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Original Articles

Inhibitions of mTORC1 and 4EBP-1 are key events orchestrated by Rottlerin in SK-Mel-28 cell killing

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ABSTRACT

Earlier studies demonstrated that Rottlerin exerts a time- and dose-dependent antiproliferative effect on SK-Mel-28 melanoma cells during 24 h of treatment, but cytotoxicity due to cell death began only after a 48 h exposure. In the current study, in order to identify the type of cell death in this cell line, which is notoriously refractory to most anticancer therapies, and to clarify the underlying mechanisms of this delayed outcome, we searched for apoptotic, necrotic/necroptotic and autophagic traits in Rottlerin-exposed cells. Although SK-Mel-28 cells are both apoptosis and autophagy competent, Western blotting analysis, caspase activity assay, nuclear imaging and the effects of autophagy, apoptosis and necroptosis inhibitors, indicated that Rottlerin cytotoxicity was due to none of the aforementioned death mechanisms. Nevertheless, in growth arrested cells, the death did occur after a prolonged treatment and most likely ensued from the observed blockage of protein synthesis that reached levels expected to be incompatible with cell survival. From a mechanistic point of view, we ascribed this effect to the documented inhibition of mTORC1 activity; mTORC1 inhibition on the one hand led to a not deadly, rather protective autophagic response but, on the other hand caused a near complete arrest of protein synthesis. Interestingly, no cytotoxicity was found towards normal skin fibroblasts, which only resulted mildly growth arrested by the drug.

Introduction

We recently presented evidence that the natural polyphenol Rottlerin is a potent autophagy inducer. In apoptosis-resistant MCF-7 breast cancer cells, the treatment, at cytotoxic doses, led to autophagic cell death [1]. Autophagy induction was AMPK- and Beclin-1- independent and mediated by direct inhibition of mTORC1 [2], a central controller of several essential cellular functions, the best understood of these being the inhibition of autophagy and the positive regulation of protein synthesis [3].

Autophagy, an important cellular homeostatic process necessary to maintain cell survival under various stresses and changes in intracellular and environmental conditions, functions as a scavenger for misfolded proteins and damaged organelles and/or as a defense against microbes and parasites. Defective autophagy, indeed, is associated with various human pathologies, such as cardiomyopathy, neurodegeneration, autoimmune diseases, infections and cancer [4]. In human cancer, autophagy has become a potential target for pharmacological interventions because it can be also a mechanism of non-apoptotic cell death (type II programmed cell death); excessive autophagy in fact may destroy essential intracellular molecules and structures to a level incompatible with cell life.

It is generally believed that cancers with constitutively activated autophagy, such as SK-Mel-28 melanoma cells used in the current study [5], are profoundly dependent on this process for tumorigenic growth and survival; therefore they should be treated with autophagy-inhibiting drugs, as in the case of pancreatic cancers treated with the autophagy blocker chloroquine (CQ) [6]. Pancreatic cancer, however, also responds to an autophagy inducer, such as gemcitabine, indicating that when autophagy is pharmacologically stimulated beyond a tolerable threshold, it could contribute to cancer cell death even in cells showing elevated basal levels of autophagy [7]. At any rate, because of the 'yin–yang' role of autophagy in survival *versus* cell death, autophagy inducers (Rottlerin included) should be used with caution in anticancer therapies.

Abbreviations: RPMI, Roswell Park Memorial Institute (RPMI); Becl-1, beclin-1; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; mTOR, mammalian target of rapamycin; raptor, regulatory-associated protein of mTOR; p70S6K, p70 ribosomal protein S6 kinase; p90RSK, p90 ribosomal S6 kinase; LKB1, liver kinase B1; SQSTM 1, sequestosome 1; LC3-II, microtubule-associated protein 1 light chain 3-II; PARP, poly(ADP-ribose) polymerase; Z-VAD- FMK, benzyloxycarbonyl-ValAla-Asp (OMe) fluoromethylketone; CQ, chloroquine; 3-MA, 3-methyladenine; Nec-1, necrostatin-1; TCA, trichloroacetic acid; SRB, sulforhodamine; PMSF, phenylmethylsulfonyl fluoride; LDH, lactate dehydrogenase.

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However, the recent literature has revealed that Rottlerin is a multitarget drug, able to interfere with both the apoptotic and the autophagic pathways and to exert, as a single agent or as part of combined therapies, a successful anticancer action *in vitro* in a variety of tumor cells [8].

