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RESEARCH

Glucocorticoid Receptor Expression and Antiproliferative Effect of Dexamethasone on Human Melanoma Cells

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Abstract Glucocorticoids, such as dexamethasone are widely used in cancer therapy and have cell type-specific pro- or antiapoptotic effects. We examined whether melanoma cells are sensitive to dexamethasone treatment. We have demonstrated for the first time that in human melanoma cell lines as well as in benign and malignant melanocytic tumors glucocorticoid receptor (GCR) is present both at mRNA and protein level. Dexamethasone applied at high doses inhibited the in vitro growth of WM983A human melanoma cells. The inhibitory effect was due to apoptosis induction. In the case of this relatively sensitive cell line dexamethasone enhanced the effect of the chemotherapeutic drug DTIC.

Keywords Apoptosis · Chemotherapy · Dexamethasone · Glucocorticoid receptor · Melanoma

Introduction

The glucocorticoid (GC) derivative dexamethasone acts as an anti-inflammatory and immunosuppressor agent. It is routinely used as co-medication in cancer therapy to counteract certain side effects of antitumor treatment (edema, nausea). In the treatment of certain hematological malignancies, especially in multiple myeloma, dexamethasone is part of all chemotherapy protocols, for its strong apoptosis inducing effect on cells of the hematological lineage. Glucocorticoids can regulate both the extrinsic and intrinsic apoptosis pathways through non-genomic actions or de novo gene expression [1]. The effects of GCs on apoptosis are cell type-specific. They can cause Fas-associated cell death like in osteocytes [2], activate the proapoptotic Bim as in murine and human T-cell lymphoma and leukemia cells [3–5], or cause indirect cell damage by increasing ROS production as in muscle cells [6].

The gene of the glucocorticoid receptor (GCR) consists of nine exons. GCR has two major isoforms produced by alternative splicing. The classic GCRα has a widespread distribution and acts as a transcription factor. It participates in several physiological processes such as glucose homeostasis, protein, lipid and carbohydrate metabolism, acting in interaction with other factors, e.g. AP-1. The GCRβ isoform localizes constitutively to the nucleus and is unable to bind steroids [7]. It acts as a negative regulator of the glucocorticoid effect [8] by blocking the binding of coactivators [9].

Resistance to glucocorticoid therapy may occur if inactive GCR isoforms are present in vast majority, if the members of the ABC-transporter family are overexpressed, or if the apoptotic pathways are inhibited (e.g. Bcl-2 overexpression) [10]. Several different variants and loss-of-function mutations may play a role as well in glucocorticoid resistance [10].

Here we demonstrate the presence of glucocorticoid receptor in human melanoma cell lines both at mRNA and protein level, however, dexamethasone inhibited in vitro cell growth only at high doses. The inhibitory effect was due to apoptosis induction. We showed that the expression

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level of GCR mRNA in melanomas is significantly lower compared to normal skin or nevus.

Materials and Methods

Tissue Samples

The expression of glucocorticoid receptor was examined on tissue specimens excised from patients treated at the National Institute of Oncology, Budapest. The molecular biological assays were performed on frozen tissue sections of primary tumor from cutaneous melanoma patients, on normal skin from the same patients and on nevus samples.

Tumor Cells and Culture Conditions

The HT168 and HT168-M1 human melanoma lines [11] are derivatives of the A2058 cell line (provided by L. A. Liotta, NCI, NIH, Bethesda, MD). HT199 melanoma line was established by our group [12]. WM35, WM983A and WM983B cell lines were gifts from M. Herlyn (Wistar Institute, Philadelphia, PA). M24met line was kindly provided by B. M. Mueller (Scripps Research Institute, La Jolla, CA). Melanoma cells were maintained in vitro as monolayer cultures in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 5% fetal calf serum (FCS; Sigma) and 50 μg/ml gentamycin at 37°C in a 5% CO_2 atmosphere.

