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Cell-type dependent response of melanoma cells to aloe emodin

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ABSTRACT

Intrinsic characteristics of melanoma cells such as expression of inducible nitric oxide synthase (iNOS), redox status, and activity of signaling pathways involved in proliferation, differentiation and cell death define the response of the cells to the diverse treatments. In this context we compared the effectiveness of herbal antaquinone aloe emodin (AE) against mouse B16 melanoma and human A375, different in initial activity of ERK1/2, constitutive iNOS expression and basal level of reactive oxygen species (ROS). Both cell lines are sensitive to AE treatment. However, while the agent induces differentiation of B16 cells toward melanocytes, in A375 cells promoted massive apoptosis. Differentiation of B16 cells, characterized by enhanced melanin production and tyrosinase activity, was mediated by H_2O_2 production synchronized with rapid p53 accumulation and enhanced expression of cyclins D1 and D3. Caspase mediated apoptosis triggered in A375 cells was accompanied with Bcl-2 but not iNOS down-regulation. In addition, opposite regulation of Akt-ERK1/2 axis in AE treated B16 and A375 cells correlated with different outcome of the treatment. However, AE in a dose-dependent manner rescued both B16 and A375 cells from doxorubicin- or paclitaxel-induced killing. These data indicate that caution is warranted when AE is administrated to the patients with conventional chemotherapy.

1. Introduction

Although naturally occurring anthracene derivatives present in the extracts of the roots, bark or leaves of numerous plants were primary known as laxatives and are intensively used for that purpose until today, in last few decades anticancer properties of these compounds are in the focus of scientific interest (Mueller et al., 1998; Fullbeck et al., 2005; Huang et al., 2005; Kim et al., 2005; Mijatovic et al., 2005a; Su et al., 2005; Esmat et al., 2006; Lee et al., 2006; Lin et al., 2006). Indeed, numerous conventional cytostatic drugs are anthracyclin-type of molecules (Caponigro et al., 2005; Wu and Hasinoff, 2005). Most important natural hydroxianthraquinones with confirmed biological potential, aloe emodin (AE) and emodin, possess antibacterial, antifungal, antiviral, diuretic, immunosuppressive, anti-inflammatory, vasorelaxant and, finally, anticancer activities (Huang et al., 1991; Barnard et al., 1992; Huang et al., 1992; Yagi et al., 1997; Kumar et al., 1998; Mueller et al., 1998; Kim et al., 2001; Basu et al., 2005; Fullbeck et al., 2005; Huang et al., 2005; Kim et al., 2005; Mijatovic et al., 2005a; Su et al., 2005; Esmat et al., 2006; Lee et al., 2006; Lin et al., 2006; Tabolacci et al., 2010). AE, present in highest concentration in the cortex of *Aloe vera* leaves, induces cell death in several tumor cell lines, as well as the regression of neuroectodermal tumors in mice with severe combined immunodeficiency without appreciable signs of acute and chronic toxicity (Pecere et al., 2000; Lee et al., 2001; Kuo et al., 2002; Wasserman et al., 2002; Yeh et al., 2003; Mijatovic et al., 2004; Lin et al., 2006; Lee et al., 2006). Also there is no confirmed genotoxicity of the compound *in vivo* in healthy animals after the high acute exposure to AE when the drug concentrations in the blood were almost in the same range as highly genotoxic concentration *in vitro* (Heidemann et al., 1996). The multiple mechanisms are involved in tumor cell growth inhibitory effect of AE. The outcome of the drug treatment has been described as induction of apoptosis (Acevedo-Duncan et al., 2004; Yeh et al., 2003; Lee et al., 2005; Lin et al., 2006), autophagic cell death Mijatovic et al., 2005a), or the cytostatic activity manifested by cell cycle arrest at the different stages of cell division (Chen et al., 2004; Lin et al., 2006).

Recently, Tabolacci et al. (2010) demonstrated the inhibitory effects of AE on B16-F10 melanoma cell proliferation, invasion and differentiation, accompanied by a remarkable increase of the

Abbreviations: AE, aloe emodin; ROS, reactive oxygen species; CFSE, carboxyfluorescein diacetate succinimidyl ester; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; iNOS, inducible nitric oxide synthase; DOXO, doxorubicin; Pct, paclitaxel; F.I.C., fraction inhibitory concentration.

