Impact of vascular endothelial growth factor release on radiation resistance

JUERGEN BRIEGER[1,2], JANA KATTWINKEL[2], MANFRED BERRES[3], JAN GOSEPATH[2] and WOLF J. MANN[2]

[1]Department of Otorhinolaryngology, Head and Neck Surgery, Molecular Tumor Biology Laboratory, University Hospital of Mainz, Langenbeckstr. 1, 55101 Mainz; [2]Department of Otorhinolaryngology, Head and Neck Surgery, Mainz Medical School, Mainz; [3]Department of Mathematics and Technology, RheinAhrCampus Remagen, Remagen, Germany

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Abstract. There is increasing evidence of an angiogenic response of irradiated tumors resulting in decreased radiation sensitivity. However, little is known about the contribution of tumor vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF)-release induced by irradiation to the individual level of resistance. In this *in vitro* study, we analysed the VEGF- and bFGF-release of six epithelial tumor cell lines before and after irradiation and correlated these data to the corresponding irradiation resistance. Two head and neck squamous cell carcinoma (HNSCC), two renal cell carcinoma (RCC), and two ovarian cancer (OC) cell lines were each exposed to 2 or 6 Gy single dose using a ^{137}Cs-source. Non-irradiated controls were processed in parallel. Survival rates were assessed by colony assays as a measure of resistance. The released VEGF and bFGF was quantified by ELISA assays. Additionally, the expression of VEGF and its respective receptors (FLK, FLT, and NRP1) was visualized by immunohistochemistry. VEGF-release was significantly increased ($p<0.05$) in all cell lines after irradiation. Release was most prominent in the RCC cell lines, less in the HNSCC cell lines and lowest in the OC cell lines. Radiation resistance correlated to the absolute level of released VEGF after irradiation as well as to its relative increase ($r>0.9$, $p<0.01$). bFGF levels were not correlated to resistance. VEGF and all three VEGF-receptors were detected in all cell lines analyzed supporting the concept of an autocrine protective mechanism. We suggest that tumor cell survival after irradiation may be enhanced by released VEGF and that the level of released VEGF directly corresponds to the resistance of the tumor to irradiation.

Correspondence to: Dr Juergen Brieger, Department of Otorhinolaryngology, Head and Neck Surgery, Molecular Tumor Biology Laboratory, Building 102, 2nd floor, Part D, University Hospital of Mainz, Langenbeckstr. 1, 55101 Mainz, Germany
E-mail: brieger@mail.uni-mainz.de

Abbreviations: VEGF, vascular endothelial growth factor; HNSCC, head and neck squamous cell carcinoma; RCC, renal cell carcinoma; OC, ovarian cancer

Key words: vascular endothelial growth factor, radiation resistance, epithelial cancer

Introduction

Radiotherapy is a common treatment strategy in epithelial tumors, however in some cases success is limited because of radiation resistance (1). Induction of proangiogenic mediators and their receptors in irradiated tumors has recently been reported (2-5). Besides angiogenic activities, activation of the respective growth factor receptors mediates radioprotective responses of endothelial and tumorous cells (2,3,6). Particularly, the involvement of VEGF (*vascular endothelial growth factor*) and bFGF (*basic fibroblast growth factor*) has been discussed (2,3,5,7,8).

Basically, three VEGF-receptors have been recognised so far: FLT/VEGF-R1, KDR/FLK/VEGF-R2 and Neuropilin-1 (9-12). All three receptors bind VEGF, but with different affinity depending on the VEGF-subtype. VEGF-receptors have been described in endothelial cells and in several tumor types, e.g., squamous cell carcinoma of the head and neck (HNSCC), ovarian cancer (OC), and renal cell carcinoma (RCC) (6,13-16). The role of these receptors on tumor cell physiology is not yet fully understood, however, they have shown to be functional (6,14). bFGF is a member of the large family of the heparin-binding fibroblast growth factors (FGFs). bFGF mediates its numerous biological activities by binding to either one of the two receptors FGFR1 and FGFR2 (17).

The level of released VEGF or bFGF prior to and during irradiation therapy might be relevant for successful treatment as these factors may contribute to irradiation resistance of epithelial tumors. However, only limited data are available concerning the relation of VEGF released in response to irradiation and radiation resistance of an individual tumor species. Therefore, in the present study we analysed the VEGF- and bFGF-levels before and after irradiation of six cell lines derived from three common epithelial tumor entities to

their individual level of irradiation resistance measured by colony assays.