Detection of GCR Expression by Immunocytochemistry

To detect GCR expression, 10^4 cells were plated on slides (Multitest Slide, MP Biomedicals, Irvine, CA) in FCS-containing medium. The following day they were fixed with 4% buffered paraformaldehyde for 15 min, then permeabilized for 5 min in 0.02% saponin. Non-specific binding sites were blocked with Image-iT FX signal enhancer (Molecular Probes, Invitrogen, Carlsbad, CA). For primary antibody we used mouse monoclonal anti-human GCR, clone 5E4, 1:50, 2 h at 37°C, kind gift from P. Németh, University of Pécs, Pécs [13]. Anti-mouse IgG-FITC (1:100; Vector Laboratories Inc., Burlingame, CA) was used as secondary antibody, propidium-iodide for staining nuclei and Prolong Gold antifade reagent (Invitrogen) for mounting. Nikon (Tokyo, Japan) EX600 fluorescent microscope was used for examining the slides.

Detection of GCR Expression by Immunohistochemistry

For the immunohistochemical detection of GCR, 5-μm-thick frozen sections were fixed in methanol and blocked with 3% BSA. The primary antibody and the development of the reactions were the same as for immunocytochemistry.

Detection of GCR Expression by RT-PCR and Quantitative PCR

Total RNA was isolated from confluent cell cultures and frozen tissue sections with TRI Reagent (Sigma) according to the manufacturer's protocol. DNA contamination was eliminated by DNase treatment (DNA-free, Ambion, Applied Biosystems, Foster City, CA). Three μg of total RNA was reverse transcribed from each sample using deoxy-NTPs (0.5 mM each), a mixture of random primer and oligo dT (final concentration 3 μM), RNasin ribonuclease inhibitor (20 U/reaction; Promega, Madison, WI), reverse transcription buffer (containing 500 mM Tris–HCl, pH 8.3, 500 mM KCl, 30 mM $MgCl_2$ and 50 mM DTT) and M-MLV reverse transcriptase (Sigma, 200 U/reaction). RNA solutions were incubated for 50 min at 37°C, then for 10 min at 85°C. The outcome of the reverse transcription and the purity of the RNA samples were monitored by the amplification of β-actin (Table 1).

For the detection of GCR nested PCR was used. The primer pairs (Table 1) were designed to recognize the same region as the anti-human GCR antibody (clone 5E4). The PCR reaction mixture contained 2.5 μl 10× PCR buffer (DyNazyme, Finnzymes, Espoo, Finland), 2 μl dNTP-mix (2.5 mM each), 0.4 μl DNA-polymerase (2 U/μl; DyNazyme, Finnzymes), 2.5 μl of each primer and 2 μl cDNA made up to 25 μl with DEPC-treated water. The reaction contained 30 cycles, the cycling parameters were 94°C (1 min), 55°C (1 min) and 72°C (1 min). PCR products were separated in 2% agarose gel, stained with ethidium-bromide and detected with Gel Doc 2000 system (Bio-Rad, Hercules, CA). To identify them, they were re-isolated from the gel (MEGA-spin Agarose Gel Extraction Kit, iNtRON Biotechnology, Korea) and sequenced (Laborigo, Budapest, Hungary).

The real-time PCR analysis was standardized by coamplifying the glucocorticoid receptor gene with the housekeeping gene β-actin. The primers are listed in Table 1. The real-time

Table 1 Primer pairs for the amplification of GCR and β-actin

Nested PCR	
GCR outer	5′-GGC TGG AAT GAA CCT GGA AG-3′
	5′-CAT AAG ATA CCT GAA GCC TGT GT-3′
GCR nested	5′-GCC ACT ACA GGA GTC TCA CA-3′
	5′-GGT CAT CCA GGT GTA AGT TCC-3′
β-actin	5′-GTG GGG CGC CCC AGG CAC CCA-3′
	5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′
Quantitative PCR	
GCR	5′-CCC AAG AGT TCA GCA TCC ACT G-3′
	5′-GCT TGC AGT CCT CAT TCG AGT T-3′
β-actin	5′-TCT GGC ACC ACA CCT TCT AC-3′
	5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′

