Placenta growth factor induces melanoma resistance to temozolomide through a mechanism that involves the activation of the transcription factor NF-κB

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Received August 5, 2010; Accepted September 15, 2010

DOI: 10.3892/ijo_00000844

Abstract. Placenta growth factor (PlGF) and its receptor vascular endothelial growth factor receptor-1 (VEGFR-1) are co-expressed in a large number of human melanoma cell lines. Moreover, a correlation between *in vivo* PlGF production and melanoma progression has been suggested. To investigate whether PlGF might have a role in protecting melanoma cells from the cytotoxic effects of the anticancer agent temozolomide (TMZ), which is used for the treatment of this malignancy, we stably transfected a doxycycline-inducible PlGF antisense mRNA into a human melanoma cell clone that secretes VEGF-A and PlGF and expresses receptors for both growth factors. Induction of PlGF antisense mRNA in the transfected cells (13443/ASP3 subclone) halved TMZ IC_{50}, and exogenous addition of PlGF to the culture medium 24 h before TMZ treatment, partially restored IC_{50} values to that of control cells. The increased sensitivity of 13443/ASP3 cells upon PlGF antisense mRNA expression was not due to down-regulation of O^6-methylguanine-DNA methyltransferase, a DNA repair protein that represents the main mechanism of resistance to TMZ. Since the activity of the transcription factor nuclear factor-κB (NF-κB) has been correlated to melanoma chemoresistance, we investigated whether NF-κB was involved in PlGF-induced melanoma cell resistance to TMZ. Induction of PlGF antisense mRNA in 13443/ASP3 cells halved the levels of active NF-κB and the specific inhibition of this transcription factor increased sensitivity of 13443/ASP3 cells to TMZ. In conclusion, our data strongly suggest that PlGF plays a role in melanoma cell resistance to TMZ through a pathway that involves NF-κB activation.

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Key words: PlGF, temozolomide resistance, NF-κB, melanoma

Introduction

The placenta growth factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family of angiogenic factors that shares with VEGF-A, the principal member of the family, and VEGF-B a transmembrane tyrosine kinase receptor (VEGFR-1). The expression of PlGF is up-regulated in several types of human cancers and is associated with a poor prognosis (1-3). PlGF is capable of transducing its own signals through the phosphorylation of tyrosine residues within VEGFR-1, which are distinct from those phosphorylated upon stimulation of the receptor by VEGF-A (4).

VEGFR-1 is expressed in endothelial cells during vessel formation and remodelling, in macrophages and in myo-epithelial cells, favouring cell migration and survival (3,5,6). Moreover, it is also frequently expressed in a variety of human tumours where it predicts poor prognosis and recurrence (2,3). In tumour cells VEGFR-1 signalling has been shown to inhibit apoptosis and to induce chemoresistance (2,7,8). Indeed, VEGFR-1 neutralization has been reported to prolong survival of tumour-bearing mice (9).

We previously demonstrated the co-expression of PlGF and VEGFR-1 in a large number of human melanoma cell lines (10) and, together with other groups, suggested that the interaction of PlGF with VEGFR-1 might modulate cellular pathways important for melanoma cell proliferation, apoptosis and invasiveness (10-14).

Malignant melanoma is a highly chemoresistant tumour. To date, no treatment options are available for melanoma patients with advanced disease that either provide sufficient response rates or significantly prolong overall survival (15,16). The methylating agent dacarbazine is still regarded as the reference drug for the treatment of metastatic melanoma even though the response rate is only about 20%. Temozolomide (TMZ) is an analogue of dacarbazine with similar efficacy. Differently from dacarbazine, TMZ does not require metabolic activation, is administered per os, penetrates the blood-brain barrier and is well tolerated (17). TMZ induces the formation

of DNA methyl adducts at different base positions (18), but cytotoxicity results primarily from the formation of O^6-methylguanine (O^6-meG) lesions (19). In fact, tumour cells with high levels of the repair protein O^6-methylguanine-DNA methyltransferase (MGMT) are resistant to TMZ (20). This enzyme is able to repair the pre-toxic DNA lesion O^6-meG by transferring the methyl group from the O^6 position of guanine to an internal cysteine residue (20). When the MGMT activity is low, unrepaired O^6-meG mispairs with thymine during DNA replication, leading to activation of the mismatch repair system with consequent induction of apoptosis and growth arrest (21).