In our earlier study, we demonstrated that Rottlerin exhibited a marked antiproliferative effect on SK-Mel-28 cells during 24 h of treatment, but cytotoxicity, culminating in cell death, occurred only after a 48 h exposure [9]. In order to identify the type of cell death and to clarify the underlying mechanisms of this delayed outcome, we searched in the current study for apoptotic, necrotic/necroptotic and autophagic traits in Rottlerin-exposed cells. A comparative cytotoxicity analysis was also performed in human skin fibroblasts, confirming the already described selectivity of Rottlerin towards cancer cells [10,11].

Materials and methods

Materials

MG132 and Rottlerin were obtained from Calbiochem, San Diego, CA. DMEM, RPMI-1640, FBS, antibiotics, DMSO, Necrostatin, Z-VAD-FMK, chloroquine (CQ), Puromycin, 3-methyladenine (3-MA), LDH assay kit and Ponceau S were from Sigma Aldrich, St. Louis, MO. Antibodies against total and phospho-AMPK (Thr172), total and phospho-ACC (Ser79), total and phospho-Raptor (Ser722/Ser792), total mTOR, total and phospho- p70S6K (Thr389), total 4EBP-1, caspase 9, caspase 8, caspase 3, PARP, Becl-1, Bcl-2, PKCδ, Livin, cyclin D1 and β-actin were obtained from Cell Signaling Technology, Danvers, MA. Antibody against SQSTM1/p62was from Santa Cruz Biotechnology, Santa Cruz, CA. Antibody against Puromycin was from Merck Millipore, Darmstadt, Germany. M-PER Mammalian Protein Extraction Reagent and Halt Protease and Phosphatase inhibitor cocktail were from Pierce, Rockford, IL. Equipment and all reagents for protein assay and western blotting analysis were from Invitrogen, Carlsbad, CA. Nitrocellulose, ECL Prime Western Blotting Detection Reagent, and Hyperfilm ECL were from GE Healthcare Life Sciences, Uppsala, Sweden.

Cells and culture conditions

Sk-Mel-28 human melanoma cells (from ATCC) were grown and maintained in 25 cm^2 tissue culture flasks in a humidified atmosphere (95% air/5% CO_2) at 37 °C in RPMI-1640 medium, containing 10% FBS, glutamine (2 mM), and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B). Stock solutions of 20 mM Rottlerin, dissolved in DMSO (20 µM final concentration) was stored in a dark colored bottle at –20 °C. After reaching subconfluence, cells were treated with Rottlerin or vehicle (DMSO) in complete medium containing 2.5% FBS for the indicated periods. Human adult dermal fibroblasts were established from healthy volunteers, after written informed consent. The primary cultures of human skin fibroblasts were initiated from a 3 mm skin punch biopsy. Cells were cultured in DMEM supplemented with 10% FBS and antibiotics (as reported for SK-Mel-28 cells) and used between passage 3 and 7. Fibroblasts, grown to near confluence, were treated with Rottlerin or vehicle (DMSO) in complete medium containing 2.5% FBS for the indicated periods. In a subset of experiments, cells were pretreated 1 h with the following substances: 2 and 20 µM Z-VAD-FMK (pan-caspase inhibitor), 25 and 50 µM CQ, 5 mM 3-MA (autophagy inhibitors), 10 and 100 µM necrostatin-1 (necroptosis inhibitor). Then, SK-Mel-28 cells were treated with 20 µM Rottlerin for 24–48 h. In a set of experiments, Puromycin was added to the cultured cells (1 µg/ml) just 10 min before cell lysis.

Cell growth and cytotoxicity

Cell growth and cytotoxicity were evaluated by the SRB colorimetric assay, as previously described [9]. The SRB dye, in moderated acid conditions, binds stoichiometrically to basic protein amino acids; the measured optical density correlates well to cell number and is a good indicator of cell growth and/or drug cytotoxicity [12]. Cells were seeded in triplicate on 96-well plates, incubated 4–6 h at 37 °C to allow adherence, and treated with 20 µM Rottlerin for 24–48 h. Following treatment, the medium was removed and the cells were washed twice with PBS and fixed with 100 µL of cold 10% TCA. The plates were incubated at 4 °C for 30 min before being gently washed four times with tap water to remove TCA and dead cells. Then the plates were air-dried and 100 µL of SRB (0.4% w/v SRB dissolved in 1% acetic acid) was added. After 30 min of staining, unbound SRB was removed by four washings with 1% acetic acid. The plates were air-dried again, and 200 µL of 10 mM aqueous Tris base (pH 10.5) was added to solubilize the cell-bound dye. The plate was mixed for 30 min by frequently pipetting up and down to dissolve the dye completely. The optical density (OD) was recorded in a microplate spectrophotometer at 550 nm.

