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Enhancement of transglutaminase activity and polyamine depletion in B16-F10 melanoma cells by flavonoids naringenin and hesperitin correlate to reduction of the *in vivo* metastatic potential

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Summary. The *in vitro* and *in vivo* effects of two flavonons, naringenin (NG) and hesperitin (HP) on the proliferation rate of highly metastatic murine B16-F10 melanoma cell were investigated. NG or HP treatment of melanoma cells produced a remarkable reduction of cell proliferation, paralleled with both the lowering of the intracellular levels of polyamine, spermidine and spermine and the enhancement of transglutaminase (TGase, EC 2.3.2.13) activity. Orally administered NG or HP in C57BL6/N mice inoculated with B16-F10 cells affected the pulmonary invasion of melanoma cells in an *in vivo* metastatic assay. The number of lung metastases detected by a computerized image analyzer was reduced, compared to untreated animals, by about 69% in NG-treated mice and by about 36% in HP-treated mice. Survival studies showed that 50% of the NG-treated animals died 38 ± 3.1 days after tumor cell injection (control group: 18 ± 1.5 days) and HP-treated mice died 27 ± 2.3 days after cell inoculation. Taken together, these findings provide further evidences for the potential anticancer properties of dietary flavonoids as chemopreventive agents against malignant melanoma.

Keywords: Flavonoids – Melanoma – Metastasis – Transglutaminase – Polyamines

Introduction

Flavonoids are widely distributed throughout the plant kingdom and are abundant in many flowers, fruits and leaves. They possess antioxidant activity and are characterized by the presence of two aromatic rings (A and B rings) that are linked by a 3-carbon bridge (to form chalcones) or by pyrane or pyrone ring (ring C). On the basis of the position and of modifications to the A, B and C rings, the >4000 flavonoids discovered to date can be classified into several classes: flavonols, flavones, flavanols (cathechins), flavanones, isoflavones and anthocyanin pigments. Flavonoids and flavonoids-rich extract have been implicated as beneficial agents in a multitude of disease states, most commonly cancer (Spencer et al., 2003; Go et al., 2003; Liu, 2004). Among the various types of flavonoids in fruit and vegetables associated with cancer prevention, the flavanones naringenin (NG) and hesperitin (HP) are found in grapefruit and oranges (Liu, 2004). Although the antioxidant effects of citrus flavonoids due to their capability to scavenge free radicals have attracted a great deal of attention, there are effects beyond antioxidation that may be important in determining the anticancer activity of phytochemicals, such as effects on cell proliferation, polyamines production and TGase activity.

Polyamines, putrescine (PUT), spermidine (SPD) and spermine (SPM), may be covalently conjugated to glutaminyl residues of polypeptides through a transamidation reaction catalyzed by TGase (Folk, 1980; Beninati and Piacentini, 2004). TGases are a widely distributed and peculiar group of enzymes that catalyse 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 at the level peptide-bound glutamine residues (Folk and Finlayson, 1977). The involvement of these enzymes in a variety of cellular physiological and/or pathological processes has been demonstrated, such as in cell differentiation (Lentini and Beninati, 2002), cell-matrix interactions, migration, polimerization of eye lens crystallins and programmed cell death (Beninati, 1997). The role of the enzyme in cancer has not well clarified, even though evidence shows that its activation positively relates to induction of cell differentiation and reduction of tumor cell proliferation and

invasion (Beninati et al., 1993; Beninati, 1995; Lentini et al., 2000, 2004; Facchiano et al., 2001; Caraglia et al., 2002, 2004). In the light of the increasing interest in possible new interventions capable to counteract the abnormal tumor cell growth and invasiveness, the aim of this work is to investigate the antiproliferative and antimetastatic role of two flavonoids, NG and HP on B16-F10 melanoma cells and the concomitant modulation of polyamine metabolism and/or TGase activity, used respectively as cell growth and differentiation markers.

Materials and methods

Materials

D-MEM, glutamine, penicillin (10,000 UI/ml) and streptomycin (10,000 µg/ml) were from Eurobio Laboratoires (Le Ulis Cedex, France). FCS was from Gibco (Grand Island, NY). [^{14}C]-methylamine (46.6 mCi/mmol) was purchased from Amersham International (Bucks, UK). NG, HP, Tris, *o*-phthaldehyde, β-mercaptoethanol, putrescine, spermidine and spermine were from Sigma Chemicals (St. Louis, MO). Acetonitrile, tetrahydrofuran and other solvents were purchased from Mallinckrodt Baker (Milan, Italy).