The transcription factor nuclear factor-κB (NF-κB) plays a key role in the regulation of cell proliferation, inflammation, angiogenesis and suppression of apoptosis, and, when constitutively activated, may be critical in the development of drug resistance in tumour cells (22,23). It consists of hetero- or homodimers of five structurally related proteins: p65 (RelA), Rel B, c-Rel, p50 (NF-κB1) and p52 (NF-κB2), the most abundant form being the p50 and p65 heterodimer (24). In quiescent cells, NF-κB is associated in the cytosol with an inhibitory protein of the IκB family. Following stimulation of a variety of cell membrane receptors, IκB is phosphorylated by the IκB kinase (IκK) complex and thus marked for proteosomal degradation (24). Degradation of IκBα results in the release of the NF-κB subunits which translocate into the nucleus and bind specific DNA sequences in the promoter region of NF-κB-regulated genes, which include, among others, anti-apoptotic gene products such as Bcl-2/Bcl-xL, Mcl-1, cIAPs and survivin (25,26).

In this study, we investigated whether PlGF might have a role in protecting melanoma cells from the cytotoxic effects of TMZ. To this end we utilized a melanoma model in which a PlGF antisense mRNA can be conditionally induced. Our results indicate that PlGF down-modulation significantly increases melanoma cell sensitivity to TMZ and that activation of the NF-κB signaling pathways is involved in PlGF-mediated melanoma cell resistance to TMZ.

Materials and methods

Reagents. Culture media were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fatty acid-free bovine serum albumin (BSA) was from Roche Diagnostics (Mannheim, Germany). VEGF-A and PlGF homodimers and polyclonal antibodies used in ELISA assays, were from R&D Systems (Abingdon, UK). TMZ, kindly provided by Schering-Plough Research Institute (Kenilworth, NJ, USA), was freshly prepared in complete medium immediately before use. O^6-benzylguanine (BG, Sigma-Aldrich) was dissolved in ethanol (2.4 mg/ml), stored as stock solution at -80˚C, and diluted in complete medium just before use. DHMEQ (dehydroxylmethyllepoxyquinomicin) was dissolved in dimethyl sulphoxide (10 mg/ml), stored in aliquots at -20˚C and diluted in complete medium just before use. The final concentration of ethanol or dimethyl sulphoxide used as solvent for BG or DHMEQ, respectively, did not affect cell growth (data not shown). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was prepared at a concentration of 5 mg/ml in phosphate-buffered saline (PBS) and stored at 4˚C.

Melanoma cell lines. The origin of the human melanoma cell lines utilised in the study (GR-Mel, M14 and 13443-Mel) and their characterization regarding the production of VEGF and PlGF and the expression of the receptors for both growth factors, have been described elsewhere (10). The 13443-N2 cell clone was obtained from the original 13443-Mel cell line by the limiting dilution method (13).

Expression of antisense PlGF mRNA sequence in melanoma cells. Inducible expression of PlGF mRNA antisense sequence in 13443/N2 cells was achieved by using the tetracycline-regulated T-REx mammalian expression system, from Invitrogen (Groningen, The Netherlands), according to the instructions furnished by the manufacturer. The plasmid pcDNA4/ASP was a generous gift of Dr A. Fiore (Laboratory of Molecular and Cellular Biology, IDI-IRCCS, Rome, Italy). To generate this plasmid, the PlGF cDNA, obtained by RT-PCR from normal human keratinocytes, was subcloned in the antisense direction into the pcDNA-TO-myc-HisA vector (pcDNA4) provided in the T-REx system kit.

13443-N2 cells were initially transfected with the plasmid pcDNA6/TR, containing the sequence encoding a Tet repressor (provided in the T-Rex system kit), and then with the pcDNA4/ASP plasmid or with the empty vector (pcDNA4), as previously described (13). Subclones of 13443-N2 cells, containing the plasmids pcDNA6/TR and pcDNA4/ASP (ASP subclones) or the plasmids pcDNA6/TR and pcDNA4 (ASC subclones), were subsequently isolated. 13443/ASP3 and 13443/ASC1 subclones were selected for further studies. These cells were maintained in RPMI-1640 medium supplemented with 10% Tet-system approved fetal calf serum (BD Biosciences, Bedford, MA, USA), 2 mM glutamine, 200 μg/ml zeocin, 5 μg/ml blasticidin and 20 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]).

Expression of PlGF mRNA antisense sequence was achieved by treating semi-confluent 13443/ASP3 cell cultures with 1 μg/ml doxycycline (a tetracycline analogue with longer half-life) for 48 h. For the evaluation of the amount of PlGF released in the culture supernatant by 13443/ASP3 cells after treatment with doxycycline, during the last 24 h of incubation, culture medium was replaced with 0.1% BSA/RPMI-1640 medium containing freshly added doxycycline.