Western blotting analysis

Cell extracts, each containing 30–40 µg of total protein, were resolved on 8% or 12% SDS polyacrylamide gel. Proteins were electrotransferred onto nitrocellulose membranes which were blocked by 5% nonfat dry milk in TBS containing 0.1% Tween 20 for 1 h at room temperature. Then the blots were probed with primary antibodies overnight at 4 °C. After washing, horseradish peroxidase-conjugated IgG was added for 1.5 h at room temperature. β-actin was used as a loading control. The blots were developed by the ECL reagent and exposed on photographic film.

Measurement of the caspase 3/7 activity

Caspase 3/7 enzymatic activity was measured as previously described [13]. Briefly, the cells were harvested, washed with PBS, suspended (106 cells/100 µL) in ice-cold lysis buffer (20 mM Hepes-NaOH, pH 7.5, containing 10% sucrose, 0.1% CHAPS, 0.2% NP-40, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (Sigma-Aldrich)), and sonicated for 10 s (Vibracell Sonicator; amplitude 60, 25 W). The lysates were centrifuged at 14,000 × g for 15 min and the supernatants were used for determination of protein concentration through the Bradford method. 100 µg of cell lysate protein were incubated at 25 °C with 100 µM of the substrate ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (ac-DEVD-AMC, Alexis) in the assay buffer (lysis buffer, where Hepes and NP-40 are 100 mM and 0.1%, respectively). Cleavage of the fluorogenic substrate was monitored by AMC release in a fluorescence spectrophotometer (excitation/emission wavelengths: 380/460 nm) and caspase activity was expressed as arbitrary units of fluorescence (AUFs)/min/mg protein.

Morphology study in fluorescent microscopy

For analysis of apoptotic cell death, Hoechst 33342 staining was used. The dye freely crosses the plasma membrane, binds specifically to the A-T base region of DNA, and emits fluorescence. SK.Mel-28 cells, cultured on 24-well plates for 24 h and 48 h in RPMI –1640, 2.5% FBS with or without Rottlerin (20 µM), were stained with Hoechst 33342 (10 µg/mL in PBS) in the dark at room temperature for 30 min. After incubation, cells were examined at 355 nm excitation and 460 nm emission by inverted fluorescence microscopy (Nikon Eclipse TE 300, Germany). Cells were also observed under phase contrast microscope and photographed using CCD camera attached to the microscope.

Measurement of proteosynthesis

The incorporation of Puromycin, which becomes covalently linked to the C- terminus of the nascent polypeptides, was used to label newly synthesized proteins, in order to monitor protein synthesis [14]. In SK-Mel-28 cells treated for 24–48 h with 20 µM Rottlerin, Puromycin was added to the medium (1 µg/ml) just 10 min before the end of the experiment. After two washes with PBS, cells were lysed and the labeled polypeptides were visualized by Western blotting analysis using an anti-Puromycin polyclonal antibody.

LDH release assay

To detect the leakage of LDH into cell culture medium, we used a two-steps method. In the first step, LDH reduces NAD to NADH when it catalyzes the oxidation of lactate to pyruvate. In the second step, the newly synthesized NADH converts tetrazolium salt to a colored formazan product that can be specifically detected by colorimetric assay (450 nm).

Statistical analysis

Values are expressed as the mean ± SD. Student's *t*-test was used to determine statistical significance with a threshold of *P* values less than 0.05.

Results

Rottlerin selectively acts towards SK-Mel-28 melanoma cells

As shown in Fig. 1A, Rottlerin cytotoxicity (100 × (control – experimental) / control) towards melanoma cells, evaluated by the SRB assay, reached almost 60% after 48 h of exposure, in agreement with previous data [9]. Similar results were obtained by the Trypan Blue exclusion test (not shown). At the same time, a comparative analysis between melanoma cells and skin fibroblasts revealed a modest toxicity (6.7%) against normal cells, for the same Rottlerin dose and exposure time. The observed cytotoxicity is mainly ascribable to the Rottlerin cytostatic effect, because, as shown in Fig. 1B, fibroblast growth (% of control at time 0) decreased from 136% in untreated to 111% in treated cells after 48 h of culture. The observed growth

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