

GENISTEIN DOES NOT INDUCE CASPASE 2 ACTIVATION *IN VITRO* ON B16 MELANOMA CELL LINES

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Abstract

The phytocompound genistein, a tyrosin protein kinase inhibitor, the aglicon of the heteroside genistin, represents the main compound in the soybean, *Glycine max* (Fam. Fabaceae). Recent studies have attributed an anticancer potential to this compound due to its pro apoptotic and anti-proliferative effects. Several papers have demonstrated that genistein is an activator of caspase 3, an effector of apoptosis on different cell lines, including murine melanoma cell lines. Few data exist however about the effect of genistein on the initiator caspase 2 expressions on different cell lines. The aim of this study was to determine if B164A5 murine melanoma cell line and its high metastatic derivate B16F10 express caspase 2, an initiator of apoptosis after exposure to different concentrations of genistein. Results have shown that, after exposure to concentrations of genistein within the range 1-150 μ M after 72 h of incubation, B164A5 cell line and its high metastatic derivative B16F10 do not express caspase 2. This result leads to the conclusion that genistein induced apoptosis is not mediated through caspase 2 signalling pathway in case of B16 murine melanoma cell lines.

Rezumat

Fitocompusul genisteină, agliconul heterozidei genistină, un inhibitor al proteinei tirozin kinază, reprezintă principalul compus activ din semințele de soia, produsul vegetal provenit de la planta *Glycine max* (Fam. *Fabaceae*). Studii recente au atribuit acestui compus un potențial anticanceros datorită proprietăților sale pro-apoptotice și antiproliferative. Mai multe lucrări anterioare au demonstrat faptul că genisteina este un activator al caspazei 3, o proteină efectorie, direct implicată în inducerea procesului de apoptoză, pe linii celulare canceroase diferite, inclusiv linia de celule de melanom murin. Puține date există însă despre efectul genisteinei asupra caspazei 2, o proteină inițitoare a apoptozei. Scopul acestui studiu este de a investiga dacă linia celulară de melanom murin B164A5, respectiv linia derivată puternic metastatică B16F10 exprimă caspaza 2 după expunerea la diferite concentrații de genisteină. Rezultatele au arătat că, în urma expunerii la concentrații de genisteină cuprinse în intervalul 1-150 μ M, după 72 h de incubare, linia celulară de melanom murin B164A5, respectiv linia derivată puternic metastatică B16F10, nu exprimă caspază 2. Concluzia acestui studiu este că efectul pro-apoptotic al genisteinei nu este mediat prin intermediul caspazei 2.

Keywords: genistein, B16 murine melanoma, caspase 2.

Introduction

Natural phytocompound, genistein, a tyrosin protein kinase inhibitor, the aglicon of the heteroside genistin, represents the main compound in the soybean, *Glycine max* (Fam. *Fabaceae*) [1, 2]. Other medicinal plants that contain important amounts of genistein are: red clover (*Trifolium pratense*-*Fabaceae*), lucerne (*Medicago sativa* - *Fabaceae*), dyer's broom (*Genista tinctoria* - *Fabaceae*) [3]. This substance presents a similar structure to the estrogen hormone which is why it is called a phytoestrogen. Due to this property, genistein is currently used to counteract the symptoms which appear at menopause. Beside this common use, it has been proved to have several biological activities such as antioxidant, anthelmintic, antineoplastic and anti-inflammatory. Genistein is also regarded as a preventive agent for coronary heart disease and osteoporosis [4-7].

Employing different murine B16 cell sublines represents one of the most popular and versatile model for experimental melanoma. The B16 model has been extensively used in order to reveal several stages in the evolution of melanoma but also for testing different active agents, candidates as anti-melanoma compounds [8-10]. B164A5 is the cell line derived from a melanoma found in the skin of the C57BL6J mouse, while B16F10 is its high metastatic version with lung metastasis tropism [11, 12].

