Hypoxia-induced epithelial VEGF-C/VEGFR-3 upregulation in carcinoma cell lines

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Abstract. Adaptation to hypoxia, a universal hallmark of carcinomas, is a critical step for tumor cell survival and growth. One of the principal regulators of hypoxia-responsive pathways is the transcription factor hypoxia-inducible factor-1α (HIF-1α). Currently, it is known that tumoral production of members of the vascular endothelial growth factor (VEGF)-family (VEGFs) may promote tumor growth and progression by acting on carcinoma cells that express the cognate receptors (VEGFRs). However, the influence of hypoxia in the formation of such a tumoral VEGF/VEGFR loop is not completely understood. In the present study we examined the potential existence of a HIF-1α/VEGF/VEGFR autocrine loop on commonly occurring carcinomas. The experiments were performed on five colorectal carcinoma cell lines, one breast (MCF7) and one lung (A549) adenocarcinoma cell line under normoxic and oxygen stress conditions using HIF-1α-EIA, VEGFs-ELISA as well as RT-PCR and immunofluorescence for VEGFRs. HIF-1α overexpression was found already after 2 h of exposure to hypoxia in all above mentioned cell lines, thus documenting that activation of the transcription factor HIF-1α is an early cellular event. Under hypoxic conditions a significant upregulation and activation of HIF-1α accompanied by an increased production of VEGF in MCF7 and A549 was observed. The well-differentiated colorectal carcinoma cell lines were ‘hypoxia-resistant’ showing unchanged levels of HIF-1α and VEGF under hypoxia. None of the cell lines used in this study expressed the VEGF receptors VEGFR-1 and VEGFR-2 under normoxia and hypoxia. Additionally, all colorectal carcinoma cell lines were negative for VEGFR-3 transcripts in both conditions. However, VEGFR-3 mRNA and protein were expressed and under hypoxia overexpressed in MCF7 and A549. Hypoxic cultures of both cell lines secreted in elevated levels the VEGFR-3 ligand VEGF-C but not VEGF-D. Our findings suggest that under hypoxic conditions an autocrine loop between VEGF-C/VEGFR-3 and HIF-1α is possible in breast carcinoma and lung carcinoma but not in colorectal carcinoma cell lines.

Introduction

Tumor cells must survive in abnormal environmental conditions characterized by hypoxia (1,2). The oxygen tension within many solid tumors such carcinomas is substantially less compared to the adjacent normal tissue. In this stress situation a cell selection takes place in favour of the most aggressive cells. In fact, some tumor cells die by apoptosis/necrosis whereas others adapt to the hypoxic conditions by a series of compensatory mechanisms initiating tumor growth and aggressiveness by promoting cell proliferation, invasiveness and angiogenesis. The exact pathomechanisms facilitating the survival of invasive tumor cells are still unclear.

A key regulatory system during hypoxia is the transcription factor, hypoxia inducible factor-1 (HIF-1) (3). HIF-1 is a heterodimer composed of one of the three α subunits (HIF-1α, HIF-2α or HIF-3α) and one HIF-1ß subunit. Although HIF-1ß is constitutively expressed, hypoxia-mediated responses are determined by HIF-1α subunits. Under hypoxic conditions HIF-1α translocates to the nucleus and binds to HIF-1 forming the active HIF-1 complex, which induces target gene expression mostly in a cell-type-specific manner. The product of one of these genes is the vascular endothelial growth factor (VEGF also referred to as VEGF-A), a highly potent pro-angiogenic (paracrine) and pro-survival (autocrine) mediator originally of endothelial cells (4-6). Under hypoxic conditions tumor cells have also been shown to secrete elevated levels of VEGF in a paracrine manner in order to attract and stimulate proliferation of endothelial cells, thus promoting angiogenesis. Interestingly, it becomes more and more apparent that VEGF can also act in an autocrine fashion as a survival factor for the tumor cells themselves and enable their own regulation of survival, growth and progression (7). The biological effects of VEGF are mediated by two related receptor tyrosine kinases, VEGFR-1...
and VEGFR-2 (8). vegf and vegfr-1 but not vegfr-2 genes harbour consensus HIF-binding sites in their promoter/enhancer regions (4,9). So far, only a few expression analyses of both receptors and their ligand VEGF have been conducted in neoplastic cells of different tumor types under hypoxia. In colorectal carcinomas VEGF-positive cases tended to have increased expression of VEGFR-2, which was significantly associated with relatively low systemic oxygenation and tumor progression (10). The VEGF/VEGFR-2 loop was found to be related with HIF-1α expression and poor prognosis in endometrial carcinomas (11). Das et al have shown, that a hypoxia-driven VEGF/VEGFR-1 autocrine loop interacts with HIF-1α in neuroblastoma and is required for cell survival, drug resistance and angiogenesis (12).

