL-15 cells by western blot analysis, under control conditions (0.5% DMSO) and after 24 h exposure to rutin. We observed that ERK1/2 phosphorylation (P-ERK1/2) levels markedly decreased with 10–100 μM rutin. However, there were no apparent changes in total ERK1/2 levels after rutin exposure.

3.2. Modulation of GL-15 cell morphology and expression of vimentin and GFAP

A morphogenic effect for rutin was investigated by phase microscopy and immunocytochemistry for the intermediary filament proteins vimentin, a marker of immature glial cells, and GFAP, an astrocyte marker. In control conditions (0.5% DMSO), as revealed by phase microscopy after 72 h treatment (Fig. 2A), GL-15 cells present a bipolar fibroblast-like phenotype with cell size varying to some extent depending on the culture density. Following treatment with rutin, GL-15 cells became very elongated and some cells exhibited cytoplasm retraction with thin cellular processes. The effect was more pronounced and appeared as early as 24 h after treatment with the highest inhibitory concentrations of rutin (50–100 μM).

Vimentin and GFAP immunolabelling, performed after a 72 h experiment, showed that confluent control GL-15 cells have a bipolar shape with a uniformly high pattern of vimentin expression but heterogeneous GFAP expression (Fig. 2A), restricted to a subpopulation of cells. After 72 h treatment with 10 μM (data not shown), and more extensively with 50 μM rutin, GL-15 cells displayed a more condensed cell body with a radial-like bipolar phenotype. Cells contained long thin processes expressing vimentin and GFAP, and a significant proportion (>50%) of cells have higher levels of GFAP expression. After exposure to 100 μM rutin, the majority of remaining cells displayed a strong retraction of their cell body and some cells had thin cellular processes expressing both vimentin and GFAP. Fig. 2B shows the quantification of vimentin and GFAP-positive cells in control and in rutin treated glioma cells.

Vimentin and GFAP expression in GL-15 cells was also examined by western blotting, and the effect of rutin was investigated. Western blot analysis showed that control GL-15 cells express more vimentin than GFAP (Fig. 2C). However, after 72 h treatment GFAP protein increased in protein extracts of GL-15 cells exposed to 10 μM of rutin, and remained higher than controls at 50 μM rutin. However, GFAP steady-state level decreased to less than control levels in cells exposed to 100 μM rutin. Under the same conditions, vimentin steady-state levels remained unchanged when rutin was present at 10–50 μM, but were elevated in cells treated with 100 μM rutin.

3.3. Induction of apoptosis in glioblastoma cells

We investigated whether rutin induces apoptosis or necrosis on glioblastoma cells, using Annexin V-FITC/Propidium iodine staining at the optimal inhibitory concentration. Fig. 3 shows an increase in apoptosis of GL-15 cells after 24 h of treatment with 100 μM rutin. The proportion of Annexin V-positive GL-15 cells exposed to 100 μM rutin was of 87.4%, compared with the control (0.5% DMSO) that displayed 1.6% labelled cells. Rutin at this concentration did not modify the percentage of necrosis for GL-15 cells when compared with control. Moreover, nuclear condensation and fragmentation were observed in 40.1 ± 4.3% of GL-15 cells by Hoechst staining after 72 h exposure to 100 μM rutin (Fig. 4A). We also investigated the capacity of rutin to induce DNA strand breaks using a single cell electrophoresis assay (comet test; Fig. 4B). After 24 h of treatment, as expected, the comet rate in positive controls (UV irradiation) was 66.01 ± 7.45%. The comet rate in negative controls (0.5% DMSO) was 6.01 ± 0.88%, which increased to 16.21 ± 1.99% in cells exposed to 100 μM rutin. After 72 h, the comet rate in the negative control was 19.72 ± 1.98%, which increased to 44.21 ± 5.90% in cells exposed to 100 μM rutin and 97.5 ± 9.0% when cells were exposed to UV irradiation.

