TGF-β upregulates tumor-associated carbonic anhydrase IX gene expression in Hep3B cells

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Abstract

Carbonic anhydrase IX (CAIX) is a membrane-associated carbonic anhydrase (CA) that is overexpressed in a variety of tumor types and associated with increased metastasis, giving a poor prognosis. Transcriptional regulation of transmembrane protein CAIX is complex. We describe further characterization of the 1.2 kb hCA9 promoter, and the effect of TGF-β on the transcriptional activity and expression of hCAIX in Hep3B cells. Transcriptional activity of different promoter regions of hCA9 promoter showed the presence of negative regulatory region between −300 bp and −500 bp of hCAIX promoter. The −116/+38 region was enough for basal transcriptional activity in Hep3B cells. TGF-β upregulates all promoter regions of hCA9 with the highest beig for -466/+38 that has a negatively regulated region. The transcriptional activation of hCA9 promoter by TGF-β is consistent with hCAIX mRNA levels revealed by RT-PCR and hCAIX protein expression levels by flow cytometry in Hep3B cells.

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1. Introduction

Carbonic anhydrases (CAs) are an family of zinc-containing enzymes, which classically participate in the maintenance of pH homeostasis in the human body via the catalysis of the reversible reaction (Kivela et al., 2005; Winum et al., 2008). CAIX, a unique transmembrane member of the CA gene family, is a tumour-associated protein thought to be involved in malignant cell invasion and adhesion. Expression of CAIX can only be detected in a few normal tissues, but it is abundant in several tumors, e.g. renal cell carcinoma, cervical, lung, colorectal, bladder and breast carcinomas (Ivanov et al., 2001; Jarvela et al., 2008; Pastorekova et al., 1997; Kaluzová et al., 1993). Although the exact mechanisms of CAIX are not known in cancer development, it is undoubtedly a promising target for anticancer treatment.

CAIX causes a reduction of the extracellular pH thereby facilitating the breakdown of the extracellular matrix together with upregulation of genes involved in invasion and migration (Giatromanolaki et al., 2001; Kyndi et al., 2008). Association of CAIX with broad range of tumors is strongly related to its transcriptional regulation by hypoxia and high cell density, which appear to activate the CA9 promoter (Jarvela et al., 2008; Kopacek et al., 2005). Induction by hypoxia occurs via the HIF-1 transcription factor, which accumulates in tissue under hypoxic condition often present in growing tumors (Jarvela et al., 2008; Svastova et al., 2004; Swietach et al., 2007; Wykoff et al., 2000). In addition to hypoxia, other stimulating factors, e.g. hormones and cytokines, induce HIF-1 accumulation and activity under normoxia. Moreover, TGF-β regulates the expression of its own converting enzyme, furin, the recently identified HIF-1-regulated gene (Blanchette et al., 1997; Jung et al., 2003; McMahon et al., 2003, 2005, 2006). Among recent

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advances underlining the contribution of TGF-β to tumor progression is because this growth factor influences the accumulation of HIF-1 in normoxic conditions.

Due to the importance of hCAIX in tumor biology, the elucidation of transcriptional regulation is very important. The initial data obtained on the transcriptional regulation of hCA9 contain a detailed functional analysis of the −173 to +31 human CA9 upstream regions. Deletion analysis of the upstream region of CA9 by Kaluz et al. (1999) showed the complex regulation of hCAIX expression on transcriptional level because of the presence of enhancer element, −1600 and two distal negative elements, around −2000 and −900 bp (Kaluz et al., 1999; Kaluzova et al., 2001). However, deletion analysis of the promoter does not include in detail the region between −446 and −1700 bp. Regulation of this region of hCA9 promoter remains unclear.