Cell cultures

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 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), supplemented with 200 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml 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. Before each treatment, cells were starved for 24 h to role out possible interferences with cell growth due to serum components and promote their synchronization.

Cell proliferation

Melanoma cells were seeded and grown in 35 mm dishes in DMEM supplemented as reported above, and treated with 10 µM of NG or HP for 24, 48 and 72 h. Contamination was excluded and viability assessed by visual control with light microscope. Then cells were scraped off with a rubber policeman and counted after Trypan Blue staining using a Neubauer modified chamber.

Polyamine assay

An HPLC method was used for the determination of polyamines in culture cell lysates (Beninati et al., 1988). Briefly, the separation was performed using a pre-column derivatization. Cell lysates was deproteinized with PCA, centrifuged (14,000 g for15 min) and supernatants were filtered. The samples were derivatized with *o*-phtalaldehyde (1:1). Determination was performed by a AKTABASIC 10 HPLC apparatus (Amersham Pharmacia Biotech., Milan, Italy).

In vivo TGase assay

TGase assays were performed on B16-F10 cells treated with 10 µM of NG or HP for 24, 48 and 72 h in the presence of [^{14}C]-methylamine (activity: 46.6 mCi/mmol, 0.5 µl/ml D-MEM). Then, cells were harvested, counted, washed twice in PBS. Proteins were precipitated in 10% TCA, washed extensively, solubilized in 0.1N NaOH at 37 °C and counted for radioactivity.

Animals

Six- to 8-week-old male C57BL/6 mice were purchased from IFFA Credo (L'Abreole, France) and were housed throughout the experiments in air-conditioned animal room. Ten to 15 animals were used each experimental group. Treated mice were fed with NG- or HP-containing food (20 mg of flavonoid/gr of pellets) starting from 7 days before tumor cell inoculation till the end of the experiment. Daily determination of plasma levels of NG or HP was performed in triplicate by HPLC analysis (Ishii et al., 1996) and flavonoid concentrations were extrapolated from standard curves.

Experimental lung metastases

Lung metastases were obtained by intravenous injection via tail vein of 2×10^5 viable B16-F10 cells suspended in 0.2 ml D-MEM into anaesthetized mice (Nembutal, 50 mg/Kg body weight). Mice were sacrificed by cervical dislocation on the 21st day after injection of tumor cells. The lungs were then removed, fixed and the total metastatic colonies recorded by a computerized image analyzer according to a published method (Lentini et al., 2000). For the survival studies, about 10 untreated, NG-treated and HP-treated mice were inoculated via tail vein with B16-F10 cells. Each experiment was repeated three times. Moribund animals were sacrificed without waiting their predictable death, according to UKCCCR guidelines. All experimental protocols were carried out following the Guidelines for the Welfare of Animals in Experimental Neoplasia and the ECC Council Directive 86/609, OJL 358, 1st December 1987.

Protein determination

Protein was measured by the method of Bradford (Bradford, 1976), using BSA as standard.

Statistical analysis

All experiments were repeated three times, and the results are expressed as the mean ± SD of three different determinations. Data were analyzed by the *t* Student's test.

Results

B16-F10 melanoma cell growth

The proliferative capacity of B16-F10 cells was found to be affected after 48 h of treatment with 10 µM NG or HP (Fig. 1). For both treatments, cell growth was reduced by about 40% after 48 h and by about 50% after 72 h of treatment ($p < 0.001$). Both NG and HP treatments did not cause cell injury, as assessed by the Trypan Blue exclusion test. In fact, cells were still 95–100% viable after 72 h of treatment (data not shown).

