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ORIGINAL ARTICLE

Hypoxia and cytokines regulate carbonic anhydrase 9 expression in hepatocellular carcinoma cells *in vitro*

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Abstract

AIM: To study the expression of carbonic anhydrase (CA) 9 in human hepatocellular carcinoma (HCC) cells.

METHODS: We studied CA9 protein, CA9 mRNA and hypoxia-inducible factor-1 alpha (HIF-1α) protein levels in Hep3B cells exposed in different parallel approaches. In one of these approaches, HCC cells were exposed to extreme *in vitro* hypoxia (24 h 0.1% O_2) without or with interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β) stimulation for the same hypoxic exposure time or exposed to normoxic oxygenation conditions without or with cytokine stimulation.

RESULTS: The tumour cell line analysed showed a strong hypoxic CA9 mRNA expression pattern in response to prolonged severe hypoxia with cell-line specific patterns and a marked induction of CA9 protein in response to severe hypoxia. These results were paralleled by the results for HIF-1α protein under identical oxygenation conditions with a similar expression tendency to that displayed during the CA9 protein expression experimental series. Continuous stimulation with the cytokines, IL-1, IL-6, TNF-α and TGF-β, under normoxic conditions significantly increased the carbonic anhydrase 9 expression level at both the protein and mRNA level, almost doubling the CA9 mRNA and CA9 and HIF-1α protein expression levels found under hypoxia. The findings from these experiments indicated that hypoxia is a positive regulator of CA9 expression in HCC, and the four signal transduction pathways, IL-1, IL-6, TNF-α and TGF-β, positively influence CA9 expression under both normoxic and hypoxic conditions.

CONCLUSION: These findings may potentially be considered in the design of anti- cancer therapeutic approaches involving hypoxia-induced or cytokine stimulatory effects on expression. In addition, they provide

evidence of the stimulatory role of the examined cytokine families resulting in an increase in CA9 expression under different oxygenation conditions in human cancer, especially HCC, and on the role of the *CA9* gene as a positive disease regulator in human cancer.

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Key words: Angiogenesis; Carbonic anhydrase 9; Hypoxia; Hypoxia-inducible factor-1 alpha; Oxygen; Radiotherapy; Transforming growth factor-beta; Tumour microenviroment

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INTRODUCTION

Hypoxia influences the behaviour of human tumour cells *via* activation of genes involved in the adaptation to hypoxic stress which represents an important indicator of cancer prognosis and is associated with aggressive tumour growth, metastasis, poor response to treatment and malignant progression[1,2]. Hypoxia-inducible factor-1 (HIF-1) is a multi-subunit protein that regulates transcription at hypoxia response elements (HREs) and is composed of two basic helix-loop-helix proteins: a subunit, HIF-1α, and the constitutively expressed HIF-1β[3,4]. HIF-1 acts as a master regulator of numerous hypoxia inducible genes related to angiogenesis, cell proliferation/survival, and glucose/iron metabolism. Among these genes, carbonic anhydrase 9 (CA9) is one of the most strongly hypoxia-inducible genes[5] and its activity is regulated by HIF-1α under these oxygenation conditions.

CA9 is a unique transmembrane member of the *CA* gene family and is a tumour-associated protein thought to be involved in malignant cell invasion and adhesion. High levels of CA9 expression in a broad range of tumours are strongly related to its transcriptional regulation by hypoxia and high cell density, which appears to be activated by the CA9 promoter[6,7]. Induction by hypoxia occurs *via* the HIF-1 transcription factor, which accumulates in tissue under hypoxic conditions which are often present in growing tumours[8-11].

In addition to hypoxia, other stimulating factors, e.g., hormones and cytokines induce HIF-1 accumulation and activity under normoxia. Moreover, transforming growth factor-beta (TGF-β) regulates the expression of its own converting enzyme, furin, *via* a recently identified HIF-1-regulated gene[12]. The expression of CA9 can only be detected in a few normal tissues, but it is abundant in several tumours, e.g., renal cell carcinoma, cervical, lung, colorectal, bladder and breast carcinomas, adenocarcinoma, hepatocellular carcinoma (HCC), lung, head and neck cancer, cervix and uteri tumours[13-16]. Although the exact mechanisms related to the functional role of CA9 underlying the contribution of TGF-β, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α)[17-20] with the exception of IL-1, are not yet known, especially their role related to tumour progression, it is known that these growth factors influence the accumulation of HIF-1 under normoxic conditions by stimulatory regulation *via* different cytokine pathways. Within this context, while the functional mechanisms related to the IL-1 induced HIF-1α regulation under hypoxia is not yet known, it is known that under normoxia it may be regulated *via* the mitogen-activated protein kinase kinase kinase pathway[21-23]. On the other hand, IL-6 regulates other HIF-1α regulated genes such as vascular endothelial growth factor[24,25], while IL-6 itself is regulated *via* the signal transducers and activators of transcription-Janus kinase pathway, and TNF-α induces HIF-1α expression through 3-phosphoinositide-dependent protein kinase-1-mediated I kappa B kinase beta[26,27] and nuclear factor "kappa-light-chain-enhancer" of the activated B-cells pathway[28] and has been shown to play a positive role in the induction of the HIF-1α regulated genes in human glioma[29,30]. In addition, enhanced levels of TGF-β are a common feature in human tumour cells[31], and TGF-β is also released by infiltrating leukocytes and induces up-regulation of HIF-1α regulated genes[18,32] most probably without the involvement of the bone morphogenetic protein family[33,34] or the Smad family[35-39] pathways, despite the fact that TGF-β uses the Smad pathway which transcriptionally represses (inhibitor of differentiation 1) Ids proteins in epithelial cells. Also, CA9 causes a reduction in extracellular pH, thereby facilitating the breakdown of the extracellular matrix together with up-regulation of the genes involved in invasion and migration[40-43].