Therefore, the aim of the study was to investigate the detailed sequence analysis and functional dissection of 1.2 kb human CA9 promoter which lead to further understanding of CA9 transcriptional regulation. The effect of TGF-β, underlining the contribution of TGF-β to HIF 1 accumulation in normoxia, was investigated on transcriptional regulation and the mRNA and protein expression level of CAIX in human hepatoma model cells, Hep3B.

2. Materials and methods

2.1. Materials

The human hepatoma Hep3B cell line was from European Collection of Animal Cell Cultures. TGF-β was from Peprotech. All the cell culture reagents and plasticware were purchased from Greiner or Gibco. All other chemicals used were analytical grade.

2.2. Genomic DNA isolation, cloning and preparation of manipulated CAIX promoter−reporter constructs

Genomic DNA isolation was performed using human blood. Restriction digestion and PCR-based approaches were used to prepare the hCA9 promoter−reporter constructs. PCR amplification from genomic DNA was performed using 200 ng genomic DNA as a template, 2 μM final concentration primers, 1X buffer (50 mM KCl, 10 mM Tris−HCl, pH9, %1(v/v) Triton-X-100) (Fermentas), 2.5 U Taq DNA polymerase (Fermentas), and 2 mM MgCl2. The reactions conditions were 95 °C for 2 min, (94 °C for 1 min, 65 °C for 45 s, 72 °C for 1 min) × 35 cycles and 72 °C for 10 min final step. The amplified fragment was gel-purified and cloned into pGEMT vector system (Promega). The sequence of PCR amplified promoter fragment was verified by automated-sequencing. Some of the promoter constructs were prepared by PCR using the following primers, 5′-ggt acc gca gaa ttc atc tct ctt ccc tca a-3′ for full length [−1251/+38] construct; 5′-ggt acc cat tac tta act cac cct cg-3′ for [−466/+38] construct; 5′-ggt acc cag aca aac ctg tga gac tt-3′ for [−116/+38] construct. In addition, the sequence of forward primer for all

constructs was 5′gct agc ctg act gtg ggg tgt ccc agc ac-3′. These primers were designed to allow directional cloning of the amplification product into the KpnI and the NheI sites of the pGL2-Basic vector. The integrity of sequence of all the constructs was verified before use in transfection experiments GenBank Accession no. Z54349. Two more constructs were prepared by restriction digestion using Sma I (−935/+38) and PvuII (−266/+38) and subcloned into SmaI and Nhe I sites of pGL2-basic vector.

2.3. Cell culture and transient transfection assays

Hep3B cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum (1 h, 56 °C) (HI-FCS), penicillin (100 U/ml) and streptomycin (100 mg/ml). The cultures were maintained at 37 °C in a humid incubator with air containing 5% (v/v) CO_2. DNA transfections were carried out by the calcium phosphate precipitation method (Kockar et al., 2001) and utilized 1 μg promoter-luciferase DNA construct, and 0.5 μg of cytomegalovirus (CMV)-β-galactosidase plasmid to provide an internal control for transfection efficiency. The RSV luciferase and pGL2 basic luciferase vectors were used as positive and negative controls, respectively. After 6 h, the cells were washed with phosphate-buffered saline and left for predetermined time-courses in fresh culture medium alone or in the presence of TGF-β. Luciferase and β-galactosidase activities in cell extracts were determined using commercially available kits (Promega). The luciferase activity was normalized to the β-galactosidase value and each transfection was repeated at least 3 times.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was prepared using the RNeasy total RNA isolation kit (Qiagen) according to the instructions from the manufacturer. Each isolated RNA sample (1 μg) was then subjected to RT-PCR. The reactions conditions for human CAIX were 96 °C for 5 min, (93 °C for 45 s, 59 °C for 45 s, 72 °C for 90 s) × 30 cycles and 72 °C for 10 min final step. For beta-2-microglobilin, 60 °C annealing temperature and 19 cycles was performed. These conditions were in the exponential phase of amplification and, therefore, provided a direct correlation between the amount of products and RNA template abundance in the samples. The sequences of the primers were 5′-tct cat ctg cac aag gaa cg-3′ and 5′- act tca gcc gct act tcc caa -3′ for 349 bp hCAIX product and 5′-ttt ctg gcc tgg agg cta tc-3′ and 5′-cat gtc tcg atc cca ctt aac t-3′ for 314 bp β-2-microglobulin product. The PCR products were size-fractionated on a 2% (w/v) agarose gel, photographed using a Gel Doc documentation system and densitometric analysis were done with Syngene GeneTools software.