Materials and methods

Tumor cell lines. HNSCC cell lines (DePt und Hun) were previously established and characterized in our laboratory (18,19). The RCC cell lines A-498 und 786-0 were obtained from the DSMZ, Braunschweig, Germany and LGC-Promochem-ATCC, Wesel, Germany, respectively. The OC-cell lines EFO-21 and EFO-27 were purchased from the DSMZ.

Cells were maintained in DMEM/Ham's F12 (PAA, Linz, Austria), supplemented with 5% FCS (Greiner, Frickenhausen, Germany), and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml, (PAA, Laboratories, Cölbe, Germany) at 37˚C in 5% CO_2.

Colony assay. Monolayers (80% confluence) of the tumor cell lines were dispersed by tryptic digestion (Biochrom, Berlin, Germany) and re-suspended in 4 ml (200 cells/ml) EBM supplemented with 1.5% FCS and antibiotics (PAA). Cells were irradiated immediately with 2 and 6 Gy using a γ source (Cs^{137}). Mock-irradiated cultures were processed in parallel. Cells were seeded into tissue culture dishes and cultured for 7-9 days. After fixation using ethanol/acetone (50%, v/v), staining with 10% giemsa (Sigma, St. Louis, MO, USA) was performed.

Colony formation was defined as a colony of ≥50 cells. Each experiment was performed in duplicate and repeated 5 times.

Colony counts were used to establish dosage/survival curves. The mock irradiated cultures (=0 Gy) were set as 100% surviving cells. We performed two linear interpolations between mean survival at 0 and 2 Gy and at 2 and 6 Gy to establish the individual dosage/survival curves. The obtained equations were used to calculate the dosage resulting in 50% surviving cells [='surviving fraction' (ED50)].

Quantification of VEGF. Tumor cell lines were isolated by trypsin digestion (Biochrom), stained with trypan blue (Sigma, Taufkirchen, Germany) and counted. Cells (10^5) (in 2 ml) were seeded per well. After 24 h, cells were γ-irradiated with 30 Gy using a Cs^{137} source. At this intensity in previous experiments the strong and reliable quantifiable induction of a radiogenic response was shown (5). After 72 h, cells were harvested, counted, and the supernatants collected for later ELISA-analysis. Controls were not irradiated but processed in parallel. Cell culture media were changed daily. Experiments were repeated eight times for each cell line and each were performed in duplicate.

The concentrations of released VEGF protein in the cell culture supernatants were quantified by ELISA (DuoSet ELISA Development System, R&D Systems, Abingdon, UK) following the manufacturer's instructions. We calculated the VEGF-release for 10^5 cells.

Immunohistochemistry. Analysis of tumor cell lines was performed by standard procedures. In brief, $7x10^4$ cells/ml were seeded in chamber slides (LAB-TEK®, Nalge Nunc Int. Naperville, USA), cultured for 24 h and fixed by icecold acetone followed by endogenous peroxidase blockage with 3% H_2O_2/methanol. After pre-incubation with 10% normal serum in 2% albumin bovine/PBS for 20 min to avoid unspecific binding, primary antibodies were overlaid overnight at 4˚C. Slides were incubated with biotinylated secondary antibody, and streptavidin peroxidase. All washing procedures were performed in PBS. Slides were counterstained by haema-toxylin. We determined the expression of VEGF, the VEGF receptors 1 and 2 and of the third VEGF-A receptor, Neuropilin-1 (NRP1). Sections of normal kidney served as positive controls and sections incubated without the primary antibody as negative controls (data not shown). All samples were stained in parallel by the same person for better comparability. For sources and working dilutions used refer to Table I.

Statistics. A potential association of survival (ED50) and release of VEGF or bFGF was analysed using Kendall's rank correlation coefficient τ. Comparison-wise p-values are shown and adjusted by Bonferroni's procedure for multiplicity.

Table I. Used antibodies and dilutions.