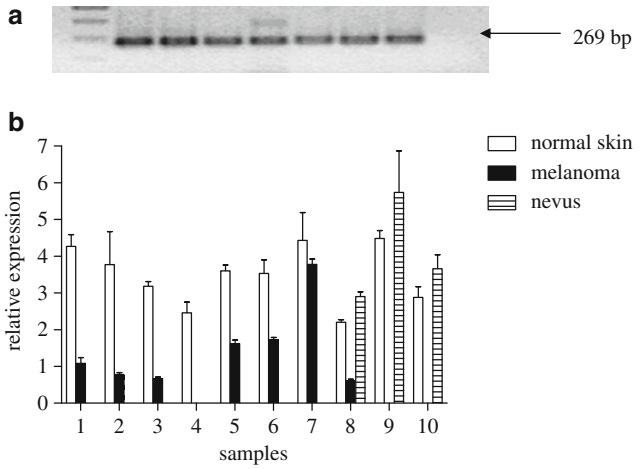

Fig. 1 Detection of GCR mRNA in human melanoma cells and in tissue samples. **a** Nested PCR in human melanoma cell lines (order of samples: 100 bp ladder, HT168-M1, HT168, A2058, HT199, M24met, WM983A, WM983B, negative control). **b** Relative amount of GCR mRNA in samples of melanoma, nevus and normal skin

PCR reaction was run on the iCycler iQ (Bio-Rad) using standard conditions, namely, optimized concentration of primers (final concentration 200 nM), iQ SYBR Green Supermix (containing 100 mM KCl, 40 mM Tris–HCl, pH 8.4, 0.4 nM of each dNTP, 6 mM $MgCl_2$, 50 U/ml iTaq DNA polymerase, SYBR Green I and 20 nM fluorescein) and 2 μl cDNA. Reaction mixtures containing water instead of cDNA were used as negative controls for every different primer pair. The cycling parameters were 95°C (10 min), 50 cycles of 95°C (30 s), 64°C (30 s) and 72°C (1 min). The starting quantity of gene expression in the sample was determined by comparing to a standard curve generated from a dilution series of template DNA of known concentration, and normalized to its own β-actin expression.

Cell Proliferation Assay

Four thousand cells were plated in flat-bottomed, 96-well tissue culture plates in RPMI/FCS, and after 24 h treated in the presence of FCS with the following concentrations of dexamethasone (Sigma): 10, 20, 40 and 80 μM (0.4% ethanol as control). The cells were incubated for 3 days, or for 5 days in the case of long-term assays. In combination experiments with the chemotherapeutic agent DTIC (Dacarbazine) we used pretreatment for 2 days with 20 or 40 μM dexamethasone, then 20, 40 or 80 μM DTIC was added for three more days parallel with dexamethasone. After incubation relative cell density was determined by a colorimetric assay: 0.5 mg/ml of the tetrazolium dye MTT (Sigma) was added to the wells and after 4 h incubation at 37°C the medium was gently removed, the plates air-dried, and the formazan crystals formed in viable cells dissolved in DMSO [14]. The absorbance was measured at 570 nm with a Bio-Rad microplate reader.

Flow Cytometric Apoptosis Assay

Cells (5×10^5) were plated in 6-well plates and treated with 20 or 40 μM dexamethasone or with 0.4% ethanol as control for 48 h. Then cells were detached from the plastic, washed with PBS and fixed with 70% ethanol. After 1 h incubation with propidium-iodide and RNase (Cystain PI Absolute T, Partec, Görlitz, Germany), we determined the amount of DNA in cells by flow cytometer (CyFlow, Partec). The percentage of the apoptotic cells was shown in the sub-G1 fraction analyzed by FlowMax software.

Modified Boyden-Chamber Migration Assay

96-well CXF8 plates (polycarbonate membrane filter with 8 μm pore size, without coating, Neuroprobe Inc., Cabin John, MD) were used. Cell suspension (10^4 cells in 20 μl medium) was placed onto the membrane in the presence or absence of dexamethasone, and the lower compartment was filled with 30 μl of fibronectin (100 μg/ml, Sigma). Cells were allowed to migrate for 6 h at 37°C, then cells on the upper surface of the filter were removed mechanically, while the migrated cells on the lower surface were fixed in methanol, stained with toluidine blue and counted under light microscope (40× magnification).