2.5. Flow cytometry

Hep3B cells were plated out 6-well plates and incubated for 72 h in fresh culture medium alone or in the presence of

500 U/ml and 1000 U/ml TGF-β. All the staining procedure was carried out on ice. Cells were scraped in PBS and centrifuged at 1000 rpm for 5 min, washed in PBS including 1% BSA. Washed cells are incubated for 40 min with the phycoerythrin-labeled monoclonal CAIX antibody (R&D system), which binds to cells expressing CAIX. Unbound antibody and any residual factors were removed by 2 washings. Finally cells were resuspended and fixed in 1% paraformaldehyde and transferred to 5 ml tubes for staining.

2.6. Statistical analysis

Statistical evaluation with respect to experiments was performed using one-way analysis of variance (ANOVA). A probability (P) of 0.05 or less was deemed statistically significant.

3. Results

3.1. Isolation and sequence analysis of hCA9 promoter clones

The identity of −1251/+38 bp human CA9 promoter isolated by PCR was confirmed by sequence analysis (2 different companies, Lark technologies, UK and REFGEN Biotechnology, Turkey) with published data Gen Bank Accession no. Z54349. BlastN searches revealed the 1289 bp promoter of CA9 to share 99.8% identity with that previously published of human CA9 upstream region. Comparison of the amplified promoter to the published CA9 promoter sequence showed that there was a trinucleotide insertion between −1176 and −1179 bases. Due to the discrepancy between sequences, the −1251/+38 promoter region was reamplified from the human genomic DNA and confirmed by sequence analysis. The computer search of hCA9 promoter region indicated that hCA9 promoter was TATA-less promoter, and did not contain Smad sites responsible for the direct regulation of TGF- β.

3.2. Analysis of hCA9 promoter activity in transfected Hep3B cells

To identify the regions in the hCA9 promoter that were important for the transcription of this gene, 5 different truncated promoter-luciferase constructs were initially prepared as above. The human Hepatoma Hep3B cell line was used for all the transfection experiments. The Hep3B cells were chosen because they are used widely as a model for the analysis of the promoter elements that are involved in both the constitutive and inducible expression of genes in hepatocytes. Basal promoter activities of all promoter constructs show dose-dependent increases (data not shown). There was a gradual activity loss between −1251/+38 and −466/+38 regions (Fig. 1). The −466/+38 region has minimal transcriptional activity. The 266/+38 promoter region had increased transcriptional activity. Kaluz et al. (1999) suggested from 9 constructs between −3500 bp and −173 bp that the highest basal activity has been reached about −1700 region, whereas

the minimum activity was due to the −446/+31 region (Jung et al., 2003).

3.3. Regulation of hCAIX by TGF-β: RT-PCR, flow cytometry and transfection experiments

Our initial aim was to determine the effect of TGF-β on transcriptional activity of hCA9 promoter region. Different concentrations of TGF-β, namely 10, 100l, 500 and 1000 U/ml were incubated with the cells transfected by 5 individual promoter regions for 24, 48 and 72 h. Although there was an increase in transcriptional activity for 24 and 48 h (data not shown), the most statistically significant increase was at 72 h (Fig. 2). Five promoter-luciferase DNA constructs were activated by 1000 U/ml TGF-β for 72 h incubation (approximate induction of 2 to 5 fold) (Fig. 2), whereas the level of stimulation of −466/+38 bp promoter region was >9 fold in 500 U/ml TGF-β for 72 h.