Two other members of the VEGF family (VEGFs), VEGF-C and VEGF-D stimulate growth of vascular and lymphatic endothelial cells by signaling through the tyrosine kinase receptors VEGFR-2 and VEGFR-3 (13). Both factors are upregulated in many malignant tumors, including colorectal, breast and lung carcinomas (14-16). VEGF-3 is mostly found on the lymphatic endothelium but it is also expressed in a variety of human malignancies (17). Recent data provide evidence for a possible role of HIF-1α in tumor lymphangiogenesis by regulating the lymphatic vascular endothelial growth factors VEGF-C in breast ductal carcinomas and esophageal squamous cell to harbour a hypoxia-responsive sequence (21). Until now the role of hypoxia-induced VEGF-D has only been investigated in breast cancer (19,22). Thus, Currie et al reported a significant HIF-1α/VEGF-D correlation in neoplastic human breast tissues (22).

Tumor cells represent a major source of VEGFs, which may form an autocrine link with VEGF receptors (VEGFRs) expressed on tumor cells themselves. Although during hypoxia such an autocrine VEGF/VEGFRs and HIF-1α loop may contribute to cell survival it is still practically unknown whether this possible link exists in diverse carcinomas. The aim of this study was to investigate the potential existence of a HIF-1α/VEGFs/VEGFRs axis in vitro in common occurring carcinomas, especially in colorectal carcinomas. For this reason, we examined the expression patterns of these three components on five colorectal carcinoma cell lines, one breast (MCF-7) and one lung (A549) adenocarcinoma cell line under normoxic and hypoxic conditions.

Materials and methods

Cell culture. Tumor cell lines (colorectal carcinoma cell lines-SW620, SW837, SW948, CX-1 and HRT18-as well as the breast carcinoma cell line MCF7 and the lung carcinoma cell line A549) were grown in RPMI-1640 medium supplemented with Glutamax (Sigma), 10% heat deactivated FCS (Gibco), line A549) were grown in RPMI-1640 medium supplemented with Glutamax (Sigma), 10% heat deactivated FCS (Gibco), breast carcinoma cell line MCF7 and the lung carcinoma cell lines SW620, SW837, SW948, CX-1 and HRT18-as well as the breast adenocarcinoma cell line MCF-7 and the lung carcinoma cell line A549). Semiquantitative reverse transcription-PCR. RNA isolation was performed using the RNeasy Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions (R&D Systems, Wiesbaden, Germany). β-actin and VEGFR transcripts were analyzed by RT-PCR. RT was performed with the help of the Omniscript RT Kit (Qiagen) in accordance with the manufacturer's manual. As a template for the specific PCR reactions 2 μl of the cDNA-pool synthesized was used. To amplify the β-actin specific fragment, 574 base pair (bp), the primers 5'-GAC CTG ACT GAC TAC CTC ATG A-3' (forward) and 5'-AGC ATT TGC GGT GGA CGA TGC AG-3' (reverse) were used. Amplification of the human VEGFR-1 specific fragment, 441 bp, was performed using the primers 5'-GCA CCT TGC TGG TGG CTG A-3' (forward) and 5'-GAG TTC CGA GGA GATG ATG GTG-3' (reverse). Amplification of the human VEGFR-2 specific fragment, 473 bp, was performed using

