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ORIGINAL ARTICLE

Role of transglutaminase 2 in quercetin-induced differentiation of B16-F10 murine melanoma cells

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Abstract Flavonoids belong to the class of plant polyphenolic compounds with over 6,000 individual structures known. These phytochemicals have attracted the interest of the scientists, because they possess a remarkable spectrum of biological activities, such as antiallergic, antiinflammatory, antioxidant, antimutagenic and anticarcinogenic. In this work, we compared the anticancer potential of two flavonoids, quercetin and pelargonidin, on highly metastatic B16-F10 melanoma murine cells. We have evaluated different parameters related to cell proliferation and differentiation, such as cell number, toxicity, intracellular content of polyamines and transglutaminase (TG, EC 2.3.2.13) activity. The higher inhibition of tumor cell growth, with respect to control, was obtained with quercetin cell treatment, i.e. 32% reduction after 48 h and 39% reduction after 72 h of incubation ($P < 0.001$). In parallel, quercetin-treated cells showed a similar decrease in polyamine content. TG activity was fourfold increased, with respect to control, after 48 h and twofold increased after 72 h ($P < 0.001$). Pelargonidin treatment did not show significant antiproliferative effects and any increase in TG activity. Proteomic approach was used to investigate changes in protein expression profiles in tumor cells following quercetin treatment. Changes in expression of 60 proteins were detected, i.e. 8 proteins were down-regulated, 35 up-regulated, 11 "de novo" synthetized proteins and 6 suppressed proteins were present in treated cells. A 80 kDa spot, identified as TG type 2 by Western blot analysis, presented a fourfold increase in intensity, confirming the key role played by TG in the induction of cancer cell differentiation.

Keywords Melanoma · Quercetin · Pelargonidin · Transglutaminase · Polyamines · Proteomics

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Introduction

Flavonoids are a group of polyphenolic compounds occurring in plants, where they play a role in many physiological process. This wide group of more than 6,000 compounds have a common phenylbenzopyrone structure (C6–C3–C6) and they are classified according to the saturation level and opening of the central pyran ring, mainly into flavonols, flavones, flavanols (cathechins), flavanones, isoflavones and anthocyanins (Cooper-Driver 2001). They possess a remarkable spectrum of biological activities, including antiallergic, antiinflammatory, antioxidant, antimutagenic and anticarcinogenic properties (Detre et al. 1986; Bomser et al. 1996; Bagchi et al. 1997; Wang et al. 1999; Atalay et al. 2003). Compelling data from in vivo and in vitro studies, epidemiological investigations and human clinical trials have evidenced the important role of flavonoids in cancer chemoprevention and therapy. The chemopreventive properties of flavonoids are generally believed to reflect their ability to scavenge endogenous ROS. However, their pro-oxidant activity rather than their antioxidant action may be an mechanism dealing with the anticancer and apoptosis-inducing properties, as ROS can mediate apoptotic DNA fragmentation (Galati and O' Brien 2004).

Some literature data reported other important effects of these phytochemicals on polyamine production and TG activity (Lentini et al. 2007). TG is a multifunctional enzyme involved in several biological processes (Folk 1980; Beninati and Piacentini 2004), included cell differentiation (Thacher and Rice 1985; Benedetti et al. 1996; Arani et al. 1997; Lentini and Beninati 2002). This enzyme catalyzes the post-translational modification of proteins by the formation of isopeptide bonds. This may occur either through protein cross-linking via ε-(γ-glutamyl)lysine bonds or through incorporation of primary amines into peptide-bound glutamine residues. It is well known that intracellular activation of soluble TG may cross-link proteins leading to the formation of envelopes in apoptotic cells (Fesus 1993; Autuori et al. 1998), whereas extracellular activation contributes to the stabilization of the extracellular matrix (ECM) and promotes cell–matrix interactions (Upchurch et al. 1987; Beninati et al. 1994). The possible mechanisms by means of flavonoids exert their antiproliferative and antimetastatic activities may be ascribed to the induction of cancer cell differentiation, the increase of intracellular cross-linking among cytoskeletal proteins that reduce cellular plasticity, and the enhancement of cell–cell and cell–ECM adhesion (Beninati et al. 1993; Beninati 1995; Lentini et al. 2000; Facchiano et al. 2001; Caraglia et al. 2002; Lentini et al. 2004).