Semi-quantitative RT-PCR technique showed the effect of TGF-β on hCAIX mRNA level in Hep3B cells. Cells were treated with different concentrations, 10−1000 U/ml TGF-β for 24, 48 and 72 h. Densitometric analysis of the data was determined by relating the intensity of the hCAIX PCR product at each time-point to the intensity of the PCR product for the housekeeping gene, Beta-2-microglobilin. hCAIX mRNA level was activated by both 100l and 500 U/ml TGF-β. The most statistically significant increase, with an 1.7 fold induction of CAIX mRNA level, was obtained with 1000 U/ml TGF-β for 72 h ($p < 0.05$: Fig. 3).

To evaluate the effect of TGF-β on hCAIX protein level that is membrane bound protein, flow cytometric analysis has been carried out. l TGF-β stimulation with 500 or 1000 U/m for 72 h also increased hCAIX protein levels on the surface of Hep3B cells (Fig. 3). TGF-β at 500 U/ml caused an statistically significant increase in hCAIX protein ($p < 0.05$).

4. Discussion

Hypoxia is a common feature of human solid tumors and causes tumor cells to undergo adaptive changes that enable them to survive and proliferate. CAIX level dramatically increases in response to hypoxia via a direct transcriptional activation of CA9 gene by hypoxia inducible factor-1 (McMahon et al., 2003; Svastova et al., 2003). CAIX helps maintain a normal pH in tumor cells in a hypoxic and acidic microenvironment, which may allow tumor cell proliferation (Pastorekova et al., 1992). Therefore, investigation of transcriptional regulation of CAIX could provide crucial information about mechanisms leading to CAIX expression and functions.

CAIX promoter (MN protein) was firstly isolated in 1996 (Opavsky et al., 1996). The first −173 bp construct was well characterized and divided into 5 functional parts named as PR1, PR2, PR3, PR4, PR5. Functional characterization showed that the highest basal activity was limited to −1700 bp region amongst 9 deletion constructs from −3500 bp to −173 bp promoter regions (Kaluz et al., 1999). Kaluz et al.
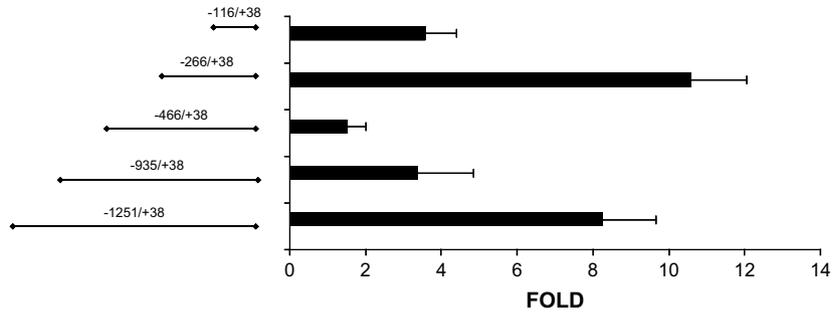
Fig. 1. The fold activity of hCA9 promoter deletion constructs against promoterless vector pGL2 basic in Hep3B cells. 1 μg hCA9 promoter constructs −1251/+38, −935/+38, −466/+38, −266/+38 and −116/+38 were transfected into Hep3B cells.

(1999) proposed that there were an enhancer element around −1600 bp and two distal negative elements, at −2000 bp and −900 bp. This information on CA9 regulation indicates that the transcriptional regulation of CA9 is more complex. However, there was lack of information on basal activity of −1700 bp and −466 bp region of CA9 promoter. Therefore, we studied this region in detail with aim of the further functional characterization of the CA9 promoter.