RT-PCR analysis. cDNA preparation and PCR amplification for evaluation of VEGFR-1 expression were performed as described (10), utilizing the following primers: VEGFR-1, forward primer 5'-CTCCTGAGTACTCTACTCCT-3', reverse primer 5'-GAGTACAGGACCACCGAGTT-3' (640 bp fragment); GAPDH, forward primer 5'-TCCCATCACCATCTTCCA-3', reverse primer 5'-CATCACGCCACAGTTTCC-3' (380 bp fragment). In both cases, primer annealing was performed for 30 sec at 58˚C.

Evaluation of VEGF and PlGF secretion. Semi-confluent melanoma cell cultures were incubated for 24 h in 0.1% BSA/RPMI-1640 medium without serum. Culture supernatants were then collected and concentrated at least 10-fold in Centriplus concentrators (Amicon, Beverly, MA, USA). Cells were detached from the flasks with PBS/EDTA and the total cell number/culture was recorded.

Quantification of the amount of VEGF and PlGF homodimers in the concentrated supernatants was performed as previously described (10), using Maxisorp Nunc immunoplates (Nunc, Roskilde, Denmark) coated with goat anti-VEGF or anti-PlGF IgGs at a concentration of 10 μg/ml in PBS. Detection of the cytokines was performed with biotinylated goat anti-VEGF or anti-PlGF IgGs and streptavidin-alkaline phosphatase conjugated (1:10,000) (Roche). Optical density at 405 nm was measured in a Microplate reader 3550-UV (Bio-Rad, Hercules, CA, USA). This assay allows detection of VEGF and PlGF polypeptides at concentrations equal to or >100 pg/ml. Cytokine secretion values were normalised by the total number of cells/culture.

Evaluation of cell chemosensitivity by the MTT assay. Cell proliferation was evaluated using the tetrazolium compound MTT, as previously described (27). Melanoma cells were suspended in complete medium at a concentration of $4x10^4$ cells/ml, dispensed in 50 μl aliquots into flat-bottom 96-well plates and allowed to adhere overnight at 37˚C. Graded amounts of TMZ or DHMEQ were then added to the wells in 50 μl of complete medium and the plates were incubated at 37˚C in a 5% CO_2 humidified atmosphere for five days. Six replica wells were used for controls and each drug concentration. Afterwards, 20 μl of the MTT solution (5 mg/ml in PBS), were added to each well, and cells were incubated at 37˚C for 4 h. Cells were then lysed with 100 μl of a buffer containing 20% SDS and 50% *N,N*-dimethylformamide at pH 4.7. After an overnight incubation, the absorbance was read at 595 nm using a 3550-UV microplate reader (Bio-Rad).

TMZ was tested at concentrations ranging between 31.2 and 1,000 μM and DHMEQ at concentrations ranging between 2.5 and 10 μg/ml. The cytotoxic effects of TMZ were evaluated also in combination with the MGMT inhibitor BG (10 μM) to prevent the repair of the methyl adducts at the O^6-G. The inhibitor was added during the overnight incubation before TMZ treatment. In the experiments aimed at testing the effect of PlGF down-regulation on cell sensitivity to TMZ, doxycycline (1 μg/ml) was included together with BG in the culture medium used to suspend the cells before dispensing them in the plates. Plates were then incubated for 48 h before adding TMZ.

Cell sensitivity to drug treatment was expressed in terms of IC_{50} (drug concentration producing 50% inhibition of cell growth, calculated on the regression line in which absorbance values at 595 nm were plotted against the logarithm of drug concentration).

Evaluation of MGMT activity. Cells were removed from continuous culture, washed twice with PBS and stored as pellets at -80˚C until used. MGMT activity in cell extracts was determined by measuring the transfer of [^{3}H]-methyl groups from a DNA substrate to the MGMT protein (28). Cell pellets ($3x10^6$ cells) were resuspended in 1 ml of lysis buffer (0.5% CHAPS, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 3 mM dithiothreitol, 100 mM NaCl, and 10% glycerol) supplemented with a cocktail of protease inhibitors (Roche Diagnostics) and incubated at 4˚C for 30 min. Cell lysates were then centrifuged at 18,000 x g for 10 min at 4˚C. Aliquots of supernatants were then diluted in 50 mM Tris-HCl buffer, pH 8.3, containing 1 mM EDTA, and 3 mM dithiothreitol, and incubated with 10 μg of ^{3}H-methylated DNA at 37˚C for 1 h. DNA was then hydrolyzed by heating samples at 75˚C for 45 min, in the presence of 1 N perchloric acid, and proteins were precipitated using 1 mg of BSA as carrier. Pellets were washed with 1 N perchloric acid, resuspended in 0.01 N NaOH, and radioactivity measured in a liquid scintillation counter (Tri-Carb 1900; Packard BioScience, CT, USA), after addition of scintillation liquid (Ultima Gold; Packard Instruments Chemical Operation, Groningen, The Netherlands). Protein concentration in cell extracts was evaluated according to the method of Bradford using the Bio-Rad dye solution and BSA as standard. MGMT activity was expressed in terms of fmoles of [^{3}H]-methyl groups transferred per mg of protein in cell extract.