One of the earliest biochemical events related to the reduction of cell proliferation is the impairment of polyamine synthesis (Tabor and Tabor 1976). Polyamines, putrescine (PUT), spermidine (SPD) and spermine (SPM), are a group of compounds involved in the regulation of cell proliferation and differentiation. Besides this, experimental evidences suggest a role for polyamines in programmed cell death. Although their exact functions have not yet been identified, it is clear that polyamines play important roles in a number of cellular processes such as replication, transcription, and translation. The importance of polyamines in cell function is reflected in a strict regulatory control of their intracellular levels. Adequate cell polyamine levels are achieved by a careful balance among their biosynthesis, degradation, and uptake.

Polyamine biosynthesis is increased by a great variety of physiological stimuli, while polyamine deficiency may be achieved by treating cells with specific inhibitors of the biosynthetic enzymes. As a consequence of polyamine deficiency, cell proliferation can be stopped and this, under certain circumstances, results in programmed cell death.

Interestingly, polyamines may be also covalently conjugated to glutamyl residues of polypeptides through a transamidation reaction catalyzed by TG (Folk 1980; Beninati and Piacentini 2004), therefore TG activation may contribute to the decrease of polyamine cytoplasmic content.

In the present work, we compared the anticancer potential of two flavonoids, quercetin and pelargonidin, belonging to flavonols and anthocyanidins families, respectively. We evaluated different parameters related to cell proliferation and differentiation, such as the evaluation of cell number, TG activity and polyamine content. Moreover, proteomic approach was used to investigate changes in protein patterns following cell treatment with the flavonoids.

Materials and methods

Materials

All reagents were Sigma-reagent grade and were used without further purification. Dulbecco's modified Eagle's medium (D-MEM), fetal calf serum (FCS), glutamine, penicillin (10,000 UI/ml) and streptomycin (10,000 μg/ml) were from Gibco Laboratories (Grand Island, NY, USA). [^{14}C]-methylamine (46.6 mCi/mmol) was purchased from Amersham International (Bucks, UK). Flavonoids quercetin and pelargonidin, sodium dodecyl sulfate (SDS), dithiotreitol, mercaptoethanol, glycerol, sodium citrate, Tris, *o*-phthaldehyde, *β*-mercaptoethanol, ethylenediaminotetraacetic acid (EDTA), acrylamide/bis-acrilamyde, putrescine, spermidine, spermine, trichloroacetic acid (TCA), perchloric acid (PCA) and dimethylsulfoxide (DMSO) were from Sigma Chemicals (St. Louis, MO, USA). Acetonitrile, tetrahydrofuran and all solvents came from Mallinckrodt Baker (Milan, Italy). All other chemicals were provided by Merck (Darmstadt, Germany).

Cell culture

Highly metastatic murine B16-F10 melanoma cell line was purchased from the Division of Cancer Treatment, Tumor Repository NIH (Frederick, MD) and propagated under standard culture conditions (Fidler 1973). Cells were cultured in D-MEM supplemented with 10% FCS, supplemented with 200 mM glutamine, 100 U/ml penicillin/streptomycin, and maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells were harvested twice a week with EDTA, re-fed every other day and used at about 80% confluence.

Cell proliferation

Melanoma cells were plated and grown in 35 mm dishes in D-MEM supplemented as above reported, and treated with 10 μM quercetin or 10 μM pelargonidin for 24, 48 and 72 h. Contamination was excluded by visual control under light microscope. Cells were detached with EDTA and

viability assessed after Trypan Blue staining. Cells were counted using a Neubauer modified chamber.

Polyamine assay

An HPLC method was used for the determination of polyamines in cultured cell lysates as previously described (Beninati et al. 1988). AKTABASIC 10 HPLC apparatus (Amersham Pharmacia Biotech., Milan, Italy), equipped with a Jasco FP-1520 fluorimeter, was used. RP-18 column (Inertsil 3 μm; 4.6 mm × 15 cm; Supelco) was used for identification of polyamines in the analytical system and detection was carried out simultaneously at 330 and 445 nm.

In vivo TG assay

TG assay was performed on B16-F10 cells treated with 10 μM of quercetin or 10 μM of pelargonidin for 24, 48 and 72 h, in the presence of [^{14}C]-methylamine (specific activity: 46.6 mCi/mmol, 0.5 μl/ml D-MEM). Then, cells were harvested and washed twice in PBS. Proteins were precipitated in 10% TCA, washed extensively, solubilized in 0.1 N NaOH at 37°C and incorporation of radiolabelled methylamine into protein was measured at a scintillation counter (TRICARB 2100, PACKARD, efficiency 70–90%).