Polyamine determination

Polyamine levels were detected during the treatment of B16-F10 cells with 10 µM NG or 10 µM HP for the time

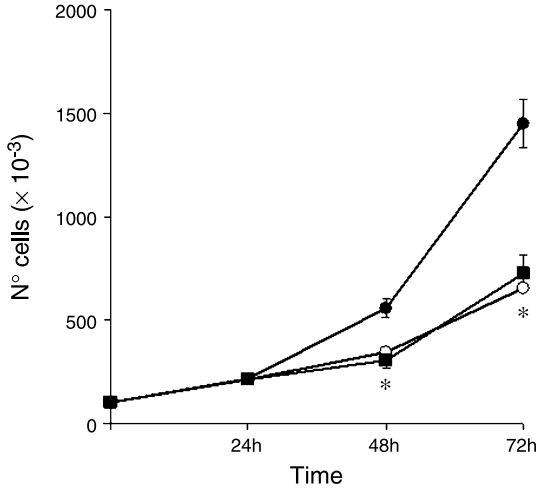

Fig. 1. Effect of NG and HP on B16-F10 melanoma cell growth. Cells were grown in DMEM supplemented with 10% FCS and serum starved for 24 h before each experiment to rule out possible interferences with cell growth due to serum components and to promote their synchronization. B16-F10 cells were challenged with 10 μM NG or HP for different experimental times (24, 48 and 72 h). Cell growth was evaluated by cell counting and data are expressed as the mean ± SD of three different determinations. ● Control; ■ NG treatment; ○ HP treatment; * highly significant versus control ($p < 0.001$)

selected**.** The results are shown in Fig. 2. Exposure to NG for 24 h did not alter polyamines content with respect to control, but significantly decreased, after 48 and 72 h, SPD and SPM levels by about 57% and by about 86%, respectively ($p < 0.001$). HP treatment for 24 h produced not changes in polyamine levels but a decrease after 48 and 72 h of SPD and SPM levels by about 31% and by about 54%, respectively ($p < 0.001$).

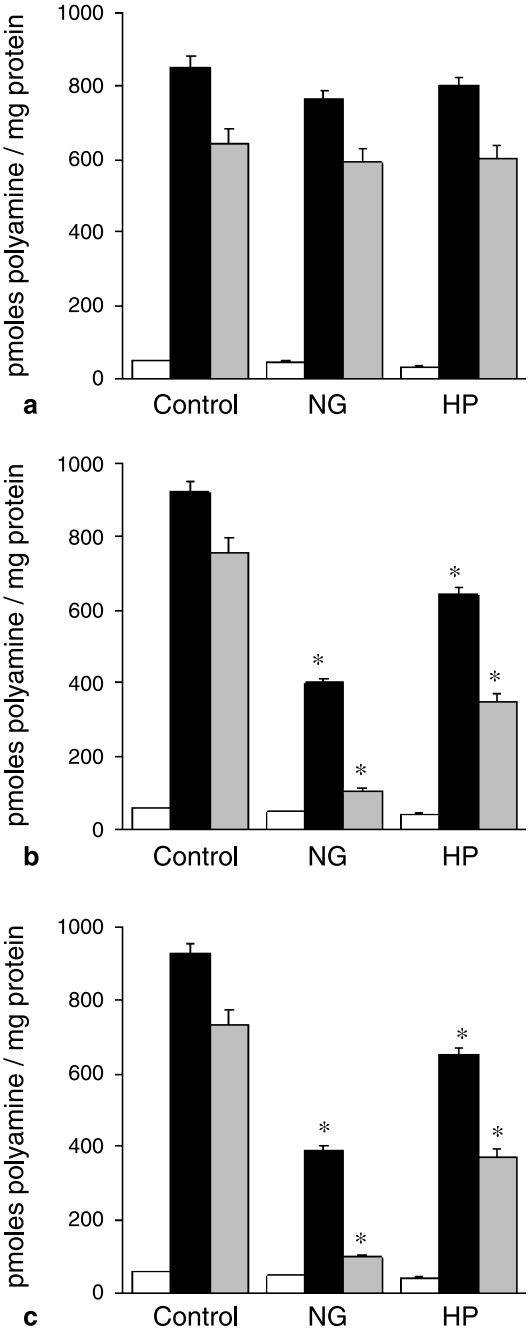

Fig. 2. Effect of NG and HP on intracellular levels of polyamines in B16-F10 melanoma cells. Cells were treated for 24 h (**a**), 48 h (**b**) and 72 h (**c**) with 10 μM flavonoid concentration. Determinations were performed by HPLC. Data represent the mean ± SD of three different determinations. □ PUT; ■ SPD; ▩ SPM; * highly significant versus control ($p < 0.001$)

TGase activity

Since TGase activity is commonly considered a differentiation marker, we checked in B16-F10 melanoma cell line the possible effect of NG and HP in the modulation of the activity of this enzyme. Figure 3 shows TGase activity of melanoma cells significantly enhanced, compared to controls, after 48 h of treatment by NG (about 65% increase), and by HP (about 35% increase) ($p < 0.001$). These increases were maintained similar after 72 h of incubation with the two flavonoids.

