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ORIGINAL ARTICLE

Transglutaminase-dependent antiproliferative and differentiative properties of nimesulide on B16-F10 mouse melanoma cells

Angelo Gismondi · Alessandro Lentini · Claudio Tabolacci · Bruno Provenzano · Simone Beninati

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Abstract The aim of this study was to collect evidences on the role of transglutaminase (TG, E.C.2.3.2.13) in the antineoplastic properties exerted by nimesulide (NMS), a non-steroidal anti-inflammatory drug, on murine B16-F10 melanoma cells. Treatment of melanoma cells with nimesulide produces a considerable reduction of cell proliferation, paralleled by a remarkable decrease of the intracellular concentration of polyamines spermidine and spermine. NMS treatment induces cancer cell differentiation, likely through the observed enhancement of TG and tyrosinase activities and increase of melanin production, well known markers of melanocyte differentiation. The overall results highlight the possibility that nimesulide acts as antineoplastic agent likely through the induction of intracellular TG activity.

Keywords Nimesulide · Polyamines · Transglutaminase · Differentiation · Tyrosinase · Melanin

Introduction

TGs belong to a class of enzymes which catalyze the post-translational modification of proteins by the formation of isopeptide bonds (Folk 1980; Beninati and Folk 1988). Increasing evidences show a direct correlation between TG activation and reduction of cancer progression, by means of stimulation of tumor cell differentiation. The linkage between the TG-dependent antineoplastic activity and the induction of cell differentiation has been well established in melanoma cells lines, as well as the involvement of the enzyme in the metastatic spread (Di Giacomo et al. 2009). In particular, melanocyte-derived tumors stimulated to differentiate express high TG and tyrosinase activity, and augmented melanin synthesis, as typical differentiation markers (Beninati et al. 1993; Lentini and Beninati 2002; Lentini et al. 2009).

Tyrosinase is a key enzyme in melanin synthesis that catalyzes three different chemical reactions; the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), the oxidation of DOPA to dopaquinone, and the oxidation of 5,6-dihydroxyindole to indole-quinone (Hearing and Tsukamoto 1991).

TGs can catalyze the covalent incorporation of several low molecular weight amines into proteins in the form of amides of the γ-carboxyl group of a peptide-bound glutamic acid. TG-catalyzed reactions are extremely specific for a particular glutamine residue in native protein substrates (Folk 1980; Greenberg et al. 1991). TG activity is particularly affected by the presence of intracellular polyamines (putrescine, PUT; spermidine, SPD; spermine, SPM), which are covalently conjugated to glutamyl residues of polypeptides through a transamidation reaction (Beninati and Piacentini 2004). Furthermore, polyamine intracellular concentration is considered a marker of cell growth. Indeed, the enhancement of polyamine intracellular concentration has been well demonstrated to up-regulate cell cycle (Heby 1981; Erwin et al. 1983).

NMS [*N*-(4-nitro-2-phenoxyphenyl)methanesulfonamide] belongs to the non-steroidal anti-inflammatory drugs (NSAIDs), which preferentially inhibit cyclooxygenase-2 (COX-2, E.C. 1.14.99.1) (Bennett and Villa 2000), an enzyme overexpressed in some tumors and linked to the stimulation of cancer cell growth and invasion (Parrett

A. Gismondi · A. Lentini · C. Tabolacci · B. Provenzano · S. Beninati (✉)
Department of Biology, University "Tor Vergata", Via della Ricerca Scientifica, 00133 Rome, Italy
e-mail: beninati@bio.uniroma2.it

et al. 1997; Zimmermann et al. 1999; Dannenberg et al. 2001; Subbaramaiah and Dannenberg 2003). Increasing evidences show that NMS is able to express antiproliferative activity on malignant cells (Fukutake et al. 1998; Denda et al. 2002; Haynes et al. 2003), likely through mechanisms involving COX-2 activity.

The purpose of this work was to investigate the possible role of TG on the antiproliferative and differentiative properties of NMS on B16-F10 melanoma cell line. The intracellular levels of polyamine were considered as proliferation markers, and the increase of TG and tyrosinase activity and melanin synthesis was assumed as a sign of tumor cell differentiation.