Protein extraction

Proteins were extracted from B16-F10 cells treated with 10 μM of quercetin for 48 h. Cells were suspended in 500 μl of lysis buffer, containing 1.5 M Tris–HCl (pH 8.0) and protease inhibitors, and sonicated for 40 s in ice. The sonicated cells were centrifuged at 13,000*g* (15 min at 4°C) and the supernatant collected. Cold acetone (1 ml) was added and the sample was kept at −20°C overnight. Samples were centrifuged (1 h, 13,000*g* at 4°C), the supernatant removed, the pellet rinsed in 1.5 ml of rinsing solution (ice-cold acetone and 0.2% DTT) without resuspending, and kept at −20°C for 1 h. A second rinse was performed and the samples were centrifuged (30 min, 13,000*g* at 4°C). Pellet was briefly dried under vacuum (SC110 Savant) and dissolved in 100 μl extraction buffer, containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamido propyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 0.8% IPG-buffer (pI 3–10 NL) and 1% DTT. Samples were shaken for 1 h at room temperature and cleared by centrifugation (30 min, 13,000*g* at 18°C). Protein concentration was determined in the supernatants by Bradford protein assay. Samples were stored at −80°C until use.

Immobilized pH gradient electrophoresis

For the first dimension of two-dimensional electrophoresis Amersham pH 3–10 NL immobilized pH gradient (IPG) strips were used to separate the cell proteins according to their isoelectric points. Proteins (100 μg) were mixed with rehydration buffer containing 6 M urea, 2 M thiourea, 0.5% CHAPS, 10% glycerol, 0.002% bromophenol blue, 0.5% IPG-buffer (pI 3–10 NL) and 0.28% DTT, to a final volume of 400 μl per each IPG strip. The IPG strips were loaded with the protein sample and passively rehydrated overnight at room temperature in a rehydration tray (Amersham Biosciences). Isoelectric focusing of the strips was performed using the Amersham Biosciences Ettan IPGphor IEF System.

Second dimension

The IPG strips were equilibrated as follows: (1) 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 50 mM Tris–HCl pH 8.0, 4.6 mM DTT for 30 min with shaking at room temperature; (2) 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 50 mM Tris–HCl pH 8, 0.22 mM iodoacetamide for 30 min on a shaker. The strips were rinsed with MQ H_2O and applied to the top of a polyacrylamide slab gels, containing 41.7% acrylamide (12.5%), 25% Tris–HCl (0.5 M, pH 8.0), 1% SDS (10%), 1% APS (10%), 0.034% TEMED and 31.3% MQ H_2O. Strips and gel were put in the vertical slab electrophoresis unit (Hoefer SE 600 Ruby, Amersham Biosciences). Intensity of the spots was expressed as integrated optical density using Gel-Pro Analyzer software.

Protein staining and analysis

The gels were fixed in 100 ml of fixing solution (50% ethanol, 12% acetic acid and 0.05% of 35% formaldehyde solution) and shaken overnight at room temperature. Proteins were stained by silver staining. Gels were scanned with a Duoscan AGFA scanner and kept under vacuum in 1% acid acetic solution. Analysis of the gels was made by the PD-Quest 2-D Analysis software (Biorad). Average gels from three independent experiments were compared to each other and spot differing at least fourfold in intensity were marked for further analysis.

Immunoblot analysis

The proteins were extract from cells treated with quercetin, separated by 2D-PAGE and transferred to a nitrocellulose membrane (Biorad) using a Trans-blot™ electrophoretic transfer cell (Bio-Rad Laboratories Ltd.) in preparation for

ECL™ Western blot analysis. Membranes were blocked 1 h with 3% w/v BSA powder in phosphate-buffered saline containing 0.1% v/v Tween 20, after which they were incubated with an anti-TG2 rabbit polyclonal antibody (kindly provided by Dr. F. Facchiano Istituto Superiore di Sanità, Rome, Italy) diluted 1:500 in a solution containing 1% w/v BSA and 0.1% v/v Tween 20 for 90 min at room temperature on a shaker. Membranes were washed with a PBS solution containing 0.1% Tween 20, and the antibody binding was detected using a peroxidase-conjugated rabbit antibody diluted 1:500 in PBS/T containing 1% w/v BSA powder (30 min) and ECL™ Western blotting detection reagents (Amersham Biosciences). Membranes were exposed o a photographic film (X-OMAT, Kodak) for spot identification.