4. Discussion

In this study, we investigated the antitumour potential of rutin, which had been extracted from seeds of the Brazilian plant *D. mollis*, in terms of growth inhibition, viability and differentiation of cultured human glioma cells. The highly proliferative human glioblastoma cell line GL-15 established by Bocchini et al. (1991) was used as a model.

The effect of rutin on the proliferation of GL-15 glioma cells was studied over a 72 h period. Proliferation of GL-15 cells was significantly inhibited by rutin over a concentration range of 10–100 μM in a dose-dependent manner (Fig. 2A), as shown by ^{3}H-thymidine incorporation and growth curves of viable cells. Maximum growth inhibition was obtained on the third day of treatment at the highest concentration tested. Such an inhibitory effect has previously been observed on rat C6 and human U138MG glioma cell lines, induced by the rutin derivative quercetin (Braganhol et al., 2006; Chen et al., 2006). ERK is activated by MAPK in response to growth stimuli and involved in diverse cellular signalling pathways including those regulating survival and proliferation (Krzeminski, 2005). We observed a rapid reduction in phosphorylation of extracellular signal-regulated kinase (P-ERK1/2). This effect has also been recently observed for the flavonoid kaempferol in relation to growth inhibition of human glioma cells (Jeong, Kim, Kim, and Kim, 2009).

In the inhibitory concentration range tested, rutin was not found to be cytotoxic for GL-15 cells after 24 h exposure, as assessed by trypan blue and Annexin V-FITC/Propidium iodine assays. Necrosis is an unregulated event that comprises the release of cellular components into the extracellular medium. In contrast,

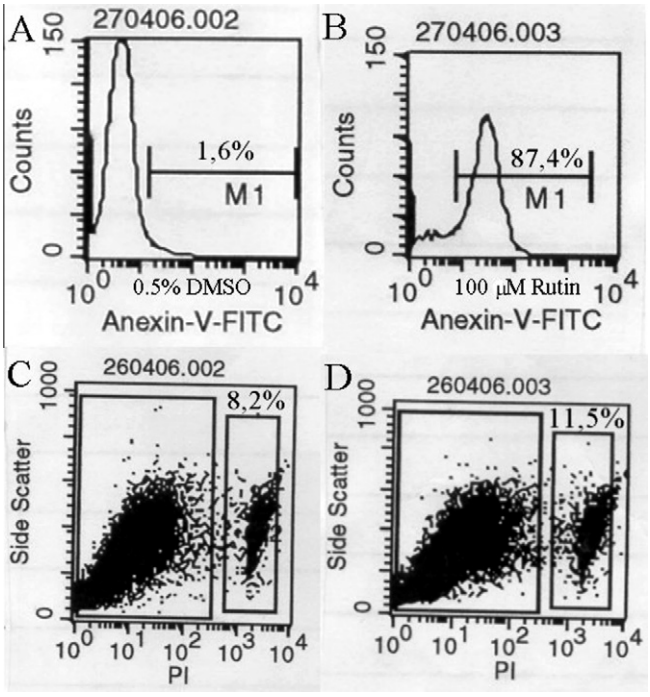

Fig. 3. Induction of apoptosis by rutin in human GL-15 cells as revealed by flow cytometry. Identification of phosphatidyl serine translocated to the outer cell membrane by Annexin V-FITC, and determination of propidium iodide-positive cells after a short time exposure (24 h).