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, USA). [^{14}C]-methylamine (46.6 mCi/mmol) was purchased from Amersham International (Bucks, UK). NMS, sodium citrate, Tris, *o*-phthaldehyde (OPA), *β*-mercaptoethanol, ethylenediaminotetraacetic acid (EDTA), PUT, SPD, SPM, diaminoctane (DAO), 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).

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 (D-MEM), supplemented with 10% fetal calf serum (FCS), 200 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and maintained in humidified atmosphere of 5% CO_2 at 37°C. Cells were harvested twice a week, re-fed every other day, and used at about 80% confluence. Before each treatment, cell line was starved for 24 h to rule out possible interferences with cell growth due to serum components and promote its synchronization.

Proliferation assay

Murine B16-F10 melanoma cells were seeded and grown in 35-mm dishes in D-MEM supplemented as reported above, and treated with NMS (10, 50 and 100 μM) for 24, 48, and 72 h. Control cells were incubated with 0.1% DMSO final concentration. Cells were harvested and counted with a Neubauer modified chamber, after Trypan Blue staining for cytotoxicity evaluation.

Determination of intracellular polyamine levels

An HPLC method was developed for the determination of polyamines in culture cell lysates. The separation was performed using a pre-column derivatization. Cell lysates were deproteinized with PCA, centrifuged (14,000*g* for 15 min), and supernatants were filtered. The samples were derivatized with OPA (1:1). The OPA reagent was prepared dissolving 4 mg in 1 ml of methanol, adding 4,740 ml of borate buffer (1 M, pH 9.5) and 10 μl of *β*-mercaptoethanol. 100 μl of mixture was injected into the HPLC. Determination was performed by AKTABASIC 10 HPLC apparatus (Amersham Pharmacia Biotech., Milan, Italy). Reverse-phase separations were conducted at room temperature in a LC-18 Supelcosyl column (150 mm × 4.6 mm, 3 μm) (Supelco, Milan, Italy). The derivatives were separated on two mobile phases: A (95% 350 mM sodium citrate, pH 4.0; 5% tetrahydrofuran) and B (45% 350 mM sodium citrate, pH 4.0; 40% acetonitrile; 15% tetrahydrofuran). The elution procedure consisted in a linear gradient from 50 to 100% of buffer B in 5 min., then an isocratic elution for 15 min., at a flow rate of 0.9 ml/min. Detection was accomplished using a spectrofluorimeter (Jasco FP-1520, Easton, MD). Fluorescence detector was set at λ_{ex} 330 nm and λ_{em} 445 nm.

TG assay

TG assay was performed by treating B16-F10 cells with 100 μM NMS in the presence of [^{14}C]-methylamine (46.6 mCi/mmol, 0.5 μl/ml DMEM). Then, cells were harvested, counted, and washed twice in PBS. Cell proteins were precipitated in 10% TCA, washed extensively, solubilized in 0.1 N NaOH at 37°C, and measured for radioactivity, according to the method of Chung and Folk (1972).

Cellular tyrosinase activity and melanin content

Tyrosinase activity on B16-F10 cells was assessed by measuring the rate of L-DOPA oxidation (Yokozawa and Kim 2007). Cells were plated in 6-well dishes and treated with 100 μM NMS. Cells were lysed with 50 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and PMSF (0.1 mM) and frozen at −80°C for 30 min. Cell lysate was thawed, mixed, and centrifuged. 80 μl of supernatant and 20 μl of L-DOPA were placed in a 96-well plate, and the adsorbance at 490 nm was measured after

40 min. at 37°C in a microplate reader. Data are expressed as percentage of the control. Determination of intracellular melanin content in both control and NMS-treated cells was performed as previously described (Lotan and Lotan 1980).

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 test; differences were considered highly significant when $p < 0.001$.