Results

Cell proliferation

The treatment of B16-F10 melanoma cells with 10 μM quercetin affected cell proliferation, in fact the treatment reduced cell growth of about 32% after 48 h and of about 39% after 72 h ($P < 0.001$). On the contrary, 10 μM pelargonidin did not show any antiproliferative effects (Fig. 1). Both treatments did not cause cell injury, as assayed by the Trypan Blue exclusion test.

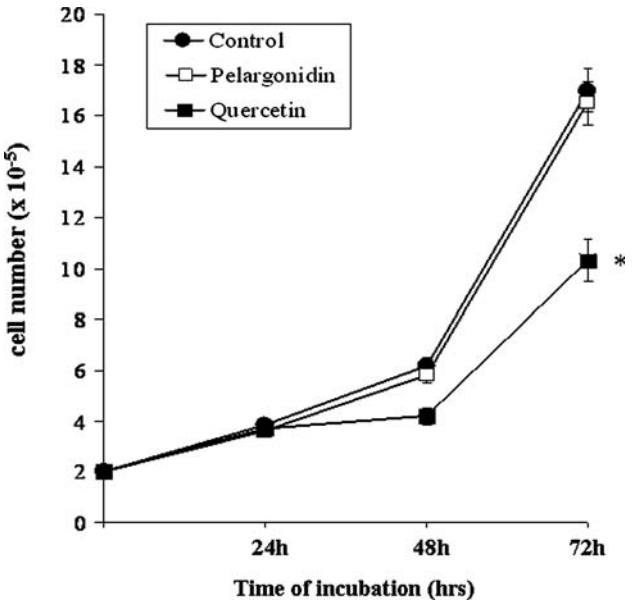

Fig. 1 Effect of flavonoids on B16-F10 melanoma cell proliferation. Cells were incubated with 10 μM quercetin or pelargonidin for 24, 48 and 72 h and proliferation was evaluated by cell counting. Cell viability was controlled by the Trypan Bleu exclusion test and found unaffected by the treatments. Data are expressed as the mean ± SD of three different determinations and differences were considered significant when $*P < 0.001$ (Student's t test)

Polyamine determination

Polyamine intracellular concentration was determined after 24, 48 and 72 h of treatment with flavonoids. PUT concentration was detected only in traces for all samples. Twenty-four-hour exposure of B16-F10 cells to quercetin (Fig. 2a) did not change SPD and SPM content while their amount was significantly decreased after 48 and 72 h with respect to control, ($P < 0.001$). SPD and SPM levels were decreased by about 29 and 45% after 48 h and about 45 and 72% after 72 h. Pelargonidin treatment (Fig. 2b) did not produce significant changes in polyamine concentration, compared to the control.