1st Antibody	Company	Concentration	2nd antibody	Concentration
VEGF (Polyclonal goat antihuman)	R&D, Germany	1:100	Biotinylated rabbit immunoglobulin	1:250
Neuropilin-1 (Polyclonal goat antihuman)	Santa Cruz, Santa Cruz, USA	1:100	Biotinylated rabbit immunoglobulin	1:250
VEGF-R1 (Polyclonal rabbit antihuman)	Santa Cruz, Santa Cruz, USA	1:100	Biotinylated goat immunoglobulin	1: 250
VEGF-R2 (Monoclonal mouse antihuman)	Santa Cruz, Santa Cruz, USA	1:100	Biotinylated goat immunoglobulin	1: 250

Table II. Release and survival of the analyzed cell lines.

A, VEGF

Cell line	Entity	Survival ED50 (Gy)	Basic VEGF release (pg/ml)	VEGF release after irradiation (pg/ml)	VEGF increase (%)
DePt	HNSCC	1.68±0.61	77±17	526±278	+583
Hun	HNSCC	1.92±0.51	99±48	835±251	+743
A498	RCC	2.21±0.43	209±79	2040±1076	+876
786-O	RCC	2.88±0.98	170±103	2580±1046	+1418
EFO-21	OC	1.68±0.19	31±6	135±40	+335
EFO-27	OC	1.41±0.14	142±34	456±99	+221

Absolute (± SD) and percentage increase of released VEGF is correlated to the survival (± SD) of tumor cells after irradiation.

B, bFGF

Cell line	Entity	Survival ED50 (Gy)	Basic bFGF release (pg/ml)	bFGF release after irradiation (pg/ml)	bFGF increase (%)
DePt	HNSCC	1.68±0.61	3.2±1.2	757±233	+23556
Hun	HNSCC	1.92±0.51	1.6±1.2	203±129	+12588
A498	RCC	2.21±0.43	1.0±0.8	73±32	+7200
786-O	RCC	2.88±0.98	1.9±1.5	82±35	+4216
EFO-21	OC	1.68±0.19	3±1.6	25±9.7	+733
EFO-27	OC	1.41±0.14	1.3±0.4	10±3.2	+669

bFGF-release (absolute ± SD) and percentage increase is not correlated to the survival (± SD) of tumor cells after irradiation.

Results

Increased VEGF and bFGF release after irradiation. We found strongly increased levels of released bFGF and VEGF after irradiation of the six analysed cancer cell lines. Increases ranged from 104 pg/ml in the EFO-21 cell line up to 2410 pg/ml in the 786-O cell line for VEGF and from 9 pg/ml in cell line EFO-27 up to 754 pg/ml in the cell line DePt for bFGF. The VEGF increase was most prominent (absolute and percentage) in the two RCC cell lines, less in the HNSCC and lowest in the ovarian cancer cell lines (Table IIA). The bFGF increase was highest in the HNSCC cell lines, less in the RCC cell lines and again lowest in the OC cell lines (Table IIB).

Resistance to irradiation-induced cell death. We then analyzed resistance of the tumor cell lines to irradiation-induced cell death as previously described. The calculated dosage resulting in 50% surviving cells (ED50) was used as measurement of the individual resistance level. Lowest ED50 levels were found in the OC cell lines followed by the HNSCC cell lines and the RCC cell lines with the highest levels indicating highest resistance (Tables IIA and B).

Survival of irradiated tumor cells correlates to released VEGF. We correlated the bFGF and VEGF-release and the respective ED50 values of each cell line. We wanted to test the hypothesis that the level of irradiation-induced growth factor release is associated to and therefore is a measure for the individual irradiation resistance of the respective cell line. We found a strong correlation of the individual increase of VEGF expressed in absolute values (p=0.022) as well as in percentage values (p=0.007, Fig. 1C and E) the latter being significant on the 5%-level after Bonferroni's adjustment. The VEGF-level prior to irradiation and the bFGF levels before and after irradiation were not predictive (Fig. 1 A,B,D,F).

Expression of VEGF, FLK-1, FLT-1 and NRP1 by immunohistochemistry. The expression of VEGF receptors is a prerequisite for an autocrine stimulating mechanism by released VEGF. We found VEGF and the three receptors expressed on all six cell lines analysed, independent of the entity (Fig. 2).

Discussion

The release of angiogenic growth factors like VEGF and bFGF have been recognized as part of the radiogenic response of epithelial tumors (2,5-8,20). Moreover, it is increasingly accepted that endothelial cells might be protected from cellular distress by these factors. Protection of tumor vessels by VEGF could thereby contribute to the radio resistance of tumors. Far less is known about the cytoprotective activities of these factors on the tumor cells themselves. However, we and others previously reported that tumor cells, like the endothelial cell linings of tumor vessels, might be protected by angiogenic factors (2,5,6).