Results

Cell growth

The proliferative activity of B16-F10 melanoma cells was found to be particularly affected after 48 and 72 h of treatment with NMS (Fig. 1). Twenty-four hours of NMS treatment did not show significant changes in cell proliferation, compared to control cells. Indeed, cells incubated with 10, 50 and 100 μM NMS for 48 h reduced their growth, with respect to the control, by about 11, 29 and 42%, respectively ($p < 0.001$). After 72 h of NMS treatment, the values of reduction in cell growth were about 26, 37 and 67%, with respect to the control ($p < 0.001$). NMS treatment 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).

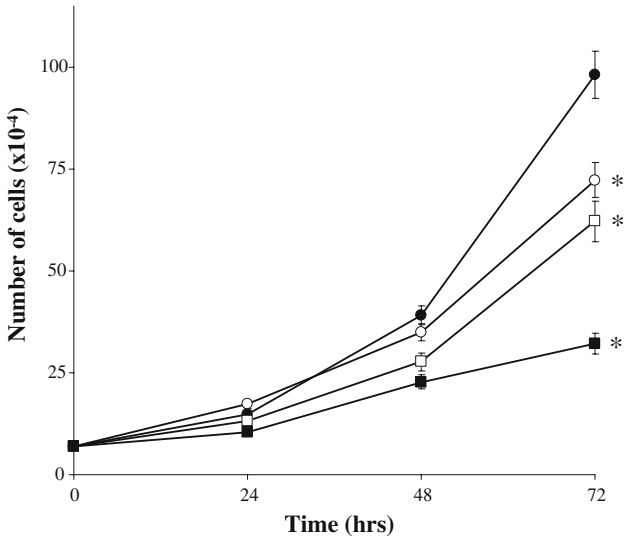

Fig. 1 Proliferation curve of murine B16-F10 melanoma cells treated with 10 μM (*open circle*), 50 μM (*open square*), or 100 μM (*filled square*) NMS after 24, 48, and 72 h. Control cells (*filled circle*) were incubated with DMSO 0.1% only. Data represent the mean of three different determinations ± SD (*$p < 0.001$)

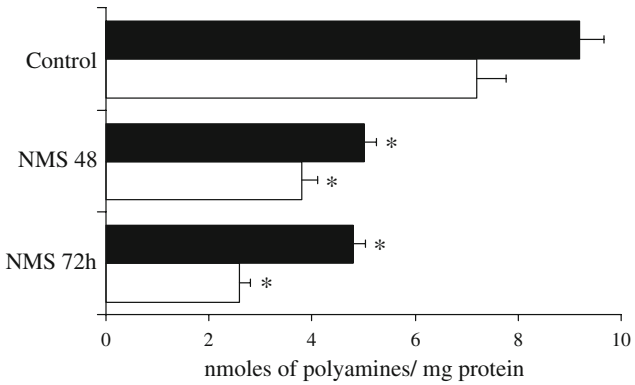

Fig. 2 Quantitative evaluation of the levels of polyamines in murine B16-F10 melanoma cells treated with 100 μM NMS. Putrescine was detected only in traces. Each point represents the mean of three different determinations ± SD (*$p < 0.001$); (*open square*) SPD; (*filled square*) SPM

Intracellular polyamine levels

As proliferation marker, polyamine levels were detected during the treatment of B16-F10 cells with 100 μM NMS for the time selected. The results are shown in Fig. 2. Exposure to NMS for 48 and 72 h caused a remarkable reduction of SPD level, respectively, by about 48% and by 63%, with respect to the control. SPM intracellular concentration also decreased, with respect to the control, by about 47% after 48 and 72 h ($p < 0.001$).

TG activity

Since TG and tyrosinase activities and melanin synthesis are commonly considered differentiation markers for melanocytes, we checked if the antiproliferative activity of NMS could be related to the stimulation of differentiation in the B16-F10 melanoma cell line. Table 1 shows that TG

Table 1 Effect of 100 μM NMS treatment of murine B16-F10 melanoma cells on TG activity

pmoles ^{14}C-methylamine/mg protein		
Control	NMS 48 h	NMS 72 h
18.30 ± 1.12	16.72 ± 1.47	29.13 ± 1.92*