Quantification of HIF-1α protein by enzyme immunoassay (HIF-1α-EIA). After exposure to the different culture conditions (normoxia, hypoxia and anoxia) the cells were fixed in buffered 3.7% paraformaldehyde for 15 min at room temperature. After three washes with PBS the tumor cells were permeabilized with PBS-buffered 0.1% Triton X-100 (for 5 min at room temperature) and incubated with blocking buffer (Boehringer Mannheim, Germany) containing 1% H2O2 at 37°C for 30 min. Subsequently, monoclonal anti-HIF-1α (IgG1, BD Transduction Laboratories, Heidelberg, Germany) was added at a dilution of 1:100 for 60 min. After three washes with PBS the cells were exposed to the second antibody, biotinylated goat-anti-mouse IgG1 (Amersham, Freiburg, Germany) at a dilution of 1:1000. Using a biotin-streptavidin-system (Amersham) an increased sensitivity was achieved. The color reaction test was performed using peroxidase-catalyzed o-phenyldiamine. The reaction was stopped by the addition of 3 M HCl. Light extinction was determined with a computer-controlled microplate reader at 492 nm. We repeated the experiments five times, to ensure the reproducibility of results.

Immunfluorescence. Tumor cells were seeded onto fibronectin-coated glass chamber-slides (LabTek II CC2, Nune, Wiesbaden, Germany). After growing to subconfluence cells were exposed to the specific cell culture conditions (normoxia, hypoxia and anoxia). After exposure time cells were fixed with buffered 3.7% paraformaldehyde (15 min, room temperature). Mouse monoclonal anti-HIF-1α (BD Transduction Laboratories) and rabbit polyclonal anti-VEGFR-3 (Chemicon) were used as primary antibodies. Alexa Fluor 488 goat anti-mouse and donkey anti-rabbit (Eugene, OR, USA) were used as secondary antibodies. Nuclear staining was performed with Hoechst 33342 (Sigma). Fluorescence labeled cells were covered with GelMount (Biomedia Corp/Natutec, Frankfurt, Germany).

VEGFs-ELISA. After an incubation time of 24 h under the different culture conditions (normoxia-hypoxia) the supernatants of the 96-well plates were harvested and VEGF-A, VEGF-C and VEGF-D ELISA was performed according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany).
the primers 5′-TAT GTC TAT GTT CAA GAT TAC-3′ (forward) and 5′-AAG TTT CTT ATG CTG ATG CTT-3′ (reverse). Amplification of the human VEGFR-3 specific fragment, 290 bp, was performed using the primers 5′-GCG ACA GGG TTCT CAT AA-3′ (forward) and 5′-CGT TGC CTC ATT GTG ATT AG-3′ (reverse). Amplification reactions were performed with the help of the Gene Amplification PCR System 2400 (Perkin Elmer, Norwalk, CT, USA) thermocycler. The PCR products were separated on agarose gels (2%) supplemented with ethidium bromide and analyzed by viewing under UV.

Statistical analysis. The analyses were performed using the Ryan-Einot-Gabriel-Welsch multiple range test. Means with different grouping letter (A, B and C) were statistically significant.