NF-κB activation assays. NF-κB activation was analyzed in nuclear extracts, prepared using the Active Motif Nuclear Extract kit (Active Motif Europe, Rixensart, Belgium) according to the manufacturer's instructions. NF-κB activity in the nuclear extracts was determined using a NF-κB p65 enzyme-linked immunosorbent assay (ELISA)-based transcription factor assay kit (TransAM assay) (Active Motif Europe) or an Electrophoretic Mobility Shift Assay (EMSA).

The TransAM assay was performed according to the manufacturer's protocol using 20 μg of nuclear extracts. The kit contains a 96-well plate with immobilized oligonucleotides encoding a NF-κB consensus site (5'-GGGACTTTCC-3') to which the active form of p65 specifically binds. The NF-κB detecting antibody recognizes an epitope on p65 that is accessible only when this polypeptide is activated and bound to its target DNA. A horseradish peroxidase-conjugated secondary antibody provides a sensitive colorimetric readout that is quantified by a Bio-Rad Microplate Reader 680 at 450 nm with a reference wavelength of 655 nm. A nuclear extract obtained from Jurkat cells stimulated with TPA and calcium ionophore provided within the Trans-AM kit, was used as internal standard in the assay. Results are expressed as arbitrary units, calculated relative to the values obtained for the Jurkat extract, according to the formula:

$$\frac{\text{(sample A450/}\mu\text{g protein in sample extract) x 100}}{\text{Jurkat A450/}\mu\text{g protein in Jurkat extract}}$$

The EMSA was performed as previously described (29). Nuclear extracts (5 μg of protein) were incubated with ^{32}P-end-labelled double-stranded NF-κB consensus oligonucleotide (Santa Cruz Biotechnologies, Santa Cruz, CA). Complexes were analyzed by non-denaturing 4% PAGE and gels were subsequently dried and autoradiographed with intensifying screens.

Results

Characterization of the melanoma cell clone expressing PlGF antisense under an inducible promoter. To investigate whether PlGF might have a role in protecting melanoma cells from the cytotoxic effects of TMZ, we utilized a Tet-on gene expression system to conditionally down-modulate this cytokine in human melanoma cells. The melanoma cell

