ANTICANCER RESEARCH *24*: 2179-2184 (2004)

Targeting Chemotherapy-induced VEGF Up-regulation by VEGF Antisense Oligonucleotides in HNSCC Cell Lines

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Abstract. *Background: Angiogenesis is increased in various human cancers, including head and neck squamous cell carcinoma (HNSCC), and correlates with tumour progression and metastasis. Vascular endothelial growth factor (VEGF) has been shown to be a key regulator of angiogenesis. Tumour treatment with anticancer agents might have an effect on the secretion of VEGF. Therefore, we determined whether certain chemotherapeutic agents stimulate VEGF secretion in HNSCC and whether VEGF antisense oligonucleotide treatment can modulate these effects in vitro. Materials and Methods: The effect of chemotherapeutic agents (Cisplatin, Carboplatin and 5-FU) on the production of VEGF was investigated on established human HNSCC cell lines at both mRNA and protein levels. By using a 21-mer VEGF antisense phosphorothioate oligonucleotide targeting the translation start site of human VEGF mRNA, we examined modulation of VEGF expression in cell line supernatants by capture ELISA. Results: The treatment of HNSCC cell lines with chemotherapeutic agents resulted in a significant induction of VEGF production. Carboplatin most prominently induced the release of VEGF from the tumour cells. VEGF antisense oligonucleotide treatment resulted in a significant reduction of chemotherapy-induced VEGF up-regulation compared to sense control. Conclusion: Induction of VEGF secretion might contribute to the frequently observed drug resistance of HNSCC to chemotherapeutic agents. This molecular effect might be reduced by the use of VEGF antisense oligonucleotides in head and neck cancer therapy.*

Head and neck squamous cell carcinoma (HNSCC), an aggressive epithelial malignancy that is the most common neoplasm arising in the upper aerodigestive tract, is an important public health problem. At the current rate, approximately 50, 000 new cases of HNSCC are reported annually in the United States and more than 500, 000 cases are diagnosed world-wide every year (1). Furthermore, the prevalence of HNSCC is increasing world-wide (2). Improved techniques in surgery, radiation and chemotherapy have increased the local control of HNSCC, but the overall survival rates have not changed significantly in 25 years (3). Despite optimal therapy, the overall survival rate is poor, with a 40% 5-year survival for those with resectable tumours and a 20% survival rate for patients whose tumours are not amenable to excision (3). More than two-thirds of the patients with HNSCC present with advanced disease (stage III and stage IV), half of those patients will go on to develop local recurrences and 30% or more will develop distant metastasis (4). Chemotherapy is generally employed in advanced cases but the response rates average only 25-40% with overall survival being measured in months (3). Thus, it is imperative to investigate the reasons for low response rates.

The platinum co-ordination complexes and 5-FU represent the most important agents now in use for head and neck cancer treatment. Cisplatin was the first platinum co-ordination drug used to treat HNSCC. Carboplatin, which was developed as a second-generation analogue of cisplatin, has demonstrated significantly less nephrotoxic and emetogenic potential in both preclinical and clinical studies (5). Recent studies described an increased release of cytokines by the tumour cells due to anti-cancer drugs (6). However, the effects on angiogenic factor secretion as a consequence of drug stimulation have not been investigated.

Angiogenesis, the process leading to the formation of new blood vessels from pre-existing ones, is required for invasive tumour growth and metastasis and constitutes an important point in the control of cancer progression (7). For this reason, tumour angiogenesis has become the focus of extensive investigation. Indeed, the intensity of angiogenesis has been shown to be increased in various human tumours, including HNSCC (8). The induction of tumour vascularization is

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Key Words: Vascular endothelial growth factor (VEGF), angiogenesis, antisense, chemotherapy, head and neck cancer.

regulated by the release of angiogenic peptides from tumour cells, macrophages and extracellular matrix (9). Vascular endothelial growth factor (VEGF) is a pivotal stimulator of angiogenesis because its binding to VEGF receptors has been shown to promote endothelial cell migration and proliferation, two key processes required for the development of new blood vessels (10,11). In addition, VEGF increases vascular permeability, which may also contribute to angiogenesis and tumour growth (12). VEGF has been shown to increase tumour growth and angiogenesis *in vivo* in a nude mouse model (13). Enhanced expression of VEGF has been detected in a large variety of malignant human tumours including HNSCC (14-16). We previously demonstrated extended VEGF serum concentration in HNSCC patients compared with healthy controls (17). VEGF expression in HNSCC tumours strongly correlated with angiogenesis (18) and was inversely correlated with apoptosis (19).