Results

Differential HIF-1α expression in diverse carcinomas under hypoxic conditions. One of the main early cellular events evoked upon exposure to hypoxia is the activation of the transcription factor HIF-1α. In this study we analyzed the expression of HIF-1α under different oxygen concentrations (normoxia: 21%, hypoxia: 3% and anoxia: nearly 0% O2) on five colorectal carcinoma cell lines, one breast (MCF7) and one lung (A549) adenocarcinoma cell line. Three colorectal carcinoma cell lines (SW620, SW837 and HRT-18), MCF7 and A549 showed a significant upregulation of HIF-1α under hypoxia and anoxia (Fig. 1). Notably, the effects of both conditions were similar except for HRT-18, in which anoxia resulted in a progressive increase of HIF-1α expression. The HIF-1α levels of two colorectal carcinoma cell lines (CX-1 and SW948) remained unchanged. Consequently, hypoxia leads to a HIF-1α upregulation in malignant cells independent of the histogenetic origin, but in this context, especially in the case of colorectal carcinoma ‘hypoxia-resistant’ cell lines also exist. Considering the kinetics of the expression process we monitored the expression pattern at four different time-points (0, 2, 4 and 8 h after hypoxia exposure) (Fig. 2). A significant upregulation of the HIF-1α level was visible already after 2 h in all ‘hypoxia-sensitive’ cell lines. In three cell lines (HRT-18, SW837 and A549) the elevated levels remained constant 4 and 8 h after hypoxic exposure. In the cell lines SW620 and MCF7, HIF-1α expression was increased gradually with exposure time. The detection of HIF-1α protein by immunofluorescence staining revealed no staining in the normoxic tumor cells (Fig. 3A). After exposure to hypoxia for 2 h a marked nuclear expression of HIF-1α was detectable in ‘hypoxia-sensitive’ cell lines (Fig. 3B-F).

Induction of VEGF expression in diverse carcinomas but not of its receptors VEGFR-1 and VEGFR-2 by hypoxia. The second link of a potential HIF-1α/VEGFs/VEGFRs axis is the tumoral production and secretion of vascular growth factors. Notably, the activation of VEGF gene transcription under hypoxia is mediated by HIF-1 binding to a hypoxia-response element (HRE) within the VEGF promoter, resulting in increased gene transcription (4). Interestingly, VEGF was detectable by ELISA in supernatants from all investigated cell cultures under normoxic conditions, although the amount was small in 6/7 tumor lines (122-273 pg/ml) (Fig. 4). After exposure of MCF7 and A549 to hypoxia or anoxia (3% and nearly 0%) for 24 h VEGF protein levels increased twice to 3-fold in an oxygen concentration-dependent manner.
(273 pg/ml vs. 495 pg/ml under hypoxia vs. 813 pg/ml under anoxia in MCF7, 122 pg/ml vs. 170 pg/ml under hypoxia vs. 245 pg/ml under anoxia in A549). In the colorectal carcinoma cell lines SW620 and SW837 an increased production of VEGF was also observed without significant differences between hypoxia and anoxia (172 pg/ml vs. 236 pg/ml under hypoxia vs. 260 pg/ml under anoxia in SW620, 247 pg/ml vs. 403 pg/ml under hypoxia vs. 445 pg/ml under anoxia in SW837). HRT-18, characterized by relatively high amounts of VEGF in normoxia secreted equal levels in the oxygen withdrawal situation (636 pg/ml vs. 556 pg/ml and 592 pg/ml under hypoxia/anoxia). It should be emphasized that neither CX-1 nor SW948, characterized by lack of HIF-1α upregulation under hypoxia/anoxia, showed modified VEGF production under these conditions (207 pg/ml vs. 248 pg/ml under hypoxia vs. 270 pg/ml under anoxia in CX-1, 230 pg/ml vs. 185 pg/ml under hypoxia vs. 159 pg/ml under anoxia in SW948). These findings suggest that hypoxia leads to a HIF-1α-associated VEGF production in malignant cells independent of the histogenetic origin. Additionally, under hypoxic conditions a significant upregulation of HIF-1α is not necessarily accompanied by an increased production of VEGF, especially in neoplastic cells with relatively high constitutive amounts of native VEGF.