Each point represents the mean ± SD of three different determinations

* $p < 0.001$

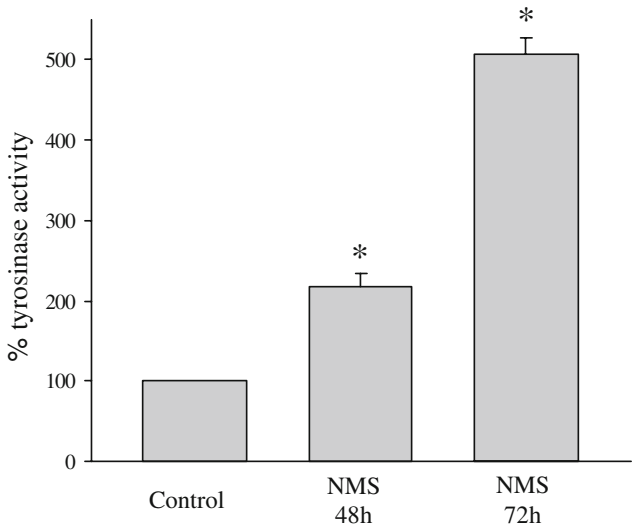

Fig. 3 Analysis of tyrosinase activity in control and 100 μM NMS-treated murine B16-F10 melanoma cells. Each point represents the mean of three different determinations ± SD (*$p < 0.001$)

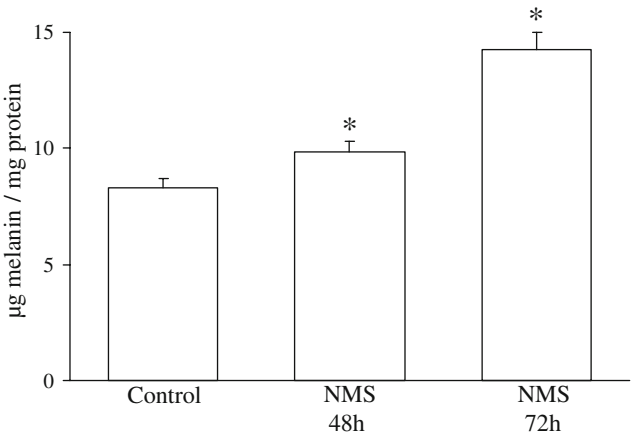

Fig. 4 Determination of melanin intracellular and extracellular concentration in 100 μM NMS-treated murine B16-F10 melanoma cells. Each point represents the mean of three different determinations ± SD (*$p < 0.001$)

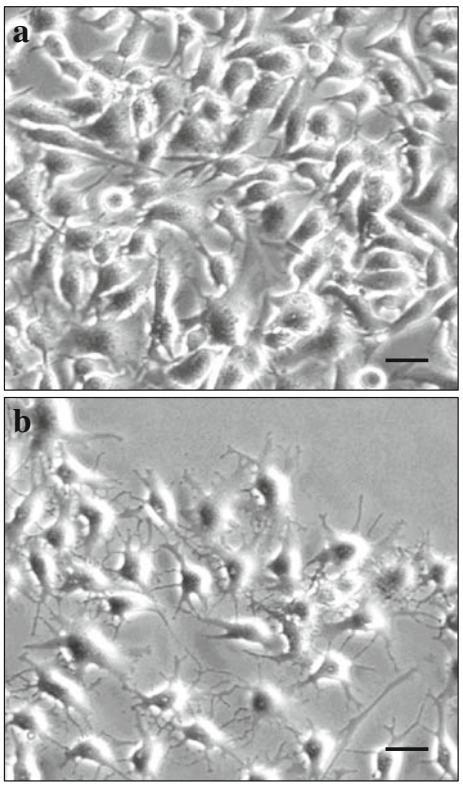

Fig. 5 Appearance of untreated **a** and 100 μM NMS-treated **b** B16-F10 melanoma cells. Typical morphological signs of differentiation appear upon NMS treatment (dendritic cells). Scale bar: 5 μm

activity increase was delayed to 72 h of incubation with 100 μM NMS (about 59%) when compared to control ($p < 0.001$).