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activity of transglutaminase 2. Melanoma represent heterogeneous group of aggressive and high metastatic tumors (Hoang and Eichenfield, 2000; Russo et al., 2009). One of the most serious barriers for their therapy is intrinsic resistance to induction of apoptosis by natural mediators of immune response and chemotherapeutic drugs (Hersey and Zhang, 2001). In the present study we reported that the cell specific response is crucial for determination of AE action. In fact, intracellular features of melanoma cells like expression of iNOS, redox status, and activity of signaling pathways crucial for proliferation, differentiation and cell death could define the response of the cells to the treatment and even convert the same treatment from cytostatic to cytotoxic (Demary et al., 2001; Tang and Grimm, 2004; Grimm et al., 2008). In this context we evaluated the molecular base of melanoma cell reaction to AE exposure and tried to explain why the same treatment in murine B16 cells resulted in cell differentiation while in human A375 line promoted massive apoptosis. We confirmed that basal level of reactive oxygen species (ROS) as well as opposite regulation of Akt-ERK1/2 axis in AE treated B16 and A375 cells is associated with the outcome of the treatment.

2. Materials and methods

2.1. Reagents, cells and animals

All chemicals used in the experiments were purchased from Sigma (St. Louis, USA), unless specified otherwise. Mayer's hematoxylin was from Bio Optica (Milano, Italy). Carboxyfluorescein diacetate succinimidyl ester (CFSE) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were from Molecular Probes (Eugene, USA). AE was stored at −20 °C at concentration of 200 μM in DMSO, and it was diluted in culture medium immediately before use. Murine melanoma B16 cell line was a kind gift from Dr. Sinisa Radulovic (Institute for Oncology and Radiology of Serbia, Belgrade, Serbia) while A375 cells were kind gift from Prof Ferdinando Nicoletti (Department of Biomedical Sciences, University of Catania, Italy). Cells were grown in HEPES-buffered RPMI 1640 medium supplemented with 5% or 10% FCS, 2 mM L-glutamine, antibiotics and 0.01% sodium pyruvate (culture medium) at 37 °C in humidified atmosphere with 5% CO_2. Cells were used for experiments after conventional trypsinization procedure. Control cell cultures contained the amount of DMSO corresponding to its content in the solution with the highest concentration of AE used in the particular experiment.

Inbred C57BL/6 mice were obtained from our own facility at the Institute for Biological Research "Sinisa Stankovic", University of Belgrade (Belgrade, Serbia) and kept under standard laboratory conditions (non specific pathogen free) with free access to food and water. The handling of animals and the study protocol were in accordance to the requirements of the European Union and approved by the Institutional Animal Care and Use Committee at the Institute for Biological Research "Sinisa Stankovic", University of Belgrade.

2.2. MTT-test and CFSE staining

The cells were seeded in flat-bottom 96-well plates (10^4cells/well) overnight, and then treated with different concentration of AE as indicated. After 24 h incubation, 3–4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test was performed as described previously (Mijatovic et al., 2005a). The results of MTT test, obtained from triplicate cultures were presented as % of control values obtained in untreated cell cultures. Rate of cell proliferation was determined using flow cytometric analysis of cells labeled with CFSE. Briefly, cells were detached and loaded with 1.5 μM CFSE for the 15 min at 37 °C, washed two times and then seeded in 6-well plates. Cells were treated for the 24 and 48 h, trypsinized and washed two times. Finally, cells were resuspended in PBS and analyzed by flow cytometry. Green fluorescence emission from cells illuminated with excitation light of 488 nm was measured with a FACSCalibur (BD, Heidelberg, Germany) and analyzed using Cell Quest Pro software (BD).

2.3. Determination of apoptosis and cell cycle analysis

Cells (2.5×10^5/well) were treated with 40 μM of AE in 6-well plate for 24 h, then trypsinized and stained with AnnV-FITC/EtBr according to the manufacturer's instruction (Biotium, Hayward, CA). Altermativly, cell cycle analysis was performed by propidium iodide (PI) staining as previously described (Mijatovic et al., 2005a; Maksimovic-Ivanic et al., 2008). Cells were analysed with FACSCalibur flow cytometer (BD, Heidelberg, Germany) using Cell Quest Pro software (BD).

2.4. Staining of cells with Mayer's hematoxylin

Cells were grown (3×10^4/well) in a chamber slide for 24 h in the presence or absence of AE or 0.5 μM of H_2O_2. Subsequently, they were fixed in 4% paraformaldehyde for 20 min at RT, washed twice in PBS and stained with Mayer's hematoxilin for 30 s. Finally, cells were washed with tap water, mounted with glycergel and analyzed under light microscope.