To determine whether VEGF may form an autocrine loop with its receptors VEGFR-1 and VEGFR-2 expressed on tumor cells themselves we examined their transcription using RT-PCR (Fig. 5). As a positive control for VEGFR-1 and VEGFR-2 expression the human colonic microvascular cell line HCMEC was used. All investigated cell lines were negative for VEGFR-1 and VEGFR-2 transcripts independent of oxygen concentration conditions. The quality of the reaction was judged upon amplification of a specific β-actin fragment. As control of the reaction (C) the human colonic microvascular endothelial cells (HCMEC) were used.
Discussion

Adaptation to hypoxia is a critical step for tumor cell survival and growth. Currently, it is known that tumoral production of cytokines of the VEGF-family may promote tumor growth and progression by acting on carcinoma cells that express the cognate receptors. However, the influence of hypoxia in the formation of such a tumoral VEGFs/VEGFRs loop is not satisfactorily investigated. In this context the present study was designed to clarify the potential existence of such a HIF-1α/VEGF/VEGFR autocrine loop in commonly occurring tumors, especially on colorectal carcinomas. Except for the well-differentiated colorectal carcinoma cell line CX-1 constitutive expression of HIF-1α protein was a general phenomenon under normoxic conditions in the investigated tumor lines. However, a simultaneous nuclear localization representing the active form of this transcription factor was not observed. These findings are consistent with the data published by other groups and support the hypothesis that genetic alterations in key suppressors or oncogenes increase synthesis or stability of HIF-1α in cancer cells, which in turn results in its expression in non-hypoxic conditions (23,24). Hypoxia-induced VEGF overexpression mediated by HIF-1α in colorectal, lung and breast carcinoma cells in vitro is well documented (25-29). In accordance with this observation in our study three of the five investigated colorectal carcinoma cell lines, the breast cancer cell line MCF7 and the lung carcinoma cell line A549 showed a significant upregulation and nuclear translocation of HIF-1α under hypoxia. HIF-1α overexpression was found already 2 h after exposure to hypoxia in all above mentioned cell lines documenting that activation of the transcription factor HIF-1α is an early cellular event. With the exception of HRT-18, these cell lines also demonstrated an increased production of VEGF. Thus, under hypoxic conditions a significant upregulation of HIF-1α is not necessarily accompanied by a significant increased production of VEGF, especially if constitutive tumoral VEGF production is relatively high. In the well-differentiated colorectal carcinoma cell lines CX-1 and SW948 hypoxia did not cause HIF-1α overexpression, nor did it provoke VEGF upregulation. HIF-α associated production of large amounts of VEGF in tumor cells cultured...
in hypoxic conditions seems to depend on the degree of differentiation of the tumor cells. In addition, Koshikawa et al have demonstrated that hypoxia markedly induced VEGF production in high-metastatic, highly-malignant lung and breast carcinoma cell lines, compared to low-metastatic, less-malignant ones (29).

Recent studies indicate that VEGFRs that are expressed on tumor cells support VEGF autocrine signaling which in turn promote tumor cell survival and progression in colorectal cancer. Lesslie et al have also found that migration of the colorectal carcinoma cell line HT-29 was promoted by a functional VEGF/VEGFR-1 interaction (30). Bates et al indentified the VEGF/VEGFR-1 autocrine loop as essential for the survival and migration of colon carcinoma cells LIM 1863 and SW837 (31). Notably, VEGFR-2 expression was not seen. In another study VEGFR-1, but not VEGFR-2, was expressed in seven colorectal carcinoma cell lines (32). In these tumor cells VEGF-induced downstream signaling led to significant motility and invasiveness. In contrast, Kim et al have shown a positive expression of VEGFR-2 in four colorectal carcinoma cell lines supporting the presence of an autocrine VEGF/VEGFR-2 growth pathway (33). Since all colorectal carcinoma cell lines used in our study were negative for the VEGFR-1 and VEGFR-2 transcripts under normoxia and hypoxia the formation of an autocrine VEGF/VEGFR-1 or VEGF/VEGFR-2 loop is not possible. Taking the results in vitro published so far it seems that autocrine VEGF/VEGFR-1 and VEGF/VEGFR-2 expression in colorectal carcinomas is cell line specific. Interestingly, contrary to the observations in vitro we and others have demonstrated a positive expression of both receptors in colorectal carcinoma tissue specimens in situ (34-36). Consequently, the in vitro expression pattern of the VEGF/VEGFR components could differ from the situation in vivo and might explain the divergent regulation mechanisms presented in the literature. We suggest that VEGF production by cancer cells is not enough for tumoral VEGFR-1 induction. In this context, VEGF production by immuneinflammatory cells of the tumor microenvironment could contribute to VEGF expression on malignant epithelia in vivo via a paracrine pathway (37). In our study, VEGFR-1 and VEGFR-2 mRNA were also undetectable in A549 and MCF7 cells. The examination of other lung adenocarcinoma cell lines and breast carcinoma cells including MCF7 in vitro provided evidence of positive VEGFR-2 expression (38,39). The conflicting results in the case of MCF7 cells may be due to use of a diverse range of MCF7 lines in different research groups.