The recognised importance of tumour-induced angiogenesis has stimulated the development of agents able to interfere with the molecules involved in this process (7). The role of VEGF as a potential target for anti-neoplastic therapy has been demonstrated in several studies in which blockage of VEGF inhibited tumour growth and vascularization *in vivo* (20,21). Tumour treatment with anticancer agents might have an effect on the secretion of VEGF. In this study, we sought to determine whether chemotherapeutic agents used in head and neck cancer therapy modulate VEGF release by the tumour cells and whether this possible effect may be reversed by abrogation of VEGF expression by antisense therapy.

Materials and Methods

Cell culture. The different UMSCC cell lines are well-described human HNSCC cell lines obtained from T. Carey (The University of Michigan, Ann Arbour, Michigan, USA). The human carcinoma cell line MDA-1483 is also a well-described HNSCC cell line derived from a tumour of the retromolar trigone region of the oropharynx (The University of Texas M.D. Anderson Cancer Centre, Houston, Texas, USA). Cell cultures were carried out in Falcon petri dishes at 37˚C in a 5% CO_2 fully humidified atmosphere using Dulbecco's modified minimum essential medium (DMEM) (Fisher Scientific Co., Pittsburgh, PA, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics (Life Technologies, Inc. [Gibco BRL], Gaithersburg, MD, USA). For antisense treatment, the medium from the cultures was aspirated and replaced with DMEM containing 5% fetal calf serum (FCS) and antibiotics followed by the addition of oligodeoxynucleotides.

VEGF determination. Secreted VEGF was measured in the supernatant of the cell lines using an ELISA technique (R&D Systems, Wiesbaden, Germany). Cell culture supernatants were collected in sterile test tubes and stored at -20˚C until used. Then, VEGF concentrations were determined by an ELISA technique (R&D Systems). The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against recombinant VEGF165. The specificity of anti-human VEGF antibodies used in the ELISA kit were examined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting (17). According to the manufacturer's directions, each ELISA assay measured 100 μl of supernatant. All analyses and calibrations were carried out in duplicate. The calibrations on each microtiter plate included recombinant human VEGF standards provided in the kit. Optical density was determined using a microplate reader at a wavelength of 450 nm. Wavelength correction was set to 540 nm and concentrations were reported as pg/ml. The coefficient of variation of inter-assay determinations reported by the manufacturer varied from 6.2% to 8.8% when VEGF concentrations ranged between 50 and 1000 pg/ml. The VEGF mRNA level was measured in 7 HNSCC cell lines using RT-PCR (VEGF-CytoXpress Multiplex PCR Kit, BioSource) according to the manufacturer's directions.

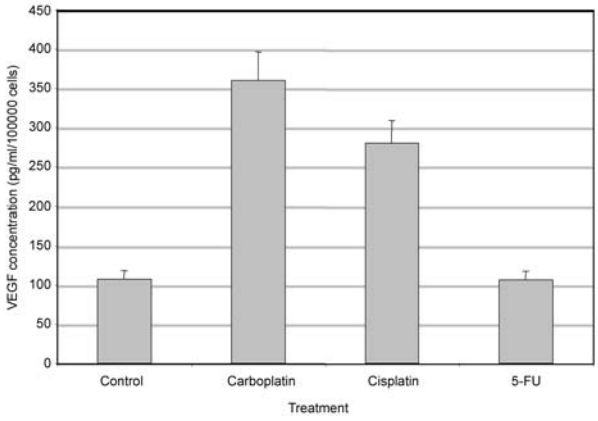

Figure 1. *UMSCC22b cells were pre-treated (2 hours) with medium (control) or medium containing chemotherapeutic agents (Cisplatin, Carboplatin and 5-FU). VEGF secretion was measured in the supernatant by ELISA assay after the cells had been cultured for 48 hours. The treatment of UMSCC22b cells with platinum-based chemotherapeutic agents resulted in a significant induction of VEGF secretion.*

Oligodeoxynucleotides. Phosphorothioated 21-mer oligodeoxynucleotides were synthesized on an Applied Biosystem 394 DNA synthesizer by means of B-cyanothylphosphoramidite chemistry to minimise degradation by endogenous nucleases. The antisense oligonucleotide (5'-CAGAAAGTTCATGGTTTCGGA-3') was directed against the translation start site (AUG codon) and surrounding nucleotides of the human VEGF cDNA. The corresponding sense oligonucleotide was 5'-TCCGAAACCATGAA CTTTCTG-3'. All experiments were performed with 12.5 μM oligodeoxynucleotides, unless otherwise stated.