The aim of the present study was to investigate CA9 transcriptional regulation in human cancer, especially HCC, under different oxygenation conditions, namely normoxic or extreme hypoxic conditions without or with TGF-β, IL-1, IL-6, and TNF-α stimulation. The results of this experimental series provide further understanding on CA9 transcriptional regulation under different physiological conditions in HCC, and potentially in other human cancer types of identical origin. Also, the stimulatory effect of TGF-β, IL-1, IL-6, and TNF-α which underlie the contribution to HIF-1α protein accumulation under normoxia and extreme hypoxia were investigated, including its transcriptional regulation at the mRNA and protein

expression level of CA9 in Hep3B HCC cells. This also provides the fundamental information necessary for the study of cytokine regulated non-hypoxic HIF-1α regulated CA9 expression in human cancer cells, especially HCC.

MATERIALS AND METHODS

Preparation of the nuclear extracts

Nuclear extracts were prepared as previously described[44-49] with modifications. Cells/mL (5×10^7) was scratched from Petri-dishes by adding 10 mL phosphate buffered saline (PBS) to the cellular film according to previous protocols[28] with minor modifications. A cell line pellet was obtained by centrifugation (Beckman CS-6R) for 4 min at 800 r/min. After two washing steps with PBS, cells were re-suspended in 1 mL PBS, transferred into a 1.5 mL tube and centrifuged at 4 ℃ for 45 s at 14 000 r/min. The cell pellet was re-suspended in 400 μL ice-cold buffer A (10 mmol/L Hepes pH 7.9, 10 mmol/L KCl, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L ethyleneglycoltetraacetic acid (EGTA), 1 mmol/L PMSF, 10 μL complete protease inhibitor cocktail (Roche) and 1 mmol/L DTT) and incubated on ice for 15 min. The cells were lysed by adding 25 μL of 10% NP-40 and homogenized with 10 strokes in a Dounce homogenizer at 4 ℃ followed by centrifugation for 1 min at 14 000 r/min for nuclei sedimentation. Supernatants were carefully removed and regarded as cytoplasmic fractions. Nuclear proteins were extracted by adding 50 μL of buffer C [20 mmol/L Hepes, pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF and 0.1 μL protease inhibitor cocktail (Roche)] and extensively shaking the tubes for 20 min at 4 ℃ in a tube shaker followed by centrifugation at 14 000 r/min and 4 ℃ for 5 min. The supernatant was removed and stored in aliquots at -80 ℃. All steps were performed on ice.

Cell and culture conditions and hypoxia treatment

Early passage Hep3B cell lines from the American Type Culture Collection (ATCC, Rockville, MD, United States) were grown on glass Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, penicillin (100 IU/mL)/ streptomycin (100 μg/mL) and 2 mmol/L L-glutamine. Cells were exposed to 0.1% O_2 for 1, 6 or 24 h in a Ruskinn Invivo2 hypoxic workstation (Cincinnati, OH, United States) as previously described[44-49] for *in vitro* hypoxia. For the reoxygenation experiments, dishes were returned to the incubator following 24 h hypoxia treatment. For cytokine stimulation, cells underwent 16 h of serum starvation before 24 h stimulation under normoxic conditions or under 24 h hypoxia exposure and stimulation with 5 ng/mL TGF-β (240B), IL-1 (200LA), IL-6 (IL-6-206) or TNF-α (210TA) all from R and D Systems, Minneapolis, MN, United States.

Isolation of total RNA from tumour cell lines and tumour tissues

Total RNA was isolated from cultured tumour cells as previously reported[47-49] and described by Kaluzová *et al*[16] including the digestion of contaminating DNA with the provided DNase. Total RNA from tumour tissues was isolated with the nucleospin RNA Ⅱ kit (Promega, Germany).