VEGFR-3 mRNA and protein were expressed and under hypoxia overexpressed in MCF7 and A549, accompanied by an increased production of its ligand VEGF-C. Notably, MCF7 cells secrete VEGF-C only under hypoxic conditions indicating that hypoxia is an essential stimulating event for an autocrine VEGF-C and VEGFR-3 interaction for this tumor line by inducing VEGF-C production and VEGFR-3 upregulation. Vgfr-3 and vegf-c genes have not been found to harbor consensus HIF-binding sites in their promoter/enhancer regions. Nevertheless, the effect of hypoxia on VEGF-C/VEGFR-3 via the transcription factor HIF-1α might be incompletely elucidated because in recently published data in breast and osophageal cancer a significant correlation between HIF-1α and VEGF-C was found (18-20). In accordance with our results in normoxia, Laakkonen et al have also demonstrated lack of VEGF-C and VEGFR-3 expression in many breast carcinoma cell lines, including MCF7 (40). A549 cells, which are known to have strong invasive activity, expressed more abundantly VEGF-C and VEGFR-3 under hypoxia than under normoxia. The functional significance of the elevated expression levels under hypoxia remains to be elucidated. VEGFR-3 and VEGF-C levels were found to be increased in the highly invasive lung cancer cells in comparison with the cells, which have low invasive activity (41). Based on these observations we assume that hypoxia could enhance cancer cell invasiveness and thereby contribute to tumor progression and aggressive behavior.

In the present study, expression of VEGFR-3 mRNA was not observed in any of the colorectal carcinoma cell lines examined. Our findings of absent VEGFR-3 expression in CRC cell lines are consistent with the data published by Onogawa et al for other human colon carcinoma cell lines (42). However, there are now many studies, including our previous study, demonstrating VEGFR-3 expression in colorectal carcinoma tissue in situ (34,43,44). These controversial results between the in vivo situation and the culture conditions underline once more the pivotal role of the tumor microenvironment in determining the expression of angiogenic factors. The receptor-ligand interactions that orchestrate the tumor progression process is also influenced by components of the intratumoral stromal tissue, including fibroblasts, endothelial cells, tumor-associated macrophages and inflammatory cells, which can express VEGF-C and/or VEGF-D (45-48).

Concerning the tumoral secretion of VEGF-D in MCF7 as well as A549 and other lung carcinoma cell lines no constitutive protein expression was seen in our study and in the analyses of other researchers (49,50). Furthermore, our current findings indicate that hypoxia can not induce VEGF-D production.

In conclusion, the present results demonstrated the presence of HIF-1α-associated VEGF-C production and induction of its receptor VEGFR-3 in breast and lung carcinomas in vitro. The role of such a possible autocrine loop between VEGF-C/VEGFR-3 and HIF-1α for tumor cell survival and progressive behavior remains to be clarified.

References