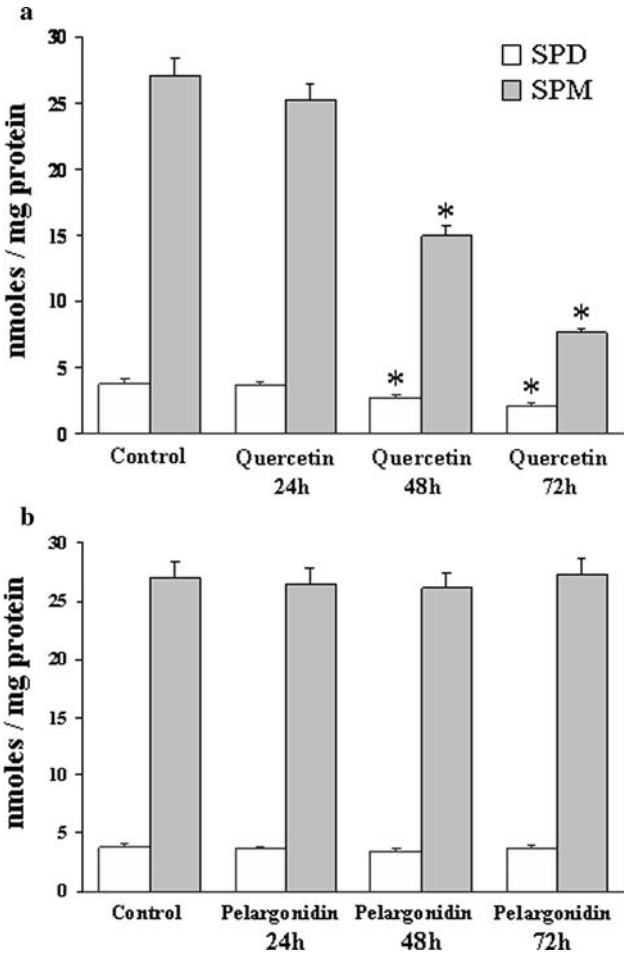

Fig. 2 HPLC chromatographic determination of the intracellular levels of polyamines in B16-F10 melanoma cells treated for 24, 48 and 72 h with 10 μM quercetin (**a**) and pelargonidin (**b**). PUT was found in traces for all treatments. Data represent the mean ± SD of three different determinations and differences were considered significant when $*P < 0.001$ (Student's t test)

TG activity

TG activity determination represents one of the methods to evaluate cell differentiation, since it is considered a differentiative biomarker. We checked in B16-F10 melanoma cell line the possible effect of quercetin and pelargonidin in the modulation of this marker, and the results are shown in Fig. 3. Twenty-four-hour incubation of B16 cells with flavonoids did not affect the enzyme activity, compared to the control. On the contrary, treatment with 10 μM quercetin significantly induced, compared to the control, a fourfold enhancement in TG activity after 48 h, and a twofold increase after 72 h of exposure ($P < 0.001$). These increases were not observed in pelargonidin-treated cells, where TG activity was similar to control (data not shown).

Protein pattern analyses

Protein synthesis in melanoma cells was affected by quercetin treatment, as revealed by the comparison of 2D patterns of untreated (Fig. 4a) or quercetin-treated cells (Fig. 4b). More than 500 protein spots could be detected. Changes in expression of 60 proteins, due to the treatments, were detected, i.e. 8 were down-regulated (Table 1) and 35 up-regulated (Table 2). Moreover, 11 "de novo" synthesis proteins and 6 suppressed proteins were present in treated cells (Table 3). The spot no. 35 in Fig. 4b (about 80 kDa), which presented a fourfold increase in intensity with respect to the control, was identified as TG by Western blot analysis (Fig. 5).

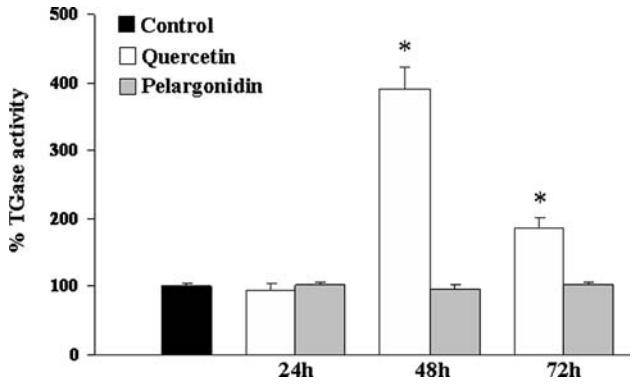

Fig. 3 Determination of TG activity in B16-F10 melanoma cells treated with 10 μM quercetin or pelargonidin, evaluated as incorporation of ^{14}C-methylamine into cell protein. Results are expressed as percentage of the control value (100%). Data represent the average ± SD of three different determinations and differences were considered significant when $*P < 0.001$ (Student's t test)