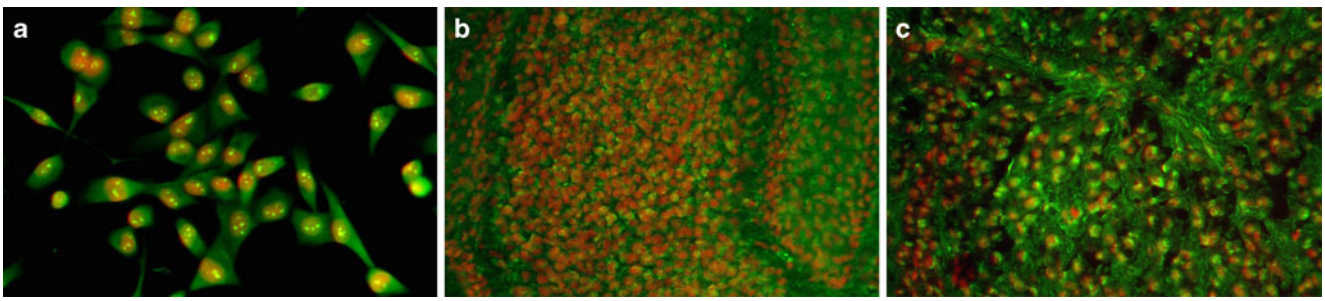

Fig. 2 Detection of GCR protein. **a** HT199 human melanoma cell line (immunocytochemistry with anti-GCR antibody clone 5E4, FITC, *green*; nucleus, PI, *red*; 400×); **b** nevus and **c** melanoma (immunohistochemistry, 200×)