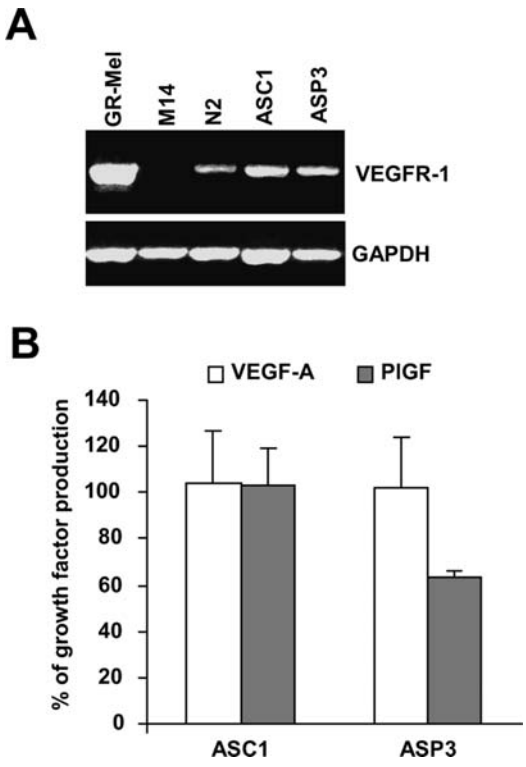

Figure 1. Characterisation of 13443/N2 subclones. (A) VEGFR-1 expression in 13443/N2 subclones. RT-PCR analysis of VEGFR-1 transcript was performed in the parental clone (N2) and the transfected subclones (ASC1 and ASP3), using the protocol and the primers indicated in Materials and methods. Melanoma cell lines GR-Mel and M14 were used as positive and negative controls, respectively. To confirm the integrity of RNA samples, the same cDNA preparations were subjected to PCR analysis of GAPDH. PCR products were separated in 0.8% agarose gels. Results are representative of one out of three different experiments. (B) Quantitative analysis of VEGF-A and PlGF released by ASC1 and ASP3 melanoma cells grown in the presence of doxycycline (1 μg/ml). After 24 h of treatment, culture medium was replaced with 0.1% BSA/RPMI-1640 medium containing freshly added doxycycline, and cell culture supernatants were collected after additional 24 h of culture. The amount of VEGF-A and PlGF released into the culture supernatant was evaluated by an ELISA assay. Data are expressed as percentage of growth factors detected in culture medium with respect to untreated controls. Each value represents the arithmetic mean of six independent determinations ± SD of the mean. Student's t-test analysis for PlGF secretion in ASP3 cells, control versus doxycycline, $p<0.001$.

clone 13443-N2, that expresses VEGFR-1 and VEGFR-2 and secretes VEGF-A and PlGF (13), was initially transfected with the regulatory pcDNA6/TR vector, which encodes the tetracycline repressor, and then with the pcDNA4/ASP vector, carrying the cDNA coding for the PlGF mRNA antisense sequence under the control of a human cytomegalovirus promoter and containing two tetracycline operator sites. As a control, 13443-N2 cells were transfected with the pcDNA6/TR plasmid and with the empty pcDNA4 vector. Two transfected subclones were selected: the 13443/ASP3 subclone (from now on referred to as ASP3 cells), containing the antisense sequence for PlGF mRNA, and the control 13443/ASC1 subclone (from now on referred to as ASC1 cells).

The selected subclones were analyzed for the expression of VEGFR-1 by RT-PCR and the results indicated that the receptor transcript was present in both ASC1 and ASP3 cells (Fig. 1A). To specifically down-regulate PlGF expression, cells were treated with doxycycline and the amount of PlGF and of VEGF, as control, released into the culture medium was measured. After 48 h treatment a 40% reduction in PlGF production was observed only in ASP3 cells, while VEGF secretion was not affected by doxycycline treatment (Fig. 1B) in both subclones.

Effect of PlGF down-modulation on melanoma sensitivity to TMZ. ASC1 and ASP3 cells were then used to investigate whether PlGF was involved in melanoma cell resistance to the cytotoxic effects of TMZ. Down-modulation of PlGF secretion in ASP3 cells was accompanied by increased sensitivity to TMZ, as shown by the reduction of the IC_{50} value for this drug from 486 to 263 μM (Table I). On the other hand, in ASC1 cells doxycyclin exposure did not significantly affect TMZ IC_{50} value (Table I). To exclude the possibility that the increased sensitivity to TMZ observed in ASP3 cells after exposure to doxycycline might be due to down-regulation of MGMT activity, we tested the level of this repair protein before and after treatment with docycycline. Both ASC1 and ASP3 subclones were found to be MGMT-proficient, being the enzyme activity higher in ASC1 cells (Table I). This finding was consistent with the higher TMZ IC_{50} value displayed

Table I. Effect of PlGF downregulation on melanoma cell resistance to TMZ.

	ASC1			ASP3		
	TMZ IC_{50}[a]			TMZ IC_{50}[a]		
Treatment	- BG	+ BG	MGMT activity[b] (fmol/mg)	- BG	+ BG	MGMT activity[b] (fmol/mg)
Control	570±6	118±9	322±35	486±7	94±1	181±3
Doxycycline	503±42	112±17	329±10	263±11	41±1	169±3

[a]Cell sensitivity to TMZ was evaluated by the MTT assay, as described in Materials and methods, 5 days after treatment with the drug, as single agent or in combination with the MGMT inhibitor BG (10 μM). Student's t-test analysis in ASP3 cells: - BG, control versus doxycycline, $p<0.002$; + BG, control versus doxycycline, $p<0.001$. [b]MGMT activity in cell extracts was determined by measuring the transfer of [^{3}H]-methyl groups from a DNA substrate to the MGMT protein, as described in Materials and methods. MGMT activity was expressed in terms of fmoles of [^{3}H]-methyl groups transferred per mg of protein in cell extract. Each value represents the arithmetic mean of three independent experiments ± SD of the mean.