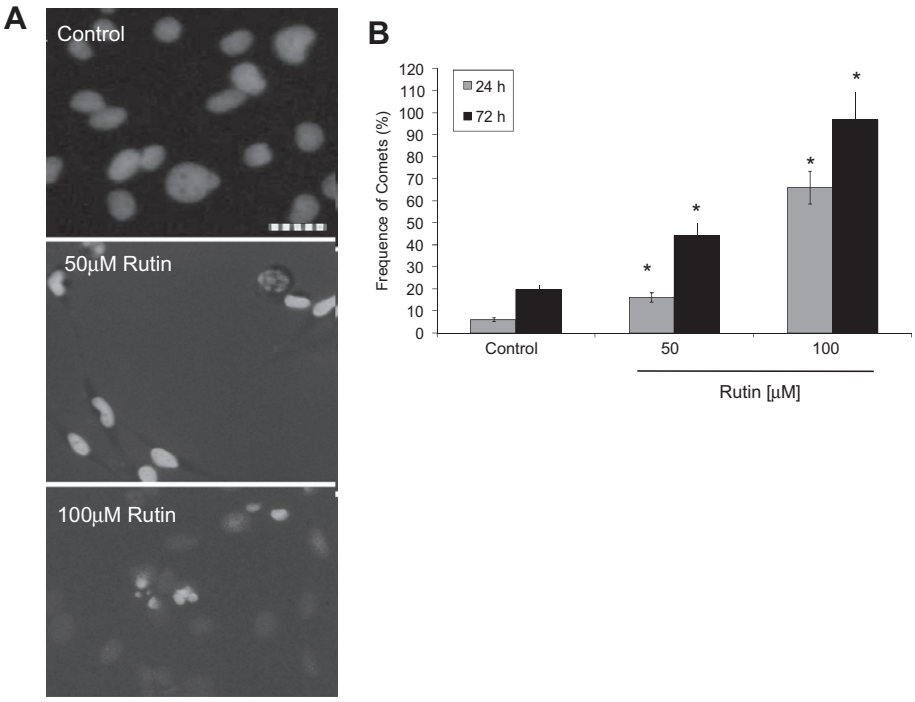

Fig. 4. Features of apoptosis in GL-15 cells after rutin exposure. (A) Morphological analysis of GL-15 cell nuclei in control conditions (0.5% DMSO) and nuclear condensation detection by Hoechst 33258 dye in cells treated with 50 or 100 μM rutin after 72 h. Arrows represents apoptotic bodies and fragmented nuclei; Objective 20 × 0.70, scale bars = 10 μm; this result is representative of three independent experiments. (B) Determination of DNA strand breaks in GL-15 cells exposed to rutin after 24 h and 72 h, using the comet assay. Cells were exposed to vehicle control (0.5% DMSO), rutin (100 μM) or UV irradiation (positive control). Data represents comet rates (mean ± SD) for at least 100 nucleoids of GL-15 cells per experimental point in three independent experiments; (*) = Significant differences from control values ($p \leqslant 0.05$).

apoptosis leads to an organised elimination of cellular components (Vaux & Strasser, 1996) and is defined by key events that are controlled by pro- and anti-apoptotic regulators. Common events in apoptosis include chromatin condensation and translocation of phosphatidyl serine to the outer cell membrane (Kokileva, 1994), as well as proteolytic activation of caspases, cytochrome c release from mitochondria, DNA fragmentation and membrane blebbing (Adrain & Martin, 2001; Vaux & Strasser, 1996). All of these events can be used as markers in the detection of apoptosis. We observed by Annexin V-FITC/Propidium iodine and Hoechst 33258 staining that rutin induces apoptosis in GL-15 glioblastoma cells. The proportion of membrane-damaged cells as determined by trypan blue staining was evident after 24 h exposure to 100 μM rutin and 72 h exposure to 50–100 μM rutin. Because apoptosis (but not necrosis) was detected after 24 h exposure to 100 μM rutin, the membrane instability observed after 72 h of rutin exposure may be attributed to cells that underwent apoptosis. Some flavonoids have the ability to interact directly with DNA and/or enhance carcinogen activation of DNA-modifying agents (Hodek et al., 2006); therefore, we tested the potential of rutin to induce DNA strand breaks in GL-15 cells. We observed that at the highest concentration adopted (100 μM), rutin induced a low but significant increase in the comet rate (i.e., frequency of DNA breaks). Because fragmentation of DNA is another event that characterises apoptosis (Heatwole, 1999), the DNA strand breaks observed in GL-15 cells exposed to rutin might be attributed to apoptosis. Associations between DNA strand breaks (revealed by the comet test), chromatin condensation and Annexin V labelling have been reported as being indicative of apoptosis in other cell systems (Morley et al., 2006). A small proportion of cells with DNA damage were observed _in control conditions, but as described by Tice et al. (2000), this result may be due to the high sensitivity of the comet test. Similar frequencies of DNA damage in control conditions have also been described in another *in vitro* study using genotoxic alkaloids (Boeira, Silva, Erdtmann & Henriques, 2001), and are probably due to the characteristics of tumour cell lines. Together, these results indicate that, in addition to growth inhibition, rutin induces apoptosis in GL-15 glioblastoma cells.