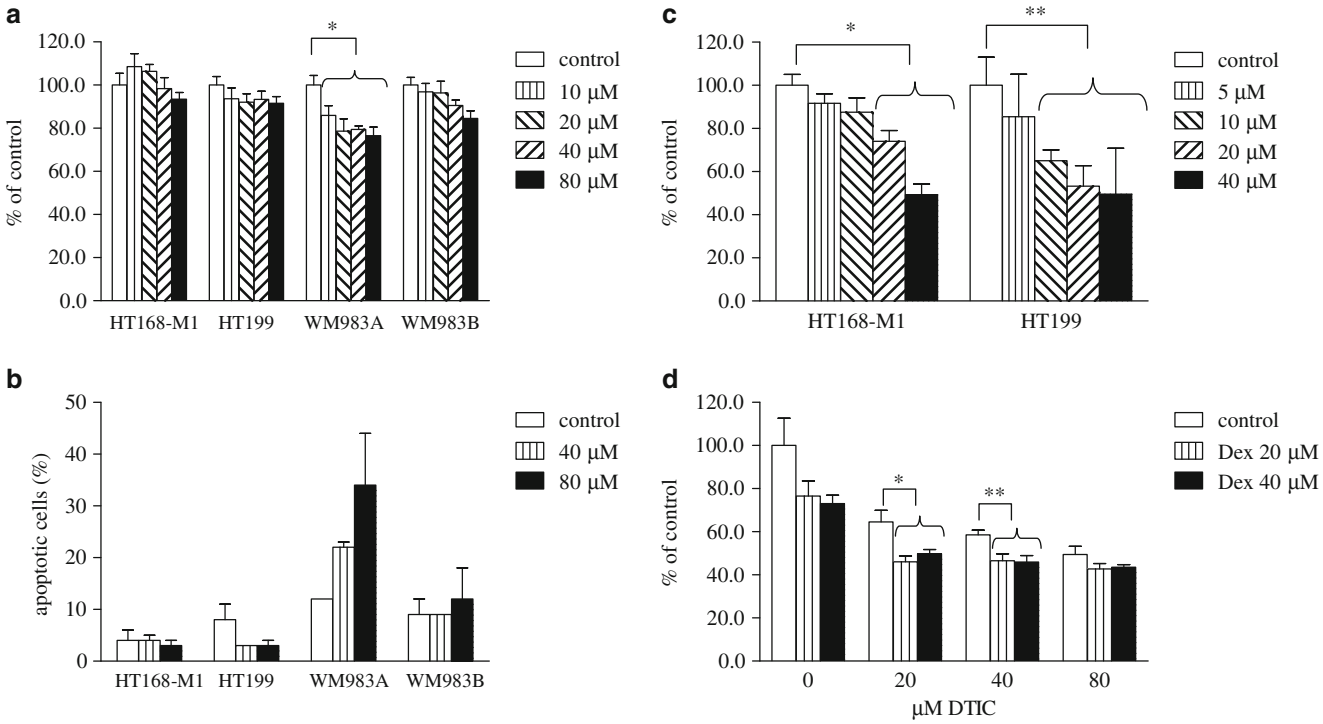

Fig. 3 Effects of dexamethasone on the proliferation and apoptosis of human melanoma cells. **a** Three-day-long treatment in proliferation test on 4 human melanoma cell lines (6 parallel samples, mean ± SD, *$p<0.0003$). **b** Measurement of the apoptosis-inducing effect of 2-day-long treatment on 4 human melanoma cell lines by flow cytometry (2 parallel samples, mean ± SD). **c** Long-term proliferation assay on HT168-M1 and HT199 cell lines (6 parallel samples, mean ± SD, *$p<0.00002$, **$p<0.002$). **d** Proliferation test using combination treatment of dexamethasone with the chemotherapeutic drug DTIC on WM983A cells (4 parallel samples, mean ± SD, *$p<0.009$, **$p<0.0002$)

Statistics

Statistical evaluation of the results of in vitro experiments was performed using Student's *t*-test. $P<0.05$ values were considered statistically significant.

Results

We examined the presence and function of glucocorticoid receptor, member of the nuclear receptor family, on human melanoma cells. Using primers to the ligand-binding domain (NCBI Reference Sequence: NG_009062.1) we detected GCR mRNA expression by nested PCR in the case of all human melanoma lines studied (Fig. 1a). We also analyzed GCR expression of the melanoma tissue, nevus and normal skin of 10 patients using quantitative PCR. We showed that the expression level of GCR in melanomas is significantly lower compared to normal skin or nevus (1.3± 1.2, 3.5±0.7 and 4.1±1.4, respectively, mean ± SD; individual values are shown in Fig. 1b). Immunocytochemical staining with antibody clone 5E4, binding to the N-terminal domain of human GCR, showed positivity in all the 8 cell lines studied (A2058, HT168, HT168-M1, HT199, WM983A, WM983B, WM35, M24met), both in the nucleus and in the cytoplasm (Fig. 2a). Furthermore, we demonstrated the expression of the receptor protein in all of the studied human nevus and melanoma tissue samples (Fig. 2b, c).

In vitro treatment of melanoma cells with the glucocorticoid derivative dexamethasone for 3 days was effective in only one of four lines studied, resulting in 25% growth inhibition in the case of WM983A cells, while no significant effect was found in the case of 3 other cell lines (Fig. 3a). The inhibitory effect was due to apoptosis induction, which was also observed only in the case of

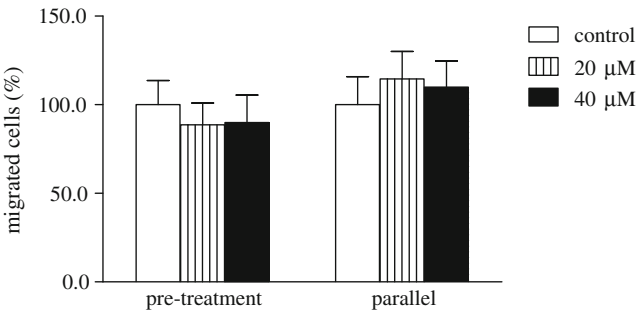

Fig. 4 Effect of dexamethasone on the migration of HT168-M1 melanoma cells (6 parallel samples, mean ± SD)

WM983A cells, but not in the other cell lines (Fig. 3b). Therefore, we have extended the treatment: 40 μM dexamethasone for 5 days inhibited the growth of HT168-M1 and HT199 cells by 50% (Fig. 3c). In combination treatment with the alkylating agent DTIC used in clinical practice, dexamethasone had a slightly additive inhibitory effect only in the case of WM983A cell line (Fig. 3d), while it was ineffective on HT168-M1, HT199 and WM983B cells (data not shown).