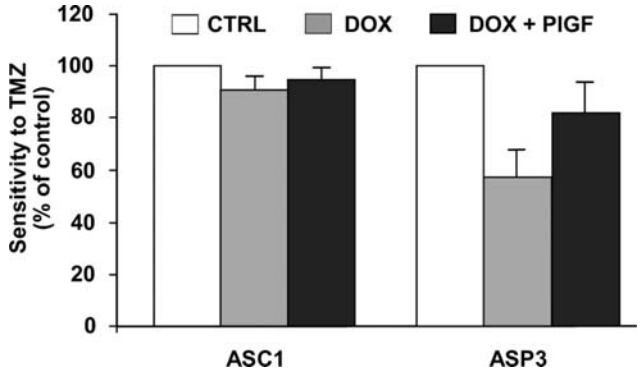

Figure 2. Exogenous PlGF counteracts the effect of doxycycline on ASP3 cell sensitivity to TMZ. IC_{50} values for TMZ were determined, as described in Materials and methods, in ASC1 and ASP3 cells either untreated (CTRL) or exposed to doxycycline (DOX, 1 μg/ml) for 48 h or to doxycycline plus 50 ng/ml PlGF, added during the last 24 h of doxycycline treatment (DOX + PlGF). Data represent the sensitivity to TMZ of ASC1 and ASP3 cells relative (%) to their respective controls not exposed to doxycycline (CTRL), calculated on the basis of the IC_{50}s values. Histograms represent the arithmetic mean of three independent experiments ± SD. Student's t-test analysis in ASP3 cells: CTRL versus DOX, p=0.008; DOX versus DOX + PlGF, p=0.007.

by ASC1 cells with respect to ASP3 cells. Moreover, MGMT activity was not affected by doxycycline treatment (Table I). Melanoma cells were also treated with the specific MGMT inhibitor BG before PlGF antisense induction and before exposure to TMZ. As illustrated in Table I, BG treatment significantly reduced TMZ IC_{50} values in both ASC1 and ASP3 cells. Notably, even in the presence of BG, doxycycline treatment was able to increase TMZ sensitivity of ASP3 cells (Table I). The effect of doxycycline was partially abrogated when exogenous PlGF was added to the culture medium during the last 24 h of treatment with the antibiotic and before TMZ addition (Fig. 2). Therefore, PlGF appears to be able to promote protection from the cytotoxic effects of TMZ in melanoma cells.

Involvement of NF-κB activity in cell resistance to TMZ mediated by PlGF. Since the activity of the transcription factor NF-κB has been correlated to melanoma chemoresistance, we investigated whether NF-κB might be involved in PlGF-induced melanoma cell resistance to TMZ.

We initially analyzed the modulation of the level of NF-κB activity in ASP3 and ASC1 cells upon treatment with doxycycline (Fig. 3). EMSA analysis revealed a strong down-modulation of NF-κB activity in ASP3 cells treated with the antibiotic (Fig. 3A). The reduction of NF-κB DNA binding activity was quantified using an ELISA-based transcription factor assay kit, which evidenced a 43% decrease of NF-κB activity in ASP3 cells exposed to doxycycline with respect to untreated ASP3 cells (Fig. 3B). This effect was not observed in the control ASC1 cells (Fig. 3).

Involvement of NF-κB activity in the ability of PlGF to promote melanoma cell resistance to TMZ was further analyzed utilizing DHMEQ, an inhibitor of the transcription factor. This inhibitor blocks the DNA-binding activity and the nuclear translocation of NF-κB (30), and reduces cell growth of hepatoma cells in a dose-dependent manner (in a