Determination of CA9 mRNA expression in vitro in HCC cell lines by semi-quantitative reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed using primers designed using published information on mRNA sequences in GenBank (sequence Accession Nos., CA9: NM_001216, β-actin NM_001101 and NM_001530.2 for HIF-1α). An aliquot of 1-5 μg of total mRNA from Hep3B cell lines was transcribed at 42 ℃ for 1 h in a 20 μL reaction mixture using 200 U RevertAidTM M-MuLV RT, oligo(dT)18 primer and 40 U ribonuclease inhibitor (all from Fermentas, Ontario, Canada). The PCR primers were designed in flanking exons with Primer3 software (available online http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi), based on the information indicated above in order to amplify and produce the following: a 342 bp CA9 product, forward primer was 50-ACCCTCTCTGAC ACCCTGTG-30 and reverse primer was 50-GGCTG-GCTTCTCAC ATTCTC-30, and produce a 668 bp amplification product of β-actin, the forward primer (F1) was 5'-CGTGCGTGACATTAAGGAGA-3' and the reverse primer (R1) 5'-CACCTTCACCGTTCCAGTTT-3' and produce a 233 bp amplification product of HIF-1α, the forward primer (F1) was 5'-TTACAGCAGC-CAGACGATCA-3' and the reverse primer (R1) 5' -CCCTGCAGTAGGTTTCTGCT-3'. The PCR was performed with 25-32 cycles with increments of five cycles using PCR systems and reagents acquired from Promega™ (Promega GmbH, Mannheim, Germany) and applied according to the manufacturer's instructions. The PCR products were separated on 1% agarose gels (Sigma-Aldrich, Steinheim, Germany) and visualized by ethidium bromide staining (0.07 μg/mL ethidium bromide; Bio-Rad, Munich, Germany).

Preparation of cell lysates and immunoblotting

Tumour cell lysates were prepared with 0.1 mL RIPA buffer (1X TBS, 1% Nonidet P-40 (Amresco, Vienna, Austria), 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors pepstatin A (1.4 μmol/L), aprotinin (0.15 μmol/L), leupeptin (2.3 μmol/L) and 100 μmol/L PMSF (all from Sigma, St. Louis, MO, United States). To inhibit protein dephosphorylation, phosphatase inhibitor mix (Sigma) was added. Using a syringe fitted with a 21-gauge needle to shear DNA, the lysates were transferred to a prechilled microcentrifuge tube, followed by 30 min incubation on ice. The cell lysate clearance was by centrifugation at 15 000 × *g* for 12 min at 4 ℃. Whole-cell lysates (20 μg) were separated on 8% polyacrylamide SDS gel. Electrophoresis was then transferred to a 0.45 μm nitrocellulose membrane (Protran BA 85, Schleicher

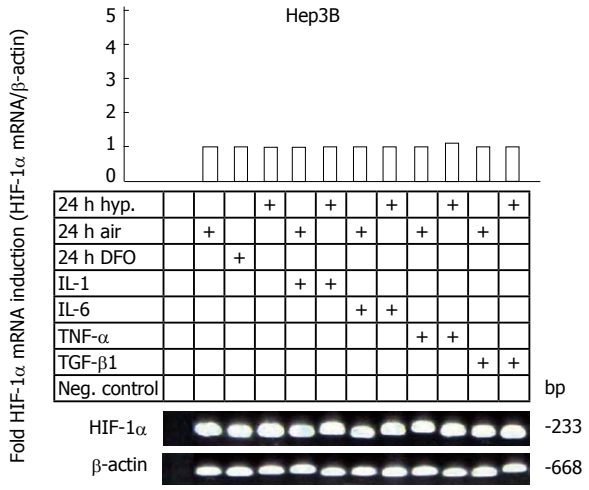

Figure 1 Hypoxia-inducible factor-1 alpha mRNA expression level determination *via* semi-quantitative reverse transcription polymerase chain reaction in the human hepatocellular carcinoma cell line Hep3B under aerobic and hypoxic conditions (0.1% O_2 for 24 h) without or with cytokine stimulation (interleukin-1, interleukin-6, tumor necrosis factor-alpha or transforming growth factor-beta) under both aeration conditions examined. β-actin was used as a loading control. Bar graphs show band intensities after densitometric evaluation. Representative experiment of three different experiments. IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; TGF-β: Transforming growth factor-beta; HIF-1α: Hypoxia-inducible factor-1 alpha.

and Schuell, Dassel, Germany). Non-specific binding was blocked by 5% non-fat milk powder in Tris-buffered saline (TBS) overnight at 4 ℃ followed by incubation with the NDRG1 primary antibody (ab8448, Abcam, Cambridge, United Kingdom), diluted 1:1000 in 2.5% non-fat milk powder in TBS for 1 h at room temperature. Blots were washed twice in TBS/0.05% Tween-20 (Bio-Rad, Munich, Germany) and then three times in TBS for 5-10 min, each. The secondary antibody, goat anti-rabbit-HRP (stock solution: 400 μg/mL, DakoCytomation, Denmark), was incubated at a dilution of 1:2000 for one additional hour at room temperature followed by five washing steps as described above. Detection of the bound antibodies was accomplished by membrane development with electroluminescence (ECL) plus a Western blotting detection system (Amersham Biosciences, Cambridge, United Kingdom) for 5 min with subsequent development of the Hyperfilm ECL (Amersham) used for detection purposes.

Determination of CA9 expression via fluorescence activated cell sorter analysis

CA9 expression was determined by flow cytofluorometry employing the fluorescence activated cell sorter (FACS) Calibur™ flow cytofluorometer (Becton-Dickinson, Heidelberg, Germany, low-power argon laser excitation at 488 nm) and CellQuest Pro™ software as cell-associated fluorescence. For each analysis, about 10.000 gated events were collected. The experimental basis was previously described[46] and was successfully applied after modification. Hep3B cells were kept under aerobic as well as hypoxic (0.1% O_2) conditions for 24 h.