We examined in modified Boyden-chamber migration assay whether dexamethasone at doses found to be ineffective in proliferation tests have any effect on the migration of HT168-M1 cells. However, 20 or 40 μM dexamethasone applied as parallel or pre-treatment of HT168-M1 cells was proved to be ineffective (Fig. 4).

Discussion

We showed for the first time the presence of glucocorticoid receptor in human melanoma cells and melanoma tissues both at mRNA and protein level. We detected a markedly lower expression level in cutaneous melanomas compared to normal skin. Although earlier studies applying biochemical methods for determining the glucocorticoid binding capabilities of human melanoma tissues gave positive results [15], the presence of GCR has not yet been demonstrated by immunocytochemistry in human melanoma lines, only in B16 mouse melanoma cells [16].

Dexamethasone at high doses was effective in inhibiting human melanoma cell growth. Earlier studies had shown inconsistent results with dexamethasone treatment of melanoma cells. It increased the doubling time of hamster melanoma cells [17], inhibited the colony-forming abilities of human and murine melanoma cells [18] and blocked the cell cycle in the G_1/S phase [19]. However, some melanoma cells are insensitive to dexamethasone treament, but there was no difference in the GC receptor specificity or affinity between dexamethasone-sensitive or dexamethasone-resistant hamster melanoma cell lines [20].

Most chemotherapeutic drugs have severe side effects, bone marrow suppression being the main dose-limiting effect. Pretreatment with dexamethasone decreased the hematopoietic toxicity of carboplatin and ifosfamide in cancer patients [21]. Dexamethasone also showed antitumor sensitizer activity on human cancer xenografts by reducing tumor interstitial fluid pressure (IFP) and, hence, increasing drug uptake [22]. In human melanoma xenografts high tumor IFP was found to be associated with the development of pulmonary and lymph node metastases [23]. Glucocorticoids encapsulated in long-circulating liposomes proved cytotoxic on mouse melanoma cells and their anti-angiogenic effects may also play a role in tumor growth inhibition [24].

Still, the antitumor activity of glucocorticoids remains a question. Recent data suggest induction of therapy resistance by glucocorticoids in solid tumors [25]. In vitro treatment with dexamethasone inhibited cisplatin- and 5-fluorouracil-induced apoptosis and promoted the growth of malignant cells [26]. In vivo results demonstrated glucocorticoid-induced chemotherapy resistance in xenografted prostate cancer, which lasted for a long time but was reversible upon removal of glucocorticoids [27]. Resistance is common for several cytotoxic treatments and for several glucocorticoid derivatives and is due to an inhibition of apoptosis, promotion of viability and cell cycle progression [27]. Slightly elevated risk of malignant melanoma was found among users of oral glucocorticoids [28]. Moreover, a clinical study demonstrating regression of visceral melanoma metastases after adrenalectomy supports the role of glucocorticoids in melanoma progression [29].

In our in vitro experiments dexamethasone failed to have biologically significant direct effects on the proliferation or migration of most human melanoma cell lines studied. Melanomas were described to be resistant to drug-induced apoptosis [30, 31], and that may be the reason why dexamethasone was relatively inactive in the majority of the studied human melanoma cell lines, though GCR was present both at mRNA and protein levels. However, prolonged exposure to dexamethasone resulted in inhibition of cell proliferation. Revealing the molecular mechanisms behind this steroid resistance may help not only to understand melanoma biology, but also to explore novel ways to melanoma therapies.

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