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 (PIGF) 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 PIGF 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 PIGF antisense mRNA into a human melanoma cell clone that secretes VEGF-A and PIGF and expresses receptors for both growth factors. Induction of PIGF antisense mRNA in the transfected cells (13443/ASP3 subclone) halved TMZ IC50, and exogenous addition of PIGF to the culture medium 24 h before TMZ treatment, partially restored IC50 values to that of control cells. The increased sensitivity of 13443/ASP3 cells upon PIGF antisense mRNA expression was not due to down-regulation of O6-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 PIGF-induced melanoma cell resistance to TMZ. Induction of PIGF 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 PIGF plays a role in melanoma cell resistance to TMZ through a pathway that involves NF-κB activation.

Introduction

The placenta growth factor (PIGF) 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 PIGF is up-regulated in several types of human cancers and is associated with a poor prognosis (1-3). PIGF 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).

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).

We previously demonstrated the co-expression of PIGF and VEGFR-1 in a large number of human melanoma cell lines (10) and, together with other groups, suggested that the interaction of PIGF 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).

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

INTERNATIONAL JOURNAL OF ONCOLOGY  38:  241-247,  2011
of DNA methyl adducts at different base positions (18), but cytotoxicity results primarily from the formation of O\textsubscript{6}-methylguanine (O\textsubscript{6}-meG) lesions (19). In fact, tumour cells with high levels of the repair protein O\textsubscript{6}-methylguanine-DNA methyltransferase (MGMT) are resistant to TMZ (20). This enzyme is able to repair the pre-toxic DNA lesion O\textsubscript{6}-meG by transferring the methyl group from the O\textsubscript{6} position of guanine to an internal cytosine residue (20). When the MGMT activity is low, unrepaired O\textsubscript{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 heteromeric 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 PI GF 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 PI GF antisense mRNA can be conditionally induced. Our results indicate that PI GF down-modulation significantly increases melanoma cell sensitivity to TMZ and that activation of the NF-κB signaling pathways is involved in PI GF-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 PI GF 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 phosphate-buffered saline (PBS) and stored at 4˚C.

Evaluation of VEGF and PlGF secretion. Semi-confluent melanoma cell cultures were incubated for 24 h in 0.1% BSA/RPMI-1640 medium containing freshly added doxycycline.
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 [3H]-methyl- 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 [3H]-methyl groups transferred per mg of protein in cell extract.

Evaluate of MGMT activity. Cells were removed from the cytotoxic effects of TMZ, we utilized a Tet-on inducible promoter 

Quantification of the amount of VEGF and PI GF homodimers in the concentrated supernatans was performed as previously described (10), using Maxisorp Nunc immunoplates (Nunc, Roskilde, Denmark) coated with goat anti-VEGF or anti-PIGF IgGs at a concentration of 10 μg/ml in PBS. Detection of the cytokines was performed with biotinylated goat anti-VEGF or anti-PIGF 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 PI GF 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% CO2 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 O6-G. The inhibitor was added during the overnight incubation before TMZ treatment. In the experiments aimed at testing the effect of PI GF 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 [3H]-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 [3H]-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 [3H]-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 an 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'-GGGACTTTC-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:

\[
\text{sample A450/μg protein in sample extract) x 100} = \frac{\text{Jurkat A450/μg protein in Jurkat extract}}{\text{Jurkat A450/μg protein in Jurkat extract}}
\]

The EMSA was performed as previously described (29). Nuclear extracts (5 μg of protein) were incubated with 32P-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 PI GF antisense under an inducible promoter. To investigate whether PI GF 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
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 doxycycline. 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 in ASP3 cells.

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

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<th>ASC1</th>
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<td>TMZ IC$_{50}$</td>
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<tr>
<td>Treatment</td>
<td>- BG</td>
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<tr>
<td>Control</td>
<td>570±6</td>
<td>118±9</td>
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<tr>
<td>Doxycycline</td>
<td>503±42</td>
<td>112±17</td>
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$^a$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.
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 IC50 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 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 IC50 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.
Data represent the mean values of three independent determinations ± SD.

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 IkB (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 PIGF 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 (RBI06P1CA9).

References