Chemotherapeutic agents. The effect of chemotherapeutic agents (Cisplatin, Carboplatin and 5-FU) on the production of VEGF was investigated on the human UMSCC22b cell line at both mRNA and protein levels. The tumour cells were plated in DMEM at a density of 10^5 cells / microtiter well in 24-well polystrene plates (Falcon). After 24 hours the cells were rinsed twice with medium and then fresh medium containing the chemotherapeutic agents (concentrations: Cisplatin: 2.5 μg/ml, Carboplatin 90 μg/ml and 5-FU

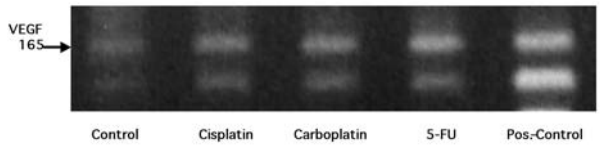

Figure 2. *VEGF mRNA in UMSCC22b cells was measured using a mRNA analysis kit (VEGF-CytoXpress Multiplex.PCR Kit, BioSource). The treatment of UMSCC22b cells with platinum-based chemotherapeutic agents resulted in a significant induction of VEGF up-regulation.*

10 μg/ml) was added for 2 hours. Cells were then washed with PBS and fresh medium as well as medium containing VEGF antisense or sense oligos was added for 48 hours. This medium was collected for VEGF determinations.

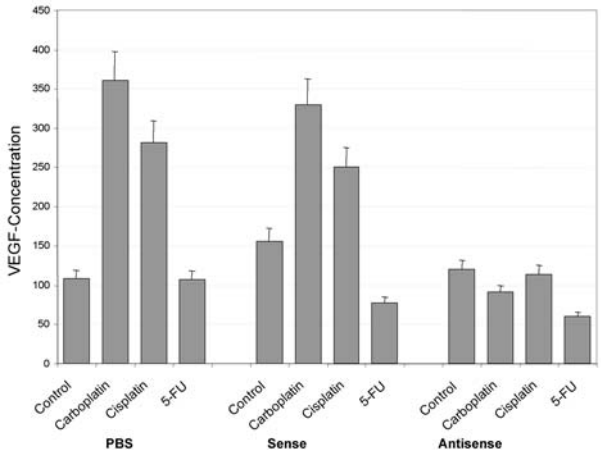

Figure 3. *The treatment of UMSCC22b cells with VEGF antisense oligonucleotide resulted in a significant reduction of chemotherapy-induced VEGF up-regulation compared to sense control.*

Results

VEGF protein was also detectable in the supernatant of all carcinoma cell lines. Values are reported as the means and standard deviation per 10^6 cells of duplicate experiments. Accordingly, RT-PCR for VEGF mRNA exhibited VEGF expression in all HNSCC cell lines. Among the head and neck cancer cell lines, a relatively high level was noted in UMSCC 22b cells (2532 ± 265 pg/ml/10^6 cells). This cell line was chosen for further study.

To quantitate VEGF secretion to the supernatant of HNSCC cell lines treated with medium (control) or medium containing chemotherapeutic agents (Cisplatin, Carboplatin and 5-FU), ELISA was performed. The treatment of UMSCC22b cells with platinum-based chemotherapeutic agents resulted in a significant induction of VEGF production at both the mRNA and protein level (Figures 1 and 2). Carboplatin most prominently induced the release of VEGF from the tumour cells. The treatment of tumour cells with 5-FU showed no effect on the secretion of VEGF.

The treatment of the tumour cells with VEGF antisense oligonucleotide resulted in a significant reduction of chemotherapy-induced VEGF up-regulation compared to the sense control (Figure 3).

Discussion

An adequate vascular response is essential for the initial development as well as the continued growth of solid tumours. Experimental evidence has demonstrated that tumour growth can be stunted by a variety of agents that have the common ability to inhibit angiogenesis (22). Therefore, targeting angiogenesis has become an attractive modality for treating and even preventing the development of malignant neoplasm. Conventional anticancer chemotherapy is thought to have various side-effects on the process of angiogenesis which, in part, might explain the drug resistance of many tumours. Miller *et al.* recently reviewed the literature on the issue of the anti-angiogenic activity of chemotherapeutics *in vitro* and found that most agents tested (a multitude of concentrations have been used), including paclitaxel, cyclophosphamide and doxorubicin, suppress 'angiogenesis' in terms of endothelial cell proliferation, migration and tubule formation. On the other hand, agents such as cisplatin and fluorouracil have demonstrated anti-angiogenic activity only in one or two of these *in vitro* assays. Relatively few chemotherapeutics have been shown to affect angiogenesis *in vivo*, however (23,24). Even so, several traditional cytotoxic agents have anti-angiogenic activity independent of their effects on the tumour cells. Anti-angiogenic therapy has been called "a treatment resistant to resistance" (25), and yet such chemotherapy has manifestly failed to cure most solid tumours. The reason for this is still unclear, though several potential mechanisms of resistance to anti-angiogenic chemotherapy should be considered. The complex paracrine microenvironment of the tumour provides a natural escape from total destruction by cytotoxic chemotherapy. Chemotherapy would enhance the hypoxia of tumour cells (26). Tumour hypoxia has long been recognised as an important mechanism of radiation resistance, but the importance of hypoxia to chemotherapy resistance is less well studied. VEGF is secreted in response to a variety of stresses, particularly hypoxia (27). So residual tumour cells might up-regulate the expression of VEGF during chemotherapy. To our knowledge, this is the first report demonstrating that various chemotherapeutic agents up-regulate angiogenic VEGF secretion activity in head and neck cancer cell lines.