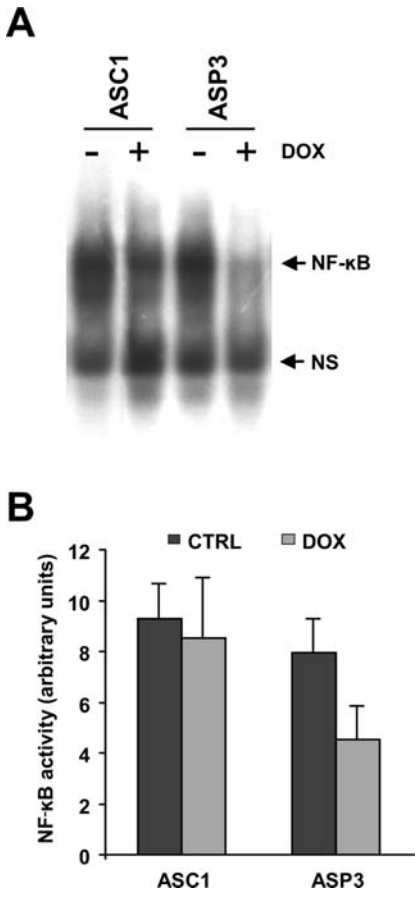

Figure 3. Effect of PlGF modulation on NF-κB activity. Nuclear extracts from ASC1 and ASP3 cells, either untreated (CTRL) or incubated for 48 h with 1 μg/ml doxycycline (DOX), were analyzed for NF-κB activity by EMSA (A) or using a quantitative Trans-AM kit (B), as described in Materials and methods. Data are representative of one out of three independent experiments with similar results (A) or represent the mean values from three independent determinations ± SD of the mean (B). NS, non-specific. Student's t-test analysis in ASP3 cells (B), CTRL versus DOX, p=0.01.

range from 5 to 20 μg/ml) (30). Initially we evaluated the effect of DHMEQ on ASP3 cell growth with the aim of finding a concentration devoid of growth inhibitory effects but still capable of inhibiting NF-κB activity to be used in combination with TMZ. Cells were exposed for 3 h (a sufficient time for its full inhibitory activity) (31) to graded concentrations of DHMEQ and, after medium replacement and incubation for additional 5 days, cell growth was analysed by the MTT assay.

The results indicated that treatment with concentrations >5 μg/ml of DHMEQ markedly impaired the growth of melanoma cells (Fig. 4A). For combination studies with TMZ, the concentration of 5 μg/ml DHMEQ was chosen since it almost halved NF-κB activity in ASP3 cells (Fig. 4B), with a minimal effect on cell growth (Fig. 4A). Preincubation with 5 μg/ml DHMEQ (at 37˚C for 3 h) of ASP3 cells before TMZ treatments resulted in a ~50% reduction in the IC_{50} value of the methylating agent (Fig. 4C).

Therefore, the results indicated that the induction of PlGF antisense mRNA in ASP3 cells halved the levels of active NF-κB and that specific inhibition of this transcription factor increased sensitivity of ASP3 cells to TMZ.

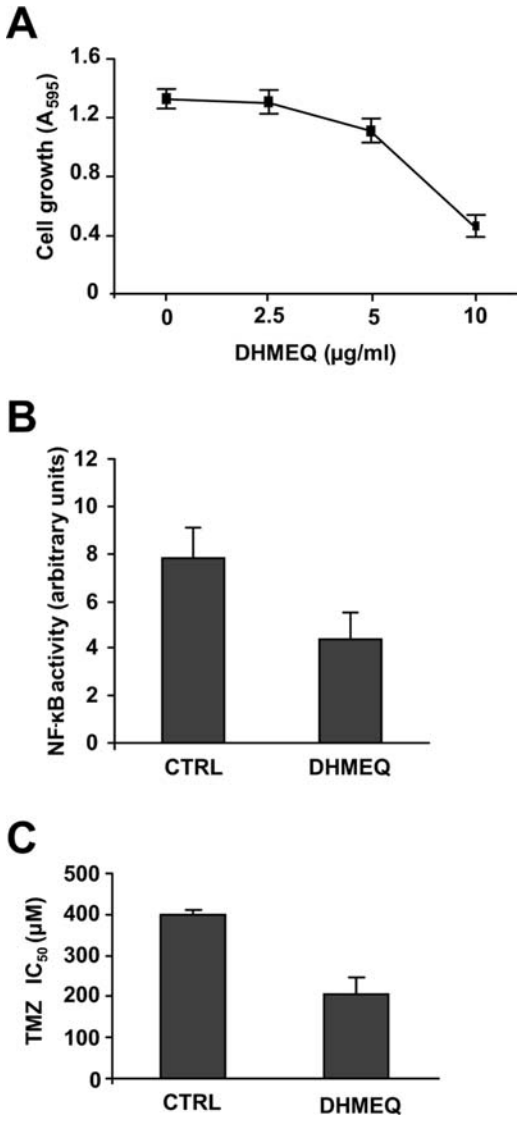

Figure 4. Effect of the NF-κB inhibitor DHMEQ on ASP3 cell sensitivity to TMZ. (A) Cells (2,000 cells/well) were allowed to adhere on 96-well plates overnight at 37˚C and then treated for 3 h with DHMEQ at the indicated concentrations. Afterwards, cells were kept in culture in fresh medium without the drug for 5 additional days and cell growth was evaluated by the MTT assay. A representative experiment, out of three with similar results, is shown. (B) Nuclear extracts obtained from ASP3 cells were incubated for 3 h with 5 μg/ml DHMEQ or with the same amount of DMSO used as solvent of the inhibitor (CTRL), at 37˚C, and analyzed for NF-κB activity utilizing a quantitative Trans-AM kit as described in Materials and methods. Data represent the mean values of three independent determinations ± SD. Student's t-test analysis, CTRL versus DHMEQ, p=0.02. (C) IC_{50} values for TMZ were determined, as described in Materials and methods, in ASP3 cells exposed to 5 μg/ml DHMEQ or to the same amount of DMSO used as solvent of the inhibitor (CRTL), for 3 h at 37˚C. Each value represents the arithmetic mean ± SD of three independent experiments. Student's t-test analysis: CTRL versus DHMEQ, p=0.02.