Tyrosinase activity and melanin synthesis

We analyzed the stimulation of tyrosinase activity in NMS-treated B16 melanoma cells, according to the procedure described in Materials and methods. Figure 3 shows that tyrosinase activity was increased by about twofold, with respect to the control, in cells treated with NMS for 48 h, and by about fivefold after 72 h of treatment ($p < 0.001$). Changes of the intracellular levels of melanin in tumor cells incubated with 100 μM NMS at different times are shown in Fig. 4. The amount of the pigment increased, with respect to the control, by about 19% after 48 h and by about 72% after 72 h of treatment ($p < 0.001$). As a further evidence of cell differentiation, morphological changes in cell shape, with formation of dendrite-like structures were observed (Fig. 5).

Discussion

Understanding the mechanisms underlying carcinogenesis provides insights necessary for the development of therapeutic strategies to prevent and cure cancer. COX-2 is a critical enzyme in fatty acid metabolism: it catalyzes the production of proinflammatory PG2. The involvement of COX-2 in development, growth, and invasion properties of cancer is well-known (Zweifel et al. 2002; Sarkar and Yiwei 2006). In fact, PG2, COX-2 mRNA and protein expression were found to be frequently elevated in cell lines derived from tumors (Molina et al. 1999; Tucker et al. 1999).

This study was focused on the role of intracellular TG in the antineoplastic activity exerted by the non-steroidal anti-inflammatory drug NMS, a COX-2 selective inhibitor

(Famaey 1997). Since TG is considered a differentiation marker, the induction of tumor cell differentiation by NMS has been further investigated. The results obtained demonstrate the antiproliferative activity of NMS, as shown in the proliferation assay and also in the reduction of the intracellular concentration of polyamines, considered mitosis markers (Russell 1977; Tabor and Tabor 1985; Beninati and Folk 1988). The role of NMS in the possible induction of cell differentiation was supported by the detection of TG and tyrosinase activation and increased melanin content in NMS-treated melanoma cells.

The molecular mechanisms underlying the antiproliferative and differentiative properties of NMS are poorly understood. Our data support the hypothesis that the antineoplastic function of NMS may be dependent on the stimulation of intracellular TG activity. This may be mainly attributed to different factors. One is the capacity of the drug to perform a selective inhibition of COX-2, whose role is essential for tumor survival (Marnett 1990; Sheng et al. 1997; Molina et al. 1999; Tucker et al. 1999; Masferrer et al. 2000; Trifan et al. 2002). As a consequence, by interfering with PG2 synthesis, NMS obstructs not only the establishment of pro-inflammatory phases, but also the activation of ornithine decarboxylase, the major enzyme regulating polyamine metabolism and cellular proliferation (Sarraf et al. 1998; Badawi 2000; Dannenberg et al. 2001; Zweifel et al. 2002). Moreover, NMS antineoplastic effects may be also mediated by the inhibition of the production of radical and mutagen species formed during COX-2 catalytic activity (Marnett 1990; Badawi 2000; Dannenberg et al. 2001).

On the other hand, NMS, by jamming the metabolism of eicosanoids, heaps up arachidonic acid. The accumulated metabolite binds to the peroxisome proliferation-activated nuclear receptors (PPARs), transcription factors, and starts them. Then, PPARs enter into the nucleus and modify gene expression. In particular, they negatively influence cellular proliferation, development of malignant cells, production of metalloproteinases, transcription of pro-inflammatory genes, and above all, they induce a TG-dependent terminal differentiation (Maier et al. 1990; Mueller et al. 1998; Chang and Szabo 2000; Haydon et al. 2002; Vosper et al. 2003). PPARs are also defined "tumor suppressors" for their functions (Wick et al. 2002).

In conclusion, these data represent the first evidence about a correlation between the antineoplastic activity of NMS and the TG-mediated stimulation of melanoma cell differentiation. Differentiation therapy is an important and rapidly evolving aspect of cancer research, based on the concept that drugs and natural substances can inhibit carcinogenesis and development of tumors through the induction of cellular terminal differentiation (Beninati 1995; Thiele et al. 2000; Lentini et al. 2007). These considerations make particularly appealing the promise of a possible association of NMS and natural molecules, that are known as TG-modulators, in cancer clinical setting.

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