As revealed by phase microscopy and by vimentin and GFAP immunocytochemistry, rutin has a morphogenic effect on GL-15 cells that is especially evident after 72 h exposure to the highest growth inhibitory concentrations of 50–100 μM (Figs. 3 and 4). Vimentin is a major cytoskeletal component of immature astrocytes (Dahl, Rueger, Bignami, & Osbom, 1981), and GFAP is a major protein of astrocyte intermediate filaments and a specific marker for these cells. Moreover, studies have reported that an increase in GFAP content is related to differentiation of malignant gliomas (Rutka et al., 1994). Thus, besides a morphogenic effect of this flavonoid, immunocytochemistry and western blots for vimentin and GFAP revealed that this flavonoid induces differentiation of GL-15 glioblastoma cells towards an astroglial phenotype. This differentiation was especially evident at 50 μM, when both, the proportion of GFAP-positive cells and GFAP steady-state levels increased. When rutin was used at 100 μM, we observed an increase in vimentin steady-state level and immunostaining after 72 h. Vimentin is highly expressed early in development in both radial glia (Sancho-Tello, 1995) and radial glia-like Bergmann cells in the mature cerebellum (Alonso, 2001). Vimentin overexpression in the GL-15 cells remaining after 100 μM rutin exposure suggests that these remaining cells may recapitulate a radial glial-like phenotype. GFAP was barely detected by western blot; its expression was however evident in the remaining GL-15 cells by immunocytochemistry, which suggests that GFAP disruption might interfere with western blot detection rather than supporting a down regulation. Sagara, Vanhnasy, and Maher (2004) investigated the differentiation potential of many flavonoids and found that the aglycone quercetin induced differentiation of rat neural PC12 cells but rutin did not, even when it was employed at 100 μM. The

authors deduced that structural determinants were needed for the differentiation activity induced by flavonoids. They observed that all active flavonoids possess a catechol structure on the B ring (C3′ and C4′), an unsaturated C ring with an oxygen keto group on C4, and a hydroxyl group on C7 in the A ring. Rutin possesses all of these chemical characteristics but, in addition, it contains a glycosyl moiety at C3 on the A ring that may interfere with its differentiation activity for rat PC12 neuronal cells but not human GL-15 glioblastoma cells.

As described, different flavonoids present considerable variation in blood–brain barrier (BBB) permeability (Fernandez et al., 2006; Youdim, Qaiser, & Begley, 2004). It was observed that flavonoids including flavonoid glycosides, naringenin, quercetin, hispidulin, hesperetin, naringenin cross the BBB, due either to their lipophilic nature or to their interactions with specific efflux transporters expressed in the BBB. Although flavonoids can access to the brain, it is clear that these substances and their metabolite forms reach lower concentrations in vivo than those recorded for small-molecule antioxidant nutrients such as ascorbic acid and α-tocopherol (Youdim et al., 2004). Consequently, the beneficial effects of flavonoid metabolites in vivo, do unlikely result from their ability to out-compete antioxidants such as ascorbate, which are present in higher concentrations. It rather supports the hypothesis that these natural polyphenol compounds might be regarded as potential neuroprotective agents in vivo. Nevertheless, it is evident that flavonoids are potent bioactive molecules, and a clear understanding of their mechanism of action is crucial to the evaluation of their therapeutic potential.

Collectively, our results have shown that the flavonoid rutin present a capacity to inhibit growth and induce differentiation of cultured glioblastoma cells, suggesting that this compound could be considered as a candidate for the treatment of malignant gliomas. Further studies are under investigation to better understand the mechanism of action of rutin on glioblastoma cells.

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