Densitometric evaluation of Western blotting and the statistical analysis of measurements

Protein expression signal strengths on Western blotting were determined with 1D Kodak Image analysis software. The signals were measured in Kodak light units (KLU) and divided by the corresponding signals of the loading controls β-tubulin and β-actin, as previously described[44-49]. The relative changes in protein expression resulting from hypoxic conditions, or hypoxic conditions with subsequent reoxygenation were analysed in relation to the 24 h normoxic value. Three to four individual experiments were always performed. The Mann-Whitney *U* test for independent samples was used to analyse these data. The Student's *t* test for unpaired samples was used to analyse overall cell numbers. In the two tests $P \leqslant 0.05$ was considered to be statistically significant. All tests were carried out using the statistical package SPSS, release 12.0.1 for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

Analysis of HIF-1α regulation by IL-1, IL-6, TNF-α, TGF-β and hypoxia by Western blotting and flow cytometry

Results of the semiquantitative RT-PCR series of experiments showed that HIF-1α mRNA was evenly expressed in the cells examined (Figure 1) which were under normoxic aeration or conditions with or without IL-1, IL-6, TNF-α or TGF-β stimulation for 24 h, and showed no HIF-1α mRNA up-regulation which is a common characteristic of tumour cells under hypoxic aeration conditions.

In contrast, and in parallel sets of experiments, HIF-1α nuclear protein expression was clearly up-regulated under severe hypoxic conditions (0.1% O_2) without IL-1, IL-6, TNF-α or TGF-β stimulation for 24 h, confirming oxygen-dependent HIF-1α expression regulation. This severe hypoxia-induced expression rate was almost doubled with IL-1, IL-6, TNF-α or TGF-β stimulation for 24 h under severe hypoxic conditions (0.1% O_2) (Figure 2).

Analysis of CA9 regulation by IL-1, IL-6, TNF-α, TGF-β and hypoxia by Western blotting and FacsScan analysis

Intracellular CA9 protein levels expressed in response to *in vitro* hypoxia in addition to CA9 protein levels from whole-cell lysates maintained under normoxic conditions were detectable in a cell type-specific fashion (Figure 3). Here, CA9 protein expression was clearly up-regulated under severe hypoxic conditions (0.1% O_2) without stimulation with IL-1, IL-6, TNF-α or TGF-β for 24 h respectively, confirming the oxygen-dependent CA9 protein regulation. This severe hypoxia induced CA9 expression rate was almost doubled with IL-1, IL-6, TNF-α or TGF-β stimulation for 24 h under severe hypoxic conditions (0.1% O_2) (Figure 3).

Furthermore, CA9 expression was determined by flow cytofluorometry employing the FACSCalibur™ flow cytofluorometer (Becton-Dickinson, Heidelberg, Germany, low-power argon laser excitation at 488 nm)

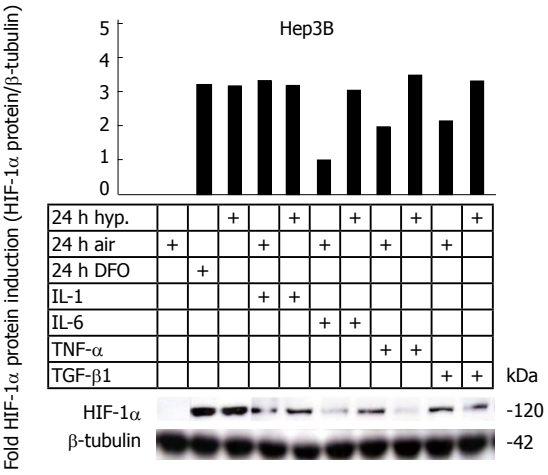

Figure 2 Western blotting analysis of hypoxia-inducible factor-1 alpha protein expression under hypoxia or cytokine stimulation (interleukin-1, interleukin-6, tumor necrosis factor-alpha or transforming growth factor-beta) in nuclear extracts of the hepatocellular carcinoma cell line Hep3B. Treatment with 100 μmol/L DFO under aerobic conditions served as a positive control. One representative Western blotting of hypoxia-inducible factor-1 alpha (HIF-1α) protein with β-tubulin as a loading control and quantification of the results *via* densitometry. IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; TGF-β: Transforming growth factor-beta.

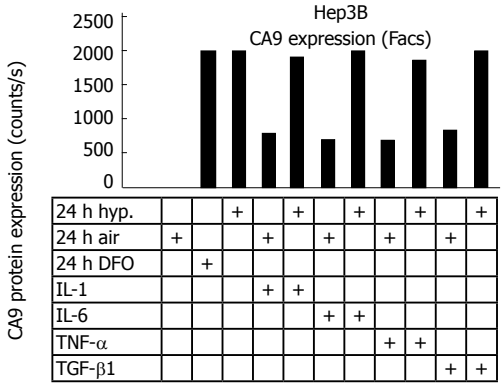

Figure 4 Representative carbonic anhydrase 9 flow cytometry with suspensions of known aerobic and hypoxic cell populations (0.1% O_2 for 24 h) or cytokine stimulation under both aeration conditions. Suspensions of hepatocellular carcinoma cell lines during hypoxia or cytokine stimulation [interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α) or transforming growth factor-beta (TGF-β)] were incubated with the anti-carbonic anhydrase 9 (CA9) antibody M75 and a fluorescein-isothiocyanate-conjugated secondary antibody.