The 1289 bp of the CA9 promoter and truncated deletion constructs were analyzed for transient transfection to determine transcriptional activity in CAIX expressing Hep3B cells (Stolze et al., 2004; Willam et al., 2006). These cells are also a good model for cytokine regulated gene expression (Kockar et al., 2001). There was a gradual decrease in activity between −1251/+38 bp and −466/+38 bp regions. The lowest activity was reached in −446/+38 bp promoter region. −266/+38 bp showed an increase in transcriptional activity, suggesting that the negatively controlled region or silencer 200 bp region between −266 bp and −466 bp might have been deleted (Fig. 1).

TGF-β, a major polypeptide that inhibits proliferation and induces apoptosis in various cell types, plays a key role in tumor cell biology. We have shown that TGF-β increases the transcriptional activity of CA9 for all promoter regions with specifically −466/+38 bp region >9 fold compared to the unstimulated transfected cells. Most interestingly, we also found that this promoter region might contain negatively regulated elements. Furthermore, TGF-β stimulates hCAIX expression ∼2-fold at mRNA level. Stimulation of TGF-β on hCAIX expression was also detected at protein level by flow cytometer. As a membrane-bound protein, CAIX protein expression occurs in several cell lines, and here we have shown CAIX expression in Hep3B cells for the first time (Fig. 3).

Many common tumors have enhanced levels of TGF-β (Pasche, 2001). This growth factor is over expressed by tumor cells and is also released by infiltrating leukocytes (Mantovani et al., 2004). TGF-β uses Smad pathway which transcriptionally repress Ids in epithelial cells. Id proteins downregulate E-cadherin and ZO-1 expression. TGF-β not only regulates Id proteins, but also other proteins (e.g. Snail family, ZEB1 ZEB2, E12/E47, LEF-1) (Ito et al., 1995; Peinado et al., 2004; Pardali and Moustakas, 2007). This regulation of TGF-β is indirect and complex. TGF-β activates hCAIX gene transcription, thereby causing an increase in mRNA and protein levels of hCAIX. This upregulation could be indirect manner since there is no Smad binding elements available in hCAIX promoter region. As reported by McMahon et al. (2006), TGF has a clear role in stabilization of HIF-1 in normoxia; in addition hCA9 promoter is transcriptionally regulated by HRE element present in all
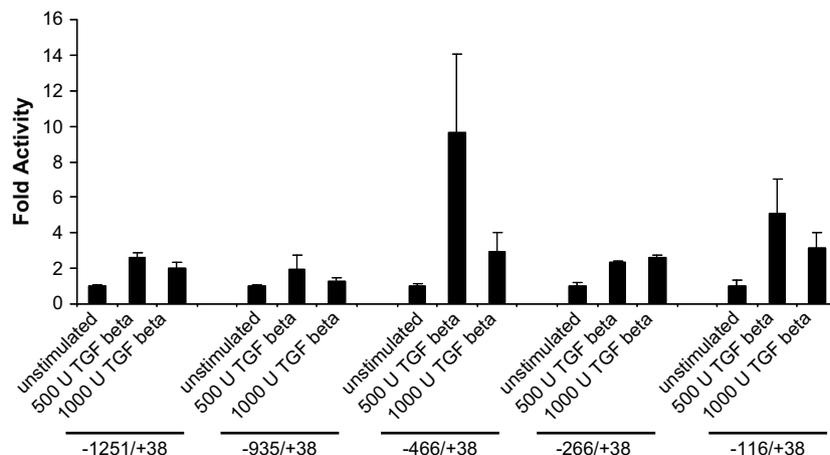