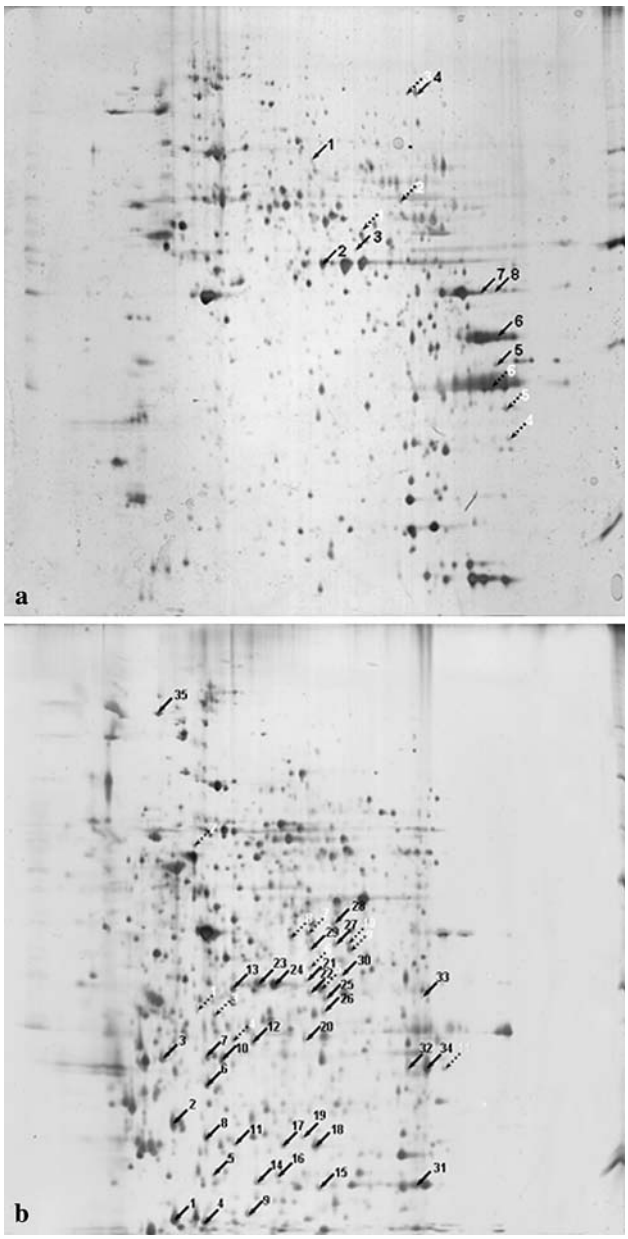

Fig. 4 2D electrophoretic gels of proteins from untreated B16-F10 melanoma cells (**a**) or treated for 48 h with 10 μM quercetin (**b**)

Discussion

Literature reports promising action for flavonoid as anticancer agents. They possess antioxidant activity and may influence cancer development by several mechanisms: free radical scavenging, modulation of oncogene expression, apoptosis induction, regulation of cellular proliferation and differentiation, antibacterial and antiviral effects and stimulation of immune system (Dragsted et al. 1993; Waladkhani and Clemens 1998 ; Sun et al. 2002; Chu et al. 2002).

Several studies have suggested a number of separate and independent mechanisms by which quercetin may act as

Table 1 Bi-dimensional gel electrophoretic analysis of down regulated proteins in quercetin-treated B16-F10 melanoma cells

Spot no	Intensity control/treated	Molecular weight (Da)	Spot no	Intensity control/treated	Molecular weight (Da)
1	4.6	70,100	5	230.8	35,630
2	16.8	51,744	6	29.4	38,688
3	6.2	54,051	7	14.6	46,347
4	24.3	83,625	8	9.6	46,340

Intensity of spots was expressed as integrated optical density by Gel-Pro Analyzer software

Table 2 Bi-dimensional gel electrophoretic analysis of up regulated proteins in quercetin-treated B16-F10 melanoma cells

Spot no	Intensity treated/control	Molecular weight (Da)	Spot no	Intensity treated/control	Molecular weight (Da)	Spot no	Intensity treated/control	Molecular weight (Da)
1	4.2	12,553	13	6.0	37,500	25	10.8	36,617
2	25.6	18,784	14	4.4	14,026	26	6.8	35,150
3	11.0	24,913	15	12.4	13,823	27	5.8	44,211
4	5.1	12,480	16	7.2	14,336	28	6.5	47,671
5	7.5	14,336	17	6.9	16,671	29	15.8	43,558
6	9.1	20,345	18	4.4	16,649	30	15.3	39,557
7	6.9	26,782	19	12.6	17,065	31	6.9	13,904
8	16.7	17,018	20	5.4	30,454	32	11.1	23,782
9	4.5	12,794	21	5.9	38,784	33	8.9	36,724
10	23.1	25,484	22	7.2	37,263	34	15.4	23,696
11	10.2	16,717	23	6.2	38,199	35	4.1	85,748
12	4.7	30,169	24	12.5	38,199			

Intensity of spots was expressed as integrated optical density by Gel-Pro Analyzer software

Table 3 Bi-dimensional gel electrophoretic analysis of de novo synthesis and suppressed proteins in quercetin-treated B16-F10 melanoma cells