and CellQuest Pro™ software as cell-associated fluorescence. For each analysis, about 10.000 gated events were collected. The experimental basis was previously described[29-33] and was successfully applied after modification. Hep3B cells were kept under aerobic as well as hypoxic (0.1% O_2) conditions for 24 h (Figure 4). CA9 protein expression was clearly up-regulated under severe hypoxic conditions (0.1% O_2) without IL-1, IL-6 and TNF-α or TGF-β stimulation for 24 h, confirming oxygen-dependent regulation of CA9. This severe hypoxia-induced expression rate was almost doubled as a consequence of IL-1, IL-6 and TNF-α or TGF-β stimulation for 24 h under severe hypoxia conditions (0.1% O_2)

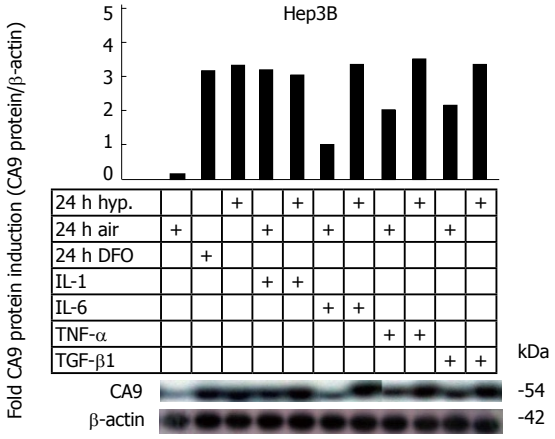

Figure 3 Western blotting of carbonic anhydrase 9 protein expression in whole-cell lysates of the hepatocellular carcinoma cell line Hep3B during hypoxia or cytokine stimulation (interleukin-1, interleukin-6, tumor necrosis factor-alpha or transforming growth factor-beta) with densitometric quantification of carbonic anhydrase 9 protein expression and β-actin as loading control. Representative experiment of three different experiments. IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; TGF-β: Transforming growth factor-beta; CA9: Carbonic anhydrase 9.

Analysis of CA9 regulation by IL-1, IL-6, TNF-α, TGF-β and hypoxia by semi quatitative RT-PCR

An O_2-concentration-dependent expression pattern of CA9 mRNA was seen in Hep3B. CA9 mRNA expression was clearly up-regulated under severe hypoxic conditions (0.1% O_2) without stimulation with IL-1, IL-6 TNF-α or TGF-β for 24 h, confirming oxygen-dependent regulation of CA9. The severe hypoxia-induced expression rate was doubled with IL-1, IL-6 and TNF-α or TGF-β stimulation for 24 h under severe hypoxia conditions (0.1% O_2) as seen in Figure 5.

DISCUSSION

Tumour hypoxia is recognized to be responsible for tumour tissue resistance to anti-cancer therapy and its fundamental role in tumorigenesis has been established. Hypoxia, by several mechanisms, induces resistance to drugs used as chemotherapeutic agents and other therapeutic modalities, e.g., radiotherapy. Some of these reflect the direct effects of hypoxia. Others reflect the indirect effects of hypoxia and the environmental perturbations associated with hypoxia on the metabolism of human tumour cells. The resistance induced by these processes will be lost when the cells are returned to a normal environment. Genes or gene products that are specifically up-regulated under hypoxic conditions are discussed as potential endogenous hypoxia markers or therapeutic targets. The hypoxia inducible factor subunit, HIF-1α, has been identified as an important mediator of the cellular response to hypoxia, promoting both cellular survival and apoptosis under different conditions, and has been extensively studied as an endogenous hypoxia marker and its mechanism of accumulation under hypoxia is well understood[14,44,50,51]. The expression of CA9, a protein strongly

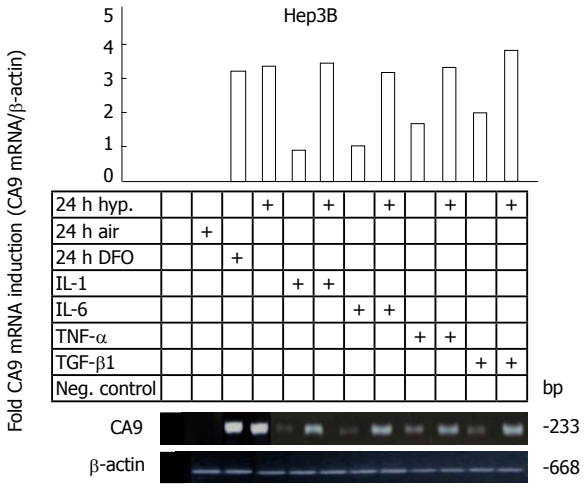

Figure 5 Regulation of hypoxia-inducible carbonic anhydrase 9 mRNA level *via* semiquantitative reverse transcription polymerase chain reaction *in vitro* in the hepatocellular carcinoma cell line Hep3B under aerobic and hypoxic conditions (0.1% O_2 for 24 h) with or without cytokine stimulation (interleukin-1, interleukin-6, tumor necrosis factor-alpha or transforming growth factor-beta) under both aeration conditions and β-actin as loading control. IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; TGF-β: Transforming growth factor-beta; CA9: Carbonic anhydrase 9.

regulated by HIF-1α, has been shown to be induced by hypoxia in various malignant cells *in vitro*.