Caspases are a class of proteases which play a capital role in apoptosis induction. Caspase-2 is a cysteine protease, one of the earliest identified caspases, which, together with caspases 8, 9 and 10 form the class

of initiator caspases. Their role is to activate effector caspases, inactive proenzymes, namely caspase 3, 6 and 7, directly responsible for the induction of programmed cell death. In terms of the sequence of molecular events that occur during apoptosis and involve caspase 2 activation it is known that this protease is primarily cleaved into three fragments of 32 kDa, 33 kDa and 14 kDa, which then transform into 18 kDa and 12 kDa active subunits [13, 14].

The aim of this study was to determine if the B164A5 murine melanoma cell line and its high metastatic derivative B16F10 express caspase 2, an apoptosis initiator, after exposure to different genistein concentrations.

Materials and Methods

Cell culture

B164A5 cells were acquired from Sigma Aldrich (ECACC and Sigma Aldrich, origin Japan stored in UK), B16F10 (ATCC CRL-6475, Naval Biosciences Laboratory), Dulbecco's Modified Eagle's Medium (DMEM), Phosphate-buffered saline (PBS), Penicillin/Streptomycin mixture, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Trypsin were purchased from Gibco. Fetal Calf Serum (FCS), Ethylenediaminetetraacetic acid (EDTA), Dimethyl sulfoxide (DMSO) was achieved from Sigma Aldrich. The complete growth medium for this cells is Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml) and 2% HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The cells were cultured by incubation at 37°C in 5% CO₂ atmosphere. When the confluence was 70-80% (every two or three days) the cells were passed using 0.25% Trypsin- 1 mM EDTA solution followed by centrifugation (5 minutes, 1200 rpm) and replated in T75 culture flasks at a subcultivation ratio of 1:10 to ensure optimal proliferation.

Immunocytochemistry

Immunocytochemistry was performed for B16 4A5 cells and B16F10 cells plated at a density of 10,000 cells/cm² in 4-well glass chamber slides (Nalgene Nunc International, New York, USA), and expanded for 24 hours in culture medium. After 24 hours new medium containing different concentrations of the active compound genistein (Extrasynthese, France)-150 µM, 100 µM, 50 µM, 30 µM, 15 µM, 5 µM, 1 µM were added. Control well contained untreated cells. Cells were maintained in culture for 72 hours after addition of substances and then prepared for immunocytochemical staining. After removing the culture medium, cells were washed, fixed with 4% paraformaldehyde (Sigma-Aldrich Company), permeabilized with 0.1%

Triton X-100 (Sigma-Aldrich Company) and then investigated for expression of the proteins of interest, using for labelling anti-h/m Caspase 2 (mCaspase 2 affinity purified rabbit IgG) (R&D Systems, Abingdon, UK). Staining protocol continued with secondary biotinylated antibody binding and substrate addition (AEC) (Dako EnVision™ + System-HRP, Dako, CA, USA) following the manufacturer protocol. After counterstaining with hematoxylin solution (Hematoxylin, Mayer's Lillie's Modification, Dako) for 30 seconds and washing with tap water the slides were mounted in an aqueous mounting media (Crystal/Mount™, Biomed, CA, USA). Microscopy analysis was performed on a Nikon Eclipse E800 microscope.

Results and Discussion

Results can be seen in Figure 1 a-h and Figure 2 a-h, respectively.