Fig. 2. The effect of TGF-β on transcriptional activity of hCA9 promoter constructs. Constructs indicated as above were transfected into Hep3B cells and treated by 1000 U/ml and 500 U/ml TGF-β for 72 h.
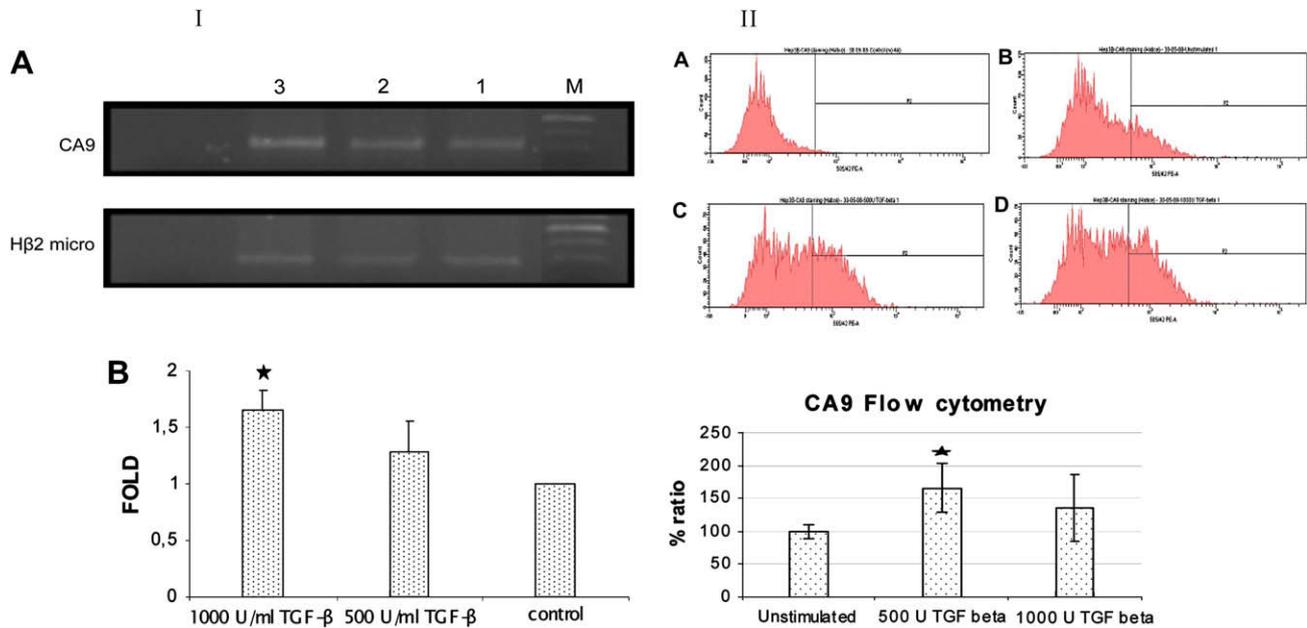
Fig. 3. I: RT-PCR analysis of hCA9 for 72 h in Hep3B cells. Cells were treated 500 U/ml and 1000 U/ml TGF-β. PCR products for CA9 and Beta-2-microglobilin fractionated by agarose gel electrophoresis are shown in Fig. 3 IA Lane1: control; Lane2: 500 U/ml TGF-β and Lane 3: 1000 U/ml TGF-β. The relative intensity of PCR products for CA9 and Beta-2-microglobilin at each time-point were determined using densitometric analysis Fig. 3 IB. II: Flow cytometry analysis of TGF-β unstimulated (B), stimulated (C and D) Hep3B cells for 72 h (A, background control). Hep3B cells were plated out 6 well plates and incubated for 72 h in fresh culture medium alone or in the presence of 500 unit/ml (C) and 1000 unit/ml (D) TGF-β.

constructs of the promoter (McMahon et al., 2006). Therefore, TGF-β beta transcriptionally upregulates all hCA9 promoter constructs. This might suggest HIFI mediated TGF upregulations on the expression of hCA9 gene. Therefore, it could be an important pathway through the explanation of high level expression of hCAIX in Hep3B cells.

We have also demonstrated that TGF-β transcriptionally upregulates hCAIX gene expression, which has an important role in hypoxia and consequently metastasis of tumors. 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. It thus appeared of interest to further explore the mechanism of the regulation for a better understanding.

Acknowledgments

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