Effect of NG and HP treatment on B16-F10 invasion of target organ

Due to the highly metastatic nature of B16-F10 cells, implantation into the target organ is rapid following intravenous injection of tumor cells via the tail vein (Lentini et al., 1998). No primary tumors were produced at the site of inoculation. Pulmonary metastases were visible from the first ten days after the injection as single black spots. The influence of oral administration of NG or HP to syngeneic mice on the experimental induction of metastasis by B16-F10 melanoma cells was evaluated. Untreated

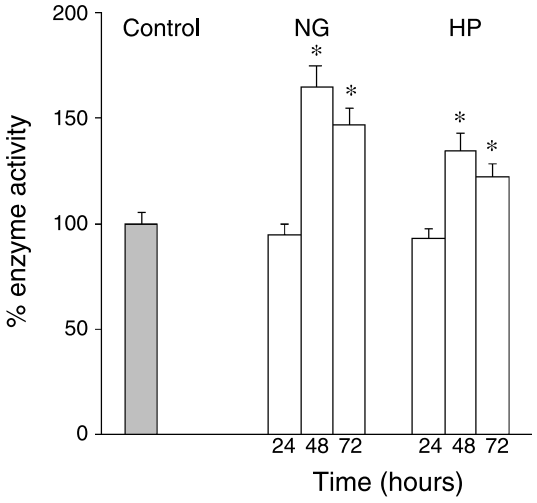

Fig. 3. Effect of NG and HP on intracellular transglutaminase activity in B16-F10 melanoma cells. Cells were treated for 24, 48 and 72 h with 10 μM flavonoid concentration. The results were expressed as the percentage with respect to the control value (100%). Data represent the mean ± SD of three different determinations; ∗ highly significant versus control ($p < 0.001$)

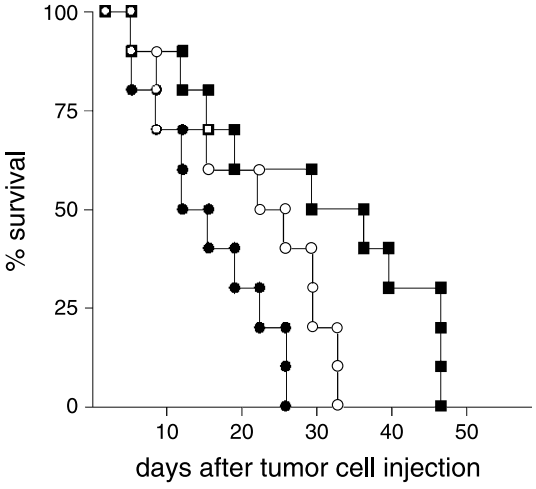

Fig. 4. Effect of NG and HP administration on the survival of B16-F10 pulmonary metastasis-bearing mice. Kaplan Meier survival curves for untreated mice (●, n = 10), NG-treated mice (■, n = 10) and HP-treated mice (○, n = 10)

Table 1. Effect of naringenin and hesperitin treatment of mice injected with B16-F10 melanoma cells on lung metastasis

Treatment	Number of lung metastases	% lung metastasis reduction
None	483 ± 37	–
Naringenin	148 ± 12*	69.3*
Hesperitin	310 ± 28*	35.8*

* $p < 0.001$

animals were injected with melanoma cells on the same day as the treated animals. Table 1 shows the effect of the different treatments of mice for 21 days from the inoculation of cancer cells on lung metastasis frequency. In NG-treated mice, the pulmonary metastatic frequencies were markedly decreased compared with control (about 69% reduction, $p < 0.001$), whereas HP treatment produced a decrease at a lesser extent (about 36% reduction, $p < 0.001$).

Effect of flavonoid treatments on mice survival

The effect of oral treatment with NG or HP on the survival of mice inoculated intravenously with B16-F10 melanoma cells was investigated. Animals were treated by oral administration of flavonoids as described in Materials and methods. The plasma concentration of NG or HP was determined daily by a HPLC assay (Ishii et al., 1996) in all experimental groups, and found stable between 2–6 μM range for the overall time of treatments (data not shown). As shown in Fig. 4, NG treatment caused a long-term delay in mortality; 50% of the NG-treated animals died by 36 ± 3.1 days from the intravenous inoculation of tumor cells (control group: 16 ± 1.5 days; $p < 0.001$), whereas HP-treated mice died by 26 ± 2.3 days after melanoma cells inoculation.