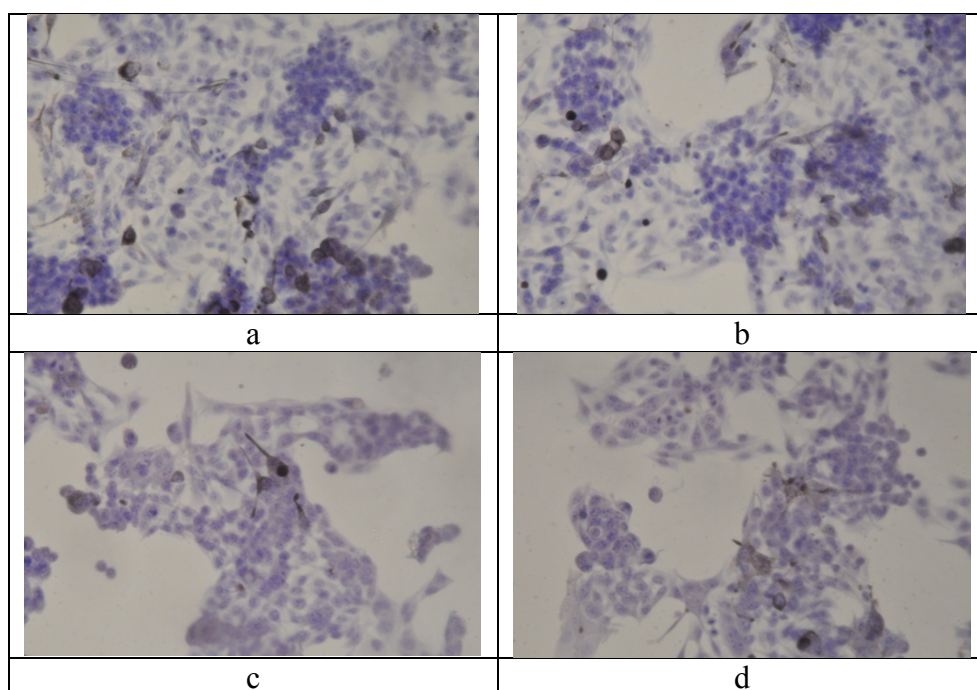


Figure 1 a – d

Caspase 2 expression for B164A5 cells analysed after 72 h of incubation with a) 0 μ M; b) 1 μ M; c) 5 μ M; d) 15 μ M;

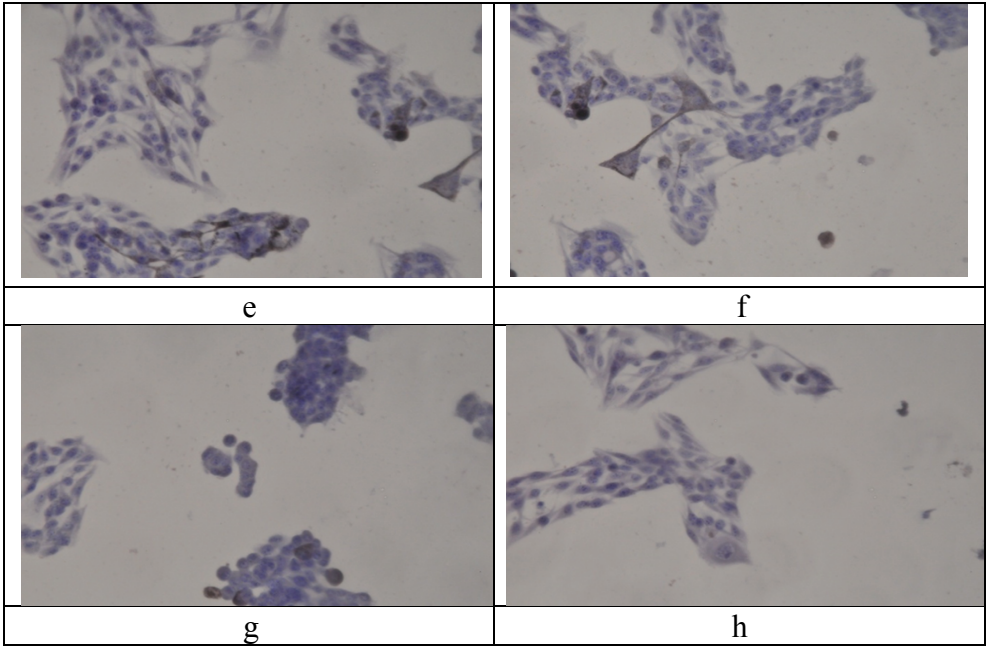


Figure 1 e – h
Caspase 2 expression for B164A5 cells analysed after 72 h of incubation with e) 30 μM; f) 50 μM; g) 100 μM; h) 150 μM genistein