We hypothesized that the differences of radiation sensitivity between the tumor entities may be partly explained

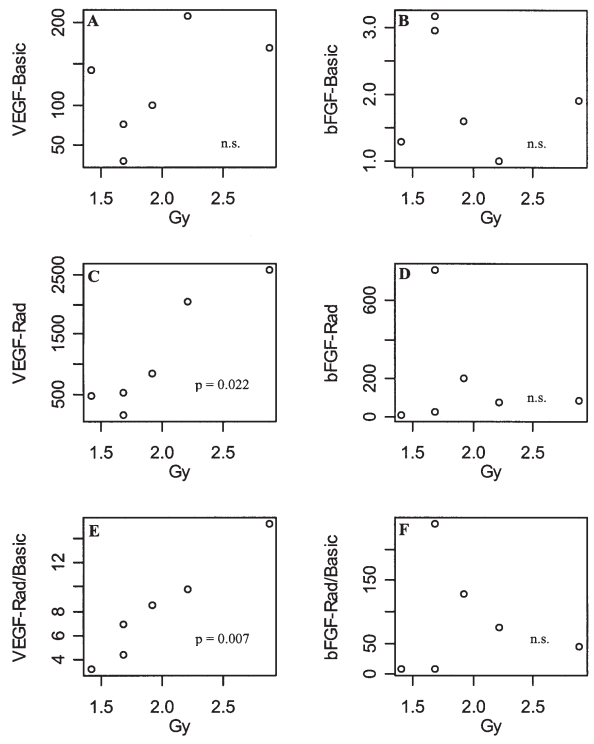

Figure 1. Correlation of VEGF and bFGF-expression and irradiation resistance. In the culture media released, VEGF and bFGF were measured before and after irradiation of the individual cell lines. These levels and the calculated percentage increases were then correlated to the colony assays to define individual irradiation resistance. Kendall's τ for correlation between ED50 for survival and release of VEGF after irradiation was 0.83 (C, exact p=0.022) and for percentage increase of VEGF after irradiation was 0.97 (E, p=0.007). Correlations for other release variables (A,B,D,F) were between -0.14 and 0.41 with p-values >0.25.

by their specific bFGF or VEGF-expression levels or their ability to release one of these factors after irradiation. For example, RCC are known to be highly vascularised tumors but are strongly radiation resistant. These tumors release high levels of VEGF resulting in the observed high blood vessel densities (21,22). The high VEGF levels may additionally contribute to blood vessel and tumor cell protection as a cause of radiation resistance. In our present study, we found that survival of all six investigated cell lines is strongly correlated to the induced and released VEGF after single dose irradiation, but not to the VEGF-levels prior to irradiation. bFGF levels were not correlated to tumor cell survival. Moreover, we found that all three VEGF-receptors are expressed on the epithelial cell lines analyzed, indicating that they are potential targets of VEGF-stimulation. Collectively, these observations support the hypothesis of a VEGF-mediated protective mechanism against radiogenic effects.

One might criticize that exposure to a single dose of radiation in our *in vitro* study does not reflect the *in vivo* situation with therapeutical dosages being applied in a fractionated manner. This is undoubtedly true, however, *in vivo*, applying a fractionated protocol, a more effective protection can be expected compared to the *in vitro* setting with a single exposure. After the first irradiation, apoptosis and VEGF release are induced. Fractionated irradiation of cell cultures results in strongly enhanced cell death as a limitation of the *in vitro* model. However, *in vivo* each subsequent irradiation and the respective VEGF-release potentially further enhances the cytoprotective effect. Therefore, we assume that our *in vitro* data with a single fraction rather underestimate the expected cellular *in vivo* response.