2.5. Staining of cells with H_2DCFDA and DAB

Cells were stained with H_2DCFDA according to the manufacturer's instruction for 30 min and then treated with AE (40 μM). After 5, 15, 30 and 45 min cells were washed and presence of reactive oxygen species was detected by flow cytometry.

Cells were exposed to AE (40 μM) in parallel with 3,3′-Diaminobenzidine (DAB, 1 mg/ml) and appearance of black precipitates was evaluated by light microscopy.

2.6. Tyrosinase activity assay and melanin determination

Tyrosinase activity was determined by measuring the rate of oxidation of L-dopa. Briefly, sub-confluent cultures in 6-well plate were lysed in 100 μl phosphate buffer pH 6.8–1% Triton X-100 and than centrifuged at 10000 rpm for 5 min. 40 μl of each extract was mixed with 100 μl of L-dopa substrate solution (2 mg/ml). Enzymatic reaction was carried out at 37 °C and the absorbance at 570 nm was read every 10 min for at least 1 h. The final activity was corrected by the total amount of protein estimated by Bradford assay. For melanin determination cells were incubated in 6-well plate for 24 h, trypsinized, counted and than lysed in 100 μl of 1 M NaOH. 400 μl of distilled water was added and samples were incubated at 60 °C for 1 h. Thereafter, absorbance of dissolved dye was measured at 492 nm.

2.7. In vivo administration of B16 cells

For *in vivo* application, B16 cells were cultivated for 24 h in the presence of 40 μM AE or adequate amount of DMSO, then trypsinized, counted and finally resuspended in PBS. Primary tumors were induced by subcutaneous (s.c.) injection of 5×10^5 melanoma cells in the dorsal right lumbosacral region of C57BL/6 mice. Tumor growth was observed every day and tumor size was measured in three dimensions. Tumor volume was calculated by the following formula: [$0.52\ ab^2$] (Maksimovic-Ivanic et al., 2008), where a is the longest and b is the shortest diameter.

2.8. Caspase detection

Cells (2.5×10^5/well) were treated with 40 μM AE for 24 h, then trypsinized and stained with Apostat (R&D Systems, Minneapolis, MN) in phenol red free RPMI 1640 (5% FCS) for 15 min at 37 °C. At the end of cultivation cells were washed and analysed with FACSCalibur flow cytometer (BD, Heildelberg, Germany) using Cell Quest Pro software (BD).

2.9. Immunoblot analysis of AKT, p-ERK, p53, cyclin D1 and D3, Bcl-2, iNOS and IκB

Cells (1×10^6) were seeded in flasks (25 cm^3), and treated with 40 μM AE for the indicated time intervals. At the end of incubation, cells were harvested in protein lysis buffer containing 62.5 mM Tris–HCl (pH 6.8 at 25° C), 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.01% w/v bromophenol blue, and subjected to electrophoresis on 12% SDS–polyacrylamide gels or 10% SDS–polyacrylamide gels for detection of iNOS. Electro-transfer to polyvinylidene difluoride membranes at 5 mA/cm^2 was done with a semi-dry blotting system (Fastblot B43, Biorad, Goettingen, Germany). The membranes were blocked with 5% w/v nonfat dry milk in PBS with 0.1% Tween-20, and blots were probed with specific antibodies to pIkB, p53, cyclin D1, cyclin D3 and β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA), iNOS, (Sigma, St. Louis, MO), AKT, p-AKT, ERK, p-ERK, Bcl-2 (all from Cell Signaling Technology), followed by incubation with secondary antibody (ECL donkey anti-rabbit HRP linked, GE Healthcare, Buckinghamshire, UK). Bands were visualized using chemiluminescence detection system (ECL, GE Healthcare).

2.10. Isobologram analysis

To determine the type of interaction between AE and conventionally used chemotherapeutic isobologram analysis was performed. Isobolograms were made from treatments with diverse concentrations of AE (40, 20, 10 μM) combined with different concentrations of doxorubicin (DOXO) 0.125–1 μM or 0.015–0.1 μM for A375 and B16, respectively, and with paclitaxel (Pct) 3.7–25 μM and 0.38–3 μM for A375 and B16, respectively. Combinations attaining 30–50% of cytotoxicity were expressed as concentration of single agent alone making this amount of toxicity. Analysis was prepared on the basis of dose–response curves of cell viability treated with AE alone, Pct or DOXO alone or their combination for 24 h. Fraction inhibitory concentration (F.I.C.): concentration of each agent in combination/concentration of each agent alone <1 is considered synergistic while F.I.C. > 1 is considered antagonistic.