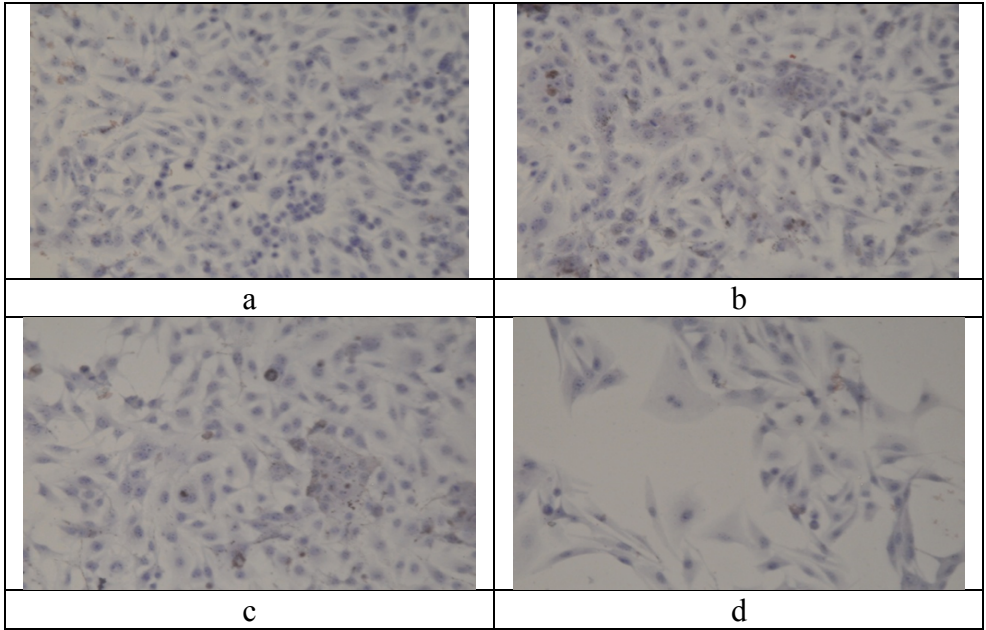


Figure 2 a – d Caspase 2 expression for B16F10 cells analysed after 72 h of incubation with a) 0 μM; b) 1 μM; c) 5 μM; d) 15 μM;