Discussion

While research generally supports the potential of flavonoids for reducing cancer risk, only a few have been widely studied. Some standouts include polyphenols, particularly EGCG (epigallocatechin-gallate), found in green tea; genistein, found in soybeans and some other legumes; quercetin, found in apples and onions; PCOs (procyanidolic oligomers, also known as proanthocyanidins), found in abundance in pine bark and grape seed extract, as well as in red wine; citrus flavonoids, including NG and HP found in oranges, grapefruits, tangerines and other citrus fruits. Researchers feel that dietary flavonoids may be the most effective cancer-preventing compound discovered to date (Hertog et al., 1995).

Searching for the molecular mechanisms by which flavonoids exert antiproliferative effects on tumor cells, several experimental evidences have been described in the literature (Kanadaswami et al., 2005). Most of authors agree with the flavonoid-induced impairment of the signal transduction pathway mediated by tyrosine kinase and/or protein kinase C (Middleton et al., 1992; Hollosy and

Keri, 2004). Some authors have observed the inhibition of the insulin-induced glucose uptake in NCF-7 breast cancer cells as a possible mechanism for the antiproliferative role of NG (Harmon and Patel, 2004), and others demonstrate a possible involvement of the anti-estrogenic activity of NG in two cancer cell lines (Totta et al., 2004). In addition, the anti-invasive role of numerous flavonoids has been well documented, as a result of inhibition of metalloproteinase activity or antiangiogenic action (Makimura et al., 1993; Jung and Ellis, 2001; Kousidou et al., 2005).

The aim of the present work was to extend our knowledge about the mechanisms involved in flavonoids-mediated antineoplastic activity, investigating the possible induction of cancer cells differentiation in B16-F10 melanoma cells treated with two citrus flavonoids: NG and HP. Our findings suggest that flavonoids, like previously observed for retinoids or methylxanthines (Lentini et al., 2004), may be considered effective activators of transglutaminases, a class of enzymes involved in cell differentiation (Thacher and Rice, 1985; Benedetti et al., 1996; Arani et al., 1997). It is well known that intracellular activation of soluble TGase may give rise to cross-linked proteins leading to the formation of envelopes in apoptotic cells (Fesus, 1993; Autuori et al., 1998), whereas extracellular activation contributes to stabilization of the extracellular matrix (ECM) and promotes cell-substrate interactions (Upchuech et al., 1987; Beninati et al., 1994). Therefore it is possible to believe that flavonoids exert their antiproliferative property by the induction of cancer cell terminal differentiation and their antimetastatic activity by the enhancement of cell-cell and cell-ECM adhesion.

The reduction of polyamine synthesis is one of the earliest biochemical events associated with the reduction of cell proliferation (Tabor and Tabor, 1976). In the light of the observed increased activity of TGase, the reduction of SPD and SPM levels in NG- and HP-treated cancer cells may give a crucial contribution to the antiproliferative capability of these flavonoids.

A full evaluation of the *in vivo* activity of potential antineoplastic molecules would require that the bioactive concentration of the drug is maintained long enough in the target organs to produce pharmacodynamic effects. Such *in vivo* evaluation necessarily needs knowledge concerning the formulation, administration, and dose-limiting host toxicity as a function of formulation, route of administration and dose scheduling. Although oral administration of NG and HP appeared to be an effective route for inhibiting melanoma metastasis, it was obviously difficult to monitor the amount of drug consumed by mice with the food intake. To overcome this difficulty, the plasma levels of flavonoids were determined daily. NG or HP reached a peak plasma concentration at about the fourth day of treatment, and this value (2–6 μM) was found to be quite stable during the experimental time. Furthermore, flavonoid treatment alone did not exert any effect on mice liver or lung weights. We have shown that NG treatment of B16-F10 melanoma-bearing mice reduced tumor implantation and significantly increased the animals' lifespan more than HP treatment.

The wider distribution and the more abundant presence of flavonoids in the plant kingdom, together with the present results, suggest that these molecules may contribute to the preventive effect of a plant-based diet on neoplastic diseases, including melanoma tumors. However, the effects of flavonoids on cancer merits further epidemiological investigation and at the moment our results can therefore not to be extrapolated directly to humans.

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