An increased VEGF production in areas of tumour hypoxia may stimulate brisk angiogenesis, essentially rescuing areas of tumour that are sublethally injured. In an *in vivo* model with rat 13,762 mammary carcinomas, treatment with cyclophosphamide resulted in tumour hypoxia with increased VEGF production and increased tumour CD31 staining detectable within 24 hours (28). However, tumour regrowth and increase in tumour cell proliferation was not associated with an increase in endothelial cell proliferation. That is, the endothelial cell labelling index decreased with cyclophosphamide treatment and remained low despite obvious tumour regrowth (29). These results suggest that cyclophosphamide decreased endothelial proliferation without affecting the existing tumour vasculature, thereby allowing tumour regrowth without further angiogenesis. Besides that, it has been demonstrated that VEGF itself induces resistance of endothelial cells to diverse anticancer drugs including taxol, cisplatin and mytomycin C (30). The mechanism underlying the multidrug resistance phenotype of endothelial cells induced by VEGF is still unknown.

As the inhibition of angiogenesis of malignant tumours has become an important strategy to be considered in novel approaches to cancer therapy, this prompted us to investigate the effect of VEGF antisense oligonucleotides on the chemotherapy-induced up-regulation of VEGF. The objective of antisense treatment is to specifically inhibit the expression of a particular gene product. The possible mechanism of action of antisense oligonucleotides includes the inhibition of transcription or translation and mRNA degradation through an RNase H cleavage mechanism (31,32). In a previous study, we were able to show that treatment of HNSCC cells with VEGF antisense oligonucleotides *in vitro* efficiently down-regulated VEGF secretion. Although the growth rate of the tumour cell lines was not affected, addition of conditioned medium from VEGF antisense-treated tumour cells resulted in a decrease of endothelial cell proliferation and migration (33). Our results are in line with other recent *in vitro* studies showing down-regulation of VEGF expression by VEGF antisense oligonucleotides in normal human keratinocytes (34) and in human glioma cells (35). In addition, we were able to show that VEGF antisense oligonucleotide treatment of HNSCC xenografts resulted in a significant tumour growth suppression (33). Accordingly, antisense oligonucleotides targeted to murine VEGF have recently been shown to inhibit retinal neovascularisation in a murine model of proliferative retinopathy (36). Taken together, these results suggest that antisense VEGF oligonucleotides may have a potential therapeutic role in the treatment of head and neck cancer. Antisense therapeutics have already been suggested for many clinical applications such as viral infections (human immunodeficiency virus, human papilloma virus, hepatitis C virus and human cytomegalovirus) and acute myelogenous leukaemia or myelodysplastic syndrome (37). In recent studies, antisense oligonucleotides have been tested for toxicity and clinical activity in a phase I evaluation in patients with advanced cancer (38).

In the present study, we have demonstrated that VEGF antisense oligonucleotide treatment of HNSCC cells resulted in a significant reduction of chemotherapy-induced VEGF up-regulation. Our results suggest evidence that platin-based chemotherapy regimens might be combined with VEGF-targeting strategies for improved anti-tumour effect. Established drug-resistant tumours have already been investigated showing complete regression following combined chemotherapeutic and anti-angiogenic therapy (TNP-470 or monoclonal neutralising antibody targeting the type 2 receptor for VEGF) (24). This strategy of combined treatment with one or more chemotherapeutic drugs and one or more anti-angiogenic agents could be the future therapeutic anticancer strategy of choice, especially as it minimises or delays problems with host toxicity and acquired drug resistance (39).

Acknowledgements

The authors would like to thank Petra Prohaska, Department of Otolaryngology, Mannheim, for her excellent technical assistance. This work was supported by the Research Fund of the Faculty of Medicine Mannheim, University of Heidelberg, Germany and the Else-Kröner-Fresenius-Stiftung, Germany.

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Received February 6, 2004
Accepted May 10, 2004