CA9 is under the control of a vHL tumour suppressor gene[52], while the loss of function of this gene may result in the up-regulation of HIF-1α and CA9 in affected cells. The observed relative (Figures 3-5, 6 respectively), differences in expression between CA9 protein and the corresponding CA9 mRNA may be explained by different post-transcriptional processing or post-transcriptional regulation of CA9 mRNA by CA9 protein levels[53,54]. High basal levels of HIF-1α and CA9 under normoxic conditions may be an adaptive response in cells with increased metabolic demands or altered signal transduction pathways which are unrelated to hypoxia, but govern HIF-1 cellular activity[55]. Using flow cytometry we were able to accurately discriminate CA9-negative and -positive cells *in vitro* (Figure 5). The measured percentages of CA9 positive cells closely reflected the hypoxic status of the cells. Cytokines belonging to the examined families regulated the HIF-1α-dependent CA9 expression under normoxic conditions and enhanced the development of this expression pattern due to the shift in oxygenation conditions towards the extreme hypoxic conditions[17-19,25,30,54-64].

Within this context, TGF-β-influenced functional CA9 regulation was indirect and complex. TGF-β activates hCA9 gene transcription, thereby causing an increase in mRNA and protein levels of hCA9. This up-regulation may occur in an indirect manner since there are no Smad binding elements available in the hCA9 promoter region. TGF has a clear role in HIF-1α stabilization under normoxia[42]; in addition, the hCA9 promoter is transcriptionally regulated by the HRE present within the promoter region[44], therefore, TGF-β1 transcriptionally up-regulates the hCA9 promoter. This might suggest that the TGF-β1-mediated HIF-1α regulated up-regulation of *hCA9* gene expression is *via* the TGF-β pathway which as a consequence is responsible of this functionally important regulation leading to the high level of *hCA9* expression in Hep3B cells.

The cytokines belonging to the examined cytokine families positively regulated the HIF-1α dependent CA9 expression under normoxic conditions, and enhanced the CA9 mRNA and protein expression level pattern due to the oxygenation conditions under extreme hypoxic conditions. Within this context, cancer cell type expression pattern or tendency specificity resulted from stimulation with cytokines and may be true for all cytokines examined, with the exception of TNF-α which might display other expression patterns or tendencies (Figure 6), as in the case of HT-29 human colon adenocarcinoma cells[65].

Until now, the detailed functional *CA9* gene regulation in human tumours, especially HCC, was unknown both under hypoxia and normoxic oxygenation conditions, and combined with the stimulatory effect of the different signal transduction pathways, especially IL-1, IL-6, TNF-α and TGF-β1, it is now partially clear in which cancer tissue they play a regulatory role[66,67]. IL-1, IL-6, TNF-α and TGF-β1 induced an increase in the CA9 protein level in HCC cells, which may suggest potential targets for new and more specific approaches to cancer treatment and prevention, since CA9 overexpression in the tumour is mainly responsible for tumour resistance to both radiotherapy and/or chemotherapy-based treatment approaches (it was indicated that IL-1 was responsible for radio-resistance in murine tissues[68], and that IL-1 regulated stimulation is responsible alone or in combination with the other cytokines due to the tumour starvation effect)[55]. The down-regulation of CA9 expression (with respect to the role of the post-translational regulatory effect on expression[69] by interfering with IL-1, IL-6, TNF-α and TGF-β1 signalling leading to basal expression level without affecting the fundamental cellular functions of the targeted HCC cells) should be respected when designing new approaches or modifying existing approaches with respect to the consequences associated with the knockout of these genes during human cancer progression. We also demonstrated that IL-1, IL-6, TNF-α and TGF-β transcription up-regulated *hCA9* gene expression in HCC which has an important role in hypoxia and consequently metastasis of tumours. This regulatory cycle is of potential importance in the induction, as well as, in the activation of numerous factors implicated in the pathogenesis of cancer. Therefore, further studies to explore the mechanism(s) involved or related to this regulative process are required. There are two possible explanations for the regulatory behaviour of the regulated HIF-1α-dependent CA9 expression under normoxic or hypoxic conditions, either it enhanced this expression or it was due to the stimulatory effect of IL-1, IL-6, TNF-α and TGF-β1, or there was dynamic interplay between hypoxia and the stimulatory