Discussion

Malignant melanoma is highly resistant to chemotherapy and once it metastasizes the prognosis is extremely poor. Only few chemotherapeutic agents have shown some efficacy against metastatic melanoma, and one of these is the methylating compound TMZ, which exerts apoptotic and cytostatic effects in melanoma cells (32). However, resistance of melanoma to TMZ is common and is frequently due to over-expression of the DNA repair protein MGMT or of anti-apoptotic genes (e.g., Bcl-2). Therefore a number of strategies are under investigation in order to overcome tumour drug resistance to TMZ (reviewed in ref. 33).

Increasing data in the literature suggest that PlGF and its receptor VEGFR-1 might regulate the apoptotic pathways in tumour cells. PlGF contributes to the angiogenic and inflammatory switch in various pathologies, such as tumour growth, ischemia and arthritis (2). Moreover, clinical studies have shown that PlGF levels of expression in tumour cells correlate with poor prognosis in various tumours types (1,2). In human melanoma cell lines, the expression of this cytokine and of VEGFR-1 has been described, and a correlation between PlGF expression and tumour progression has been suggested (10-14).

In this study we demonstrated for the first time that PlGF, through the activation of VEGFR-1, plays a role in melanoma resistance to TMZ, using a melanoma model in which a PlGF antisense mRNA can be conditionally induced. To this purpose, we produced a melanoma cell line, which secretes PlGF and expresses VEGFR-1, stably transfected with a doxycycline-inducible PlGF antisense mRNA (ASP3 subclone). Treatment of ASP3 cells with doxycycline caused a down-modulation of PlGF production, thus interrupting the PlGF/VEGFR-1 autocrine loop in this system. Notably, the decrease of PlGF expression resulted in increased *in vitro* chemosensitivity, reducing by 50% the TMZ IC_{50}.

Following the engagement of a variety of membrane receptors, the transcription factor NF-κB is translocated to the cell nucleus as a result of the activation of several signal transduction pathways. One of them is the mitogen-activated protein kinase (MAPK) pathway (34), which is also triggered by PlGF after binding to VEGFR-1 (4). NF-κB activation results in the induction of several antiapoptotic gene products that might be responsible for the resistance to antitumour drugs such as TMZ. Thus, to further shed light on the possible mechanism underlying the effect of PlGF on melanoma cell resistance to TMZ, activity levels of NF-κB were analysed. The results indicated that induction of PlGF antisense mRNA was associated with a significant decrease of NF-κB activity.

Our results also demonstrate that specific inhibition of this transcription factor resulted in increased sensitivity of melanoma cells to TMZ, suggesting that the activation of the NF-κB signalling pathway might be involved in the mechanism underlying melanoma chemoresistance mediated by PlGF. To further investigate the role of NF-κB in this process, we used the recently developed synthetic NF-κB inhibitor DHMEQ. Differently from other NF-κB antagonists, DHMEQ specifically inhibits NF-κB activity by covalent binding to the Rel family proteins p65, RelB, cRel and p50, without hampering the degradation of IκB (35). This covalent binding of DHMEQ hinders the DNA binding site of NF-κB components. The association of the NF-κB inhibitor with TMZ reduced the IC_{50} of the methylating agent to an extent similar to that observed upon PlGF down-modulation (36).

Recent studies performed in the murine model have shown that neutralizing antibodies against PlGF or VEGFR-1 are able to inhibit the growth and metastatic process of several

tumours and to enhance the efficacy of chemotherapy without causing significant side-effects (3,37). Our *in vitro* results could, at least in part, explain the mechanism underlying these *in vivo* observations. Overall, our data suggest that inhibition of PlGF signal transduction pathway might represent a novel therapeutic strategy to counteract the resistance of melanoma to TMZ.

Acknowledgements

The authors would like to thank Daniele Bartoloni for the artwork. This study was supported by the Italian Ministry of Health and by a FIRB grant (RBIP061.CA9).

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