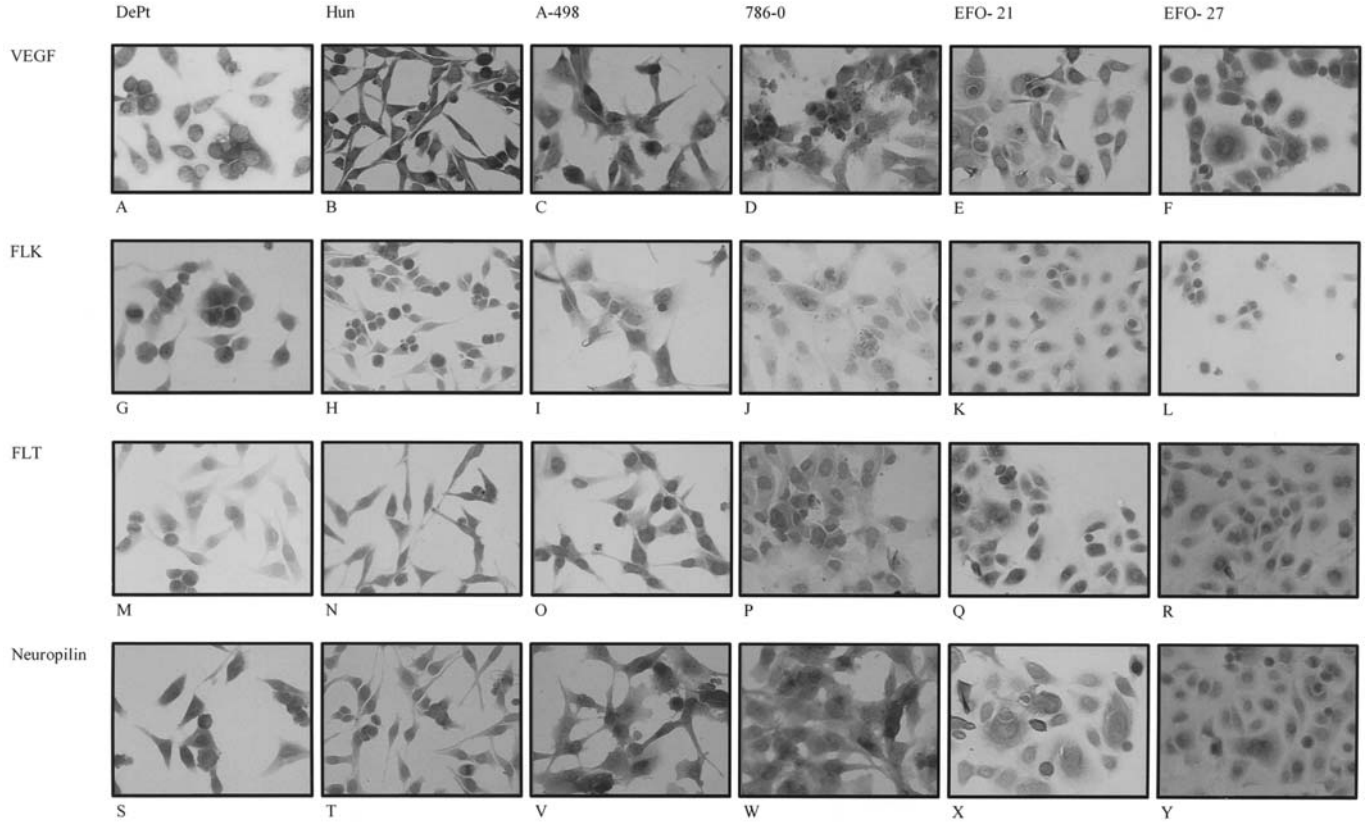

Figure 2. Immunohistochemical detection of VEGF and the VEGF-receptors FLK, FLT and Neuropilin. Stainings of the six analyzed cell lines are shown. All tumor cell lines showed positive stainings for VEGF and the three VEGF-receptors. Magnification (x400).

Following our data, if the tumor VEGF-response at an early stage of irradiation is correlated to irradiation resistance *in vivo*, the measurement of this mediator could gain predictive value for the response to a radiation protocol. Moreover, the tumors with inducible high endogenous VEGF levels can be classified as prime candidates for combined anti-angiogenic/irradiation therapy protocols (23,24).

In conclusion, we believe that the release of VEGF by epithelial tumor cells after irradiation is a common response mechanism. We now report that tumor cell survival is strongly correlated to the inducibility of VEGF by irradiation and suggest that the induction of VEGF may result in the direct protection of cancer cells. Therefore, we recommend further evaluation of VEGF-monitoring as a potential prognostic marker of individual radio-sensitivity of malignant epithelial tumors. Testing of VEGF-levels could be helpful in defining suitable candidates for a combined anti-angiogenic/radiation-protocol.

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