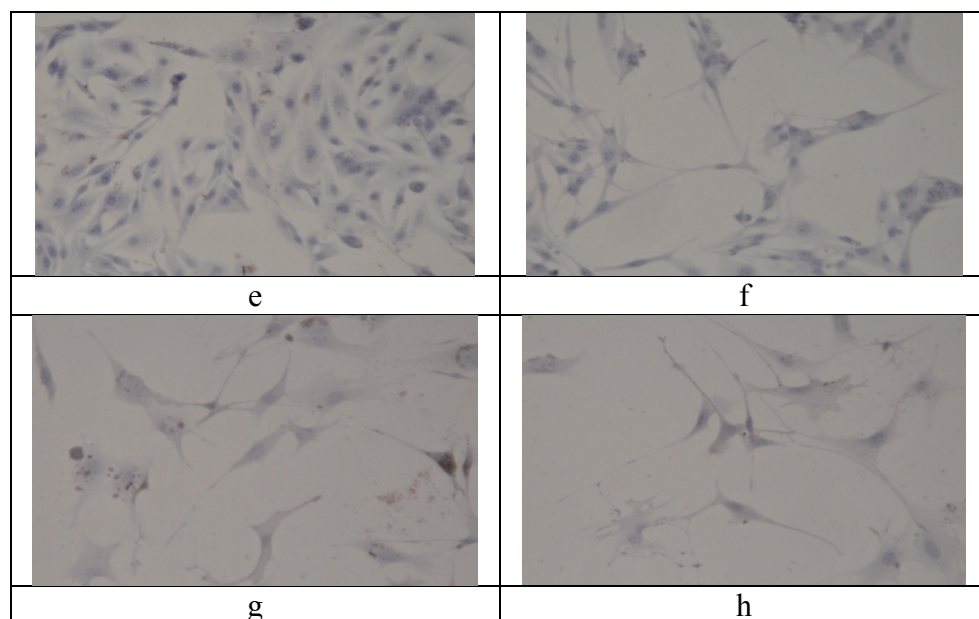


Figure 2 e – h Caspase 2 expression for B16F10 cells analysed after 72 h of incubation with e) 30 μM ; f) 50 μM ; g) 100 μM ; h) 150 μM genistein

Note that genistein does not induce caspase 2 activation, in none of the two B16 melanoma cell lines tested, after 72 h of incubation and analysing a wide range of doses (1 μM - 150 μM), as revealed by immunocytochemical detection. Another important finding is that genistein influences cell proliferation. Microscopic pictures showed an obvious decreased number of cells in the selected fields as compared to control, for the highest tested concentrations, 100 μM and 150 μM , respectively, for both B164A5 and B16F10 cell lines. Signs of an anti-proliferative effect can be observed starting from the 5 μM concentration of genistein, after 72 h of incubation.

Apoptosis or programmed cell death is a process in which cells, following a response to a signal, trigger their self-destruction. Apoptosis is a physiological process required for the normal development and functioning of multicellular organisms, providing continuous tissue renewal [15]. The caspase dependent apoptotic pathway involves events that have mitochondria as primary headquarters. The initiator caspases 2, 8, 9 and 10 activate the effector caspases 3, 6 and 7 which then lead to the loss of cell membrane integrity, nuclear fragmentation and condensed chromatin filaments [16, 17]. Under normal, physiological conditions, apoptosis is in a perfect equilibrium with cell proliferation while cancerous cells, via aberrant mutations, undergo excessive survival proliferation because they are not subject to programmed cell death [18]. That is why finding active agents that induce apoptosis is a

classical approach for fighting against cancerous cells. The literature reports genistein as a pro-apoptotic compound on different cancerous cell lines: e.g. breast cancer cells, mammary epithelial cells, hepatocellular carcinomas, prostate cancer cells [19-22]. Some of the apoptosis mechanisms have been studied and in terms of caspase activation it is well known that executor ones, caspase 3 or/and caspase 6 or/and caspase 7, are activated by genistein in some tumour cell lines (e.g. LNCaP prostate cancer cells, human breast cancer MCF-7 cells, TM 4 testicular cells, human melanoma cells) [23-26]. However, few data exist on the effect of genistein on initiator caspase 2 on different cell lines. B16A5 murine melanoma cell line and its high metastatic derivate B16F10 do not express caspase 2, after 72 h of incubation and exposure to different concentrations of genistein.

Conclusions

In this study we have investigated the effect of this natural compound on caspase 2 expression on B16A5 murine melanoma cell line and its high metastatic derivate B16F10. Using a wide range of concentrations (1-150 μ M genistein) we concluded that, after 72 h of incubation, caspase 2 is not expressed. Literature reports that genistein, at a concentration of 60 μ M, after 72 h of incubation, activates caspase 3, being a pro apoptotic agent [27]. This finding leads us to the conclusion that genistein actually induces apoptosis in case of B16 murine melanoma cell lines, yet not caspase 2 mediated.

Acknowledgements

The first author, Corina Danciu benefitted by a grant, supported by UMFT 15250/19.12.2012.

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