De novo synthesis				Suppressed	
Spot no	Molecular weight (Da)	Spot no	Molecular weight (Da)	Spot no	Molecular weight (Da)
1	36,457	7	45,998	1	58,118
2	61,889	8	45,781	2	64,415
3	34,850	9	44,449	3	83,625
4	30,895	10	45,001	4	26,946
5	37,754	11	23,159	5	31,481
6	40,239			6	33,585

anti-tumor agent. Quercetin was found to downregulate mutant p53 protein (Avila et al. 1994), tyrosine kinase (Ferry et al. 1996) and ras protein expression (Ranelletti et al. 2000), to arrest the cell cycle in the G1 phase (Yoshida et al. 1992), to induce the type II estrogen receptor, which possesses growth-inhibitory capability (Piantelli et al. 1995) and to inhibit production of heat shock proteins (Koishi et al. 1992; Elia et al. 1996; Hansen et al. 1997).

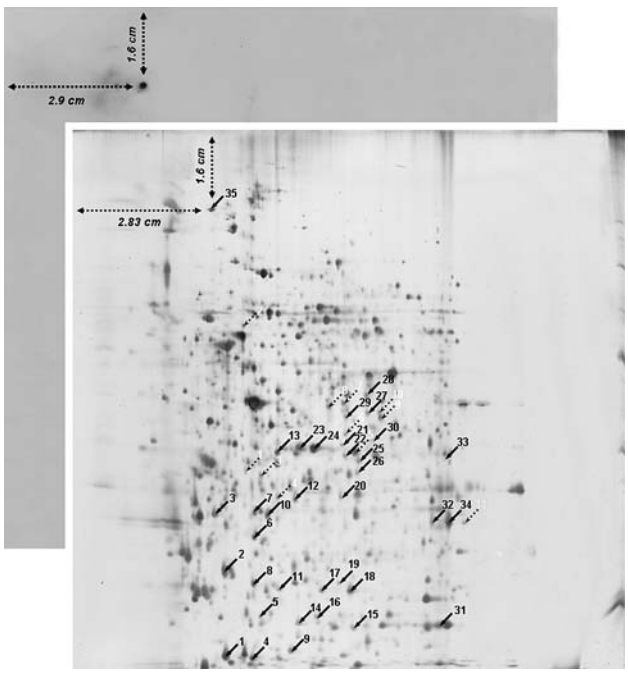

Fig. 5 Western blot analysis of proteins from quercetin-treated B16-F10 melanoma cells. The spot no. 35 (Fig. 4b) was identified as tissue TG

We are reporting that not all flavonoids share the same antiproliferative activity, indeed the flavonol quercetin was observed as more effective than the anthocyanidin pelargonidin. The proliferation data were confirmed by the reduction of a typical proliferation marker, the polyamine intracellular content. In fact, in quercetin treated-cells we observed a remarkably reduction of SPD and SPM levels with respect to the control, whereas pelargonidin had no effect.

Our study also investigated the role of quercetin and pelargonidin on melanoma cell differentiation. This paper is the first report on the effect of quercetin on TG 2, one of the markers of cell differentiation.

The reduced B16-F10 melanoma cell growth, induced by quercetin, was paralleled with a significant increase of TG activity, with respect to the control, after 48 and 72 h of exposure with the flavonol. At the same time, pelargonidin had not effect. These data support the idea that the antiproliferative effect of quercetin is associated with cell differentiation. Quercetin incubation of B16-F10 cells modified the expression of 60 proteins. Among them TG2-expression was enhanced of about fourfold. This finding appears to support the observed increase of TG activity in B16-F10 cells treated with quercetin. We may conclude that this molecule exerts its effect on melanoma cells proliferation through the modulation of both polyamine metabolism and TG expression.

Actually, "differentiation therapy" is an important and rapidly evolving aspect of cancer research. It is based on the concept that some natural substances can inhibit carcinogenesis and development of cancer through the induction of terminal differentiation of tumor cells (Beninati 1995; Thiele et al. 2000; Lentini et al. 2007). This therapeutical approach possess a lower citotoxicity compared to that exerted by drugs used in chemotherapy.

Flavonoids are widely distributed throughout the plant kingdom and are abundant in many flowers, fruits and leaves. They may be considered, as observed in this report, promising agents for cancer prevention.

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