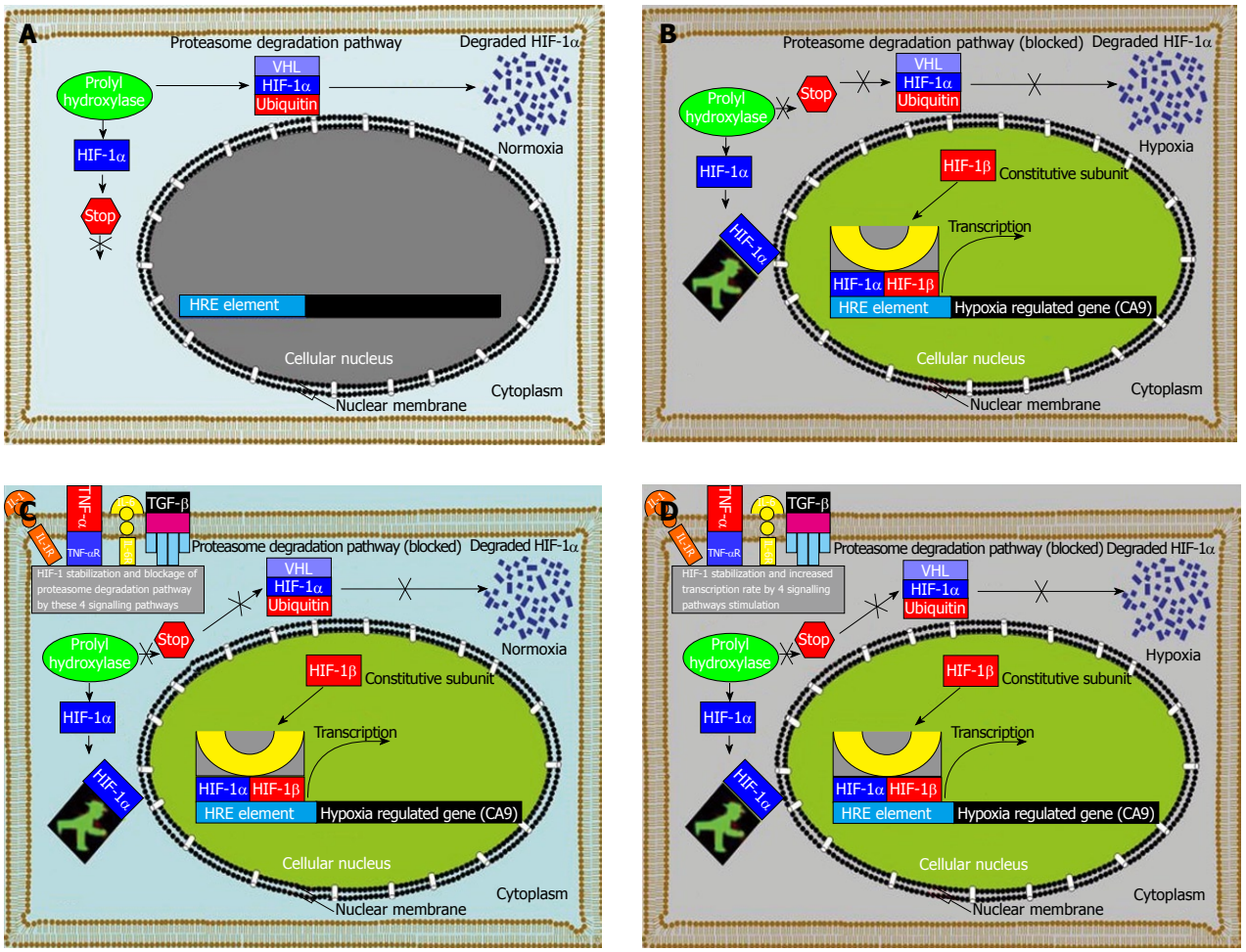

Figure 6 Hypoxia-inducible factor-1 alpha induced regulation of hypoxia-induced carbonic anhydrase 9 expression in human tumor cells without or with stimulation. A: Under normoxic conditions in the tumour cell microenvironment, hypoxia-inducible factor-1 alpha (HIF-1α) is rapidly degraded *via* the von Hippel-Lindau tumour suppressor gene product (pVHL) - mediated proteasome pathway; B: Following a shift in tumour environment aeration conditions from normoxic to hypoxic aeration conditions, HIF-1α subunit becomes stable and translocates into the cellular nucleus and interacts with co-activators of which its transcription machinery consists if e.g. p300/CBP to modulate the transcriptional activity of numerous hypoxia inducible genes, such as carbonic anhydrase 9 (CA9) in our case, and about 61 other hypoxia induced genes[51]; C: When the cells are stimulated under normoxia with either interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α) or transforming growth factor-beta (TGF-β), the transcription factor HIF-1α subunit becomes stable despite the oxygenation status of the tumour environment and translocates into the cellular nucleus and interacts with co-activators of which its transcription machinery consists of e.g. p300/CBP to modulate the transcriptional activity of CA9 with a similar expression to that under hypoxia; D: Experimental stimulation with either IL-1, IL-6, TNF-α or TGF-β1 under hypoxia increases the CA9 level to almost double the expression rate under hypoxic conditions with the stimulation of these cytokines due to increased HIF-1α translocation into the nucleus and increased binding rate to the hypoxia response element element within the CA9 promoter region.

effect of IL-1, IL-6, TNF-α and TGF-β1.

In conclusion, we showed in HCC Hep3B cell lines that CA9 protein and CA9 mRNA are generally up-regulated due to stimulation with IL-1, IL-6, TNF-α and TGF-β1, and exposure to prolonged severe (0.1% O_2) hypoxic or normoxic conditions. CA9 pathway up-regulation might be accomplished by the effect of cytokine stimulation on hypoxia induced and regulating genes might represent an interesting aspect of gene conditions under these environmental conditions. Thus, in cancer development, it is undoubtedly a promising target for anti-cancer treatment. The results of this series of experiments are useful for the potential optimization of applied tumour therapeutic approaches against HCC. Since tumour tissue oxygenation conditions are dynamic and CA9 expression in such tissues is chronic this feature is involved in the radio-resistance of this type of cancer tissue.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is the most prominent type of liver cancer, while alcoholism is the most common cause of hepatic cirrhosis. This is a result of hepatic fibrogenesis which is characterized by increased and altered deposition of newly generated extracellular matrix in response to injury. Transdifferentia-

tion of hepatic stellate cells (HSCs) is driven by an array of cytokines of which transforming growth factor beta (TGF-β) has proven to be the fibrogenic master cytokine stimulating HSCs by autocrine and paracrine mechanisms. As a result of treatment *via* hepato-surgery and because only 10%-20% of HCCs can be removed completely, patients have a short life expectancy of 3 mo to 6 mo.

Research frontiers

Carbonic anhydrase 9 (CA9), is a hypoxia induced gene and acts as a tumour hypoxia marker as well as an indicator and a potential therapeutic target in different human cancers. Transcriptional regulation of transmembrane protein CA9 is complex and the transcriptional activation of CA9 by TGF-β and other cytokine families is consistent with hCA9 mRNA levels revealed by reverse transcription polymerase chain reaction and human CA9 protein expression levels by flow cytometry in Hep3B cells in a cell-specific manner. The findings from this series of experiments showed that besides hypoxia as a positive regulator of CA9 expression in HCC, the four signal transduction pathways, interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α) and TGF-β, positively influence CA9 expression in HCC both under normoxic and hypoxic conditions. These findings may potentially be considered in the design of anti-cancer therapeutic approaches involving the CA9 gene as a positive disease regulator in human cancers.

Innovations and breakthroughs

Due to the regulatory effect of CA9 on the physiological condition of the human tumor tissue microenviroment, therapeutic approaches used against these tumors are not normally successful due to resistance to treatment modalities such as radiotherapeutic or chemotherapeutic modalities. The results of this experimental series highlight the functional regulatory role of IL-1, IL-6, TGF-β1 and TNF-α which led to an increase in the expression of CA9 in tumor tissue cells. As a consequence, these important data provide functional points of interaction for the inhibition or down-regulation of CA9 expression *via* tailored gene therapeutic modalities such as siRNA, adenoviral or retroviral vectors carrying genes acting as functional down-regulators. This is a pre-requisite step for the application of therapeutic approaches aiming to optimize the outcome of these therapies and provide a better quality of life for cancer patients undergoing treatment.

Applications

The results of this study show the regulatory events of the hypoxia induced gene CA9 both *via* oxygenation deprivation in tumor cells and *via* the stimulatory effect of different cytokine families with related expression patterns.

Terminology

Hypoxia is a pathological condition in a certain region of the body, namely the tissues of organs are deprived of adequate oxygen due to the failure to deliver oxygen to target tissues. The difference between normal oxygen supply and demand at the cellular level may result in hypoxic conditions. The oxygenation state where oxygen is absent is called anoxia. Normoxia, normal oxygen concentration as a result of a normal or adequate oxygen supply at the cellular level is typically 20%-21% in the atmosphere or 2%-3% in the physiological context. HCC accounts for most liver cancers and differs from metastatic liver cancer, which starts in another body organ such as breast or lung and disseminates towards the liver. The cause of liver cancer is usually the unidirectional development of liver fibrosis into liver cirrhosis. Different factors, besides genetic predisposition, favour this development and include; alcohol abuse, autoimmune diseases of the liver, hepatitis B or C virus infection, chronic liver inflammation, and hemochromatosis; cytokines are small cell-signalling non-hormonal protein molecules functioning in the intercellular communication. Cytokines can be classified as proteins, peptides, or glycoproteins and include a large family of regulators produced by cells of different embryonic origin; tumour therapy refers to the approaches applied for various cancers in humans. These include radiation therapy, surgical removal of cancer tissue, drugs or other substances that block cancer growth and spread by interfering with specific molecules involved in tumour growth and progression including agents which interfere with cell growth signalling or tumour blood vessel development, cancer cells specific death promotion, stimulating the immune system to destroy specific cancer cells, and toxic chemical agents delivered into cancer cells as well as gene therapeutic modalities; Tumour microenvironment: The extracellular environment present in a very small region of a solid tumour. Cells in different areas of solid tumours will have markedly different microenvironments; Angiogenesis: The formation of new blood vessels.

Peer review

Due to its clear regulatory behaviour under hypoxic condition in human tumor cells, NDRG1 represents an additional diagnostic marker for brain tumor detection, due to the role of hypoxia in regulating this gene, and it can represent a potential target for tumor treatment in human glioblastoma.

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