Transcriptional regulation of carbonic anhydrase II by retinoic acid in the human pancreatic tumor cell line DANG

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Abstract Carbonic anhydrase II (CA II) generates bicarbonate in human pancreatic duct cells. We have developed the human pancreatic duct cell line DANG as a model to study the effects of *all-trans*-retinoic acid (ATRA) on CA II gene expression. ATRA treatment resulted in a time- and dose-dependent inhibition of CA II mRNA concentrations in DANG cells. These inhibitory effects were paralleled by a time-dependent decrease of CA II protein concentrations. Nuclear run on analysis revealed that the decrease of CA II mRNA concentrations was due to a decreased rate of CA II gene transcription. These data show that ATRA transcriptionally modulates CA II gene expression in human pancreatic carcinoma cells.

Key words: Carbonic anhydrase II; Retinoic acid; Pancreas; DANG cell

1. Introduction

The carbonic anhydrase gene family (CA, EC 4.2.1.1) is encoded by at least seven genes, designated CA I-VII, which are characterized by tissue-specific expression and varying physiological functions [1]. In general, this enzyme family catalyzes the interconversion of carbon dioxide and water to bicarbonate and protons [2]. In the mammalian nontransformed pancreas, CA II is the predominant isozyme which is exclusively expressed in the exocrine ductal cells. CA II thus provides bicarbonate anions required for the elaboration of the bicarbonate-rich pancreatic secretion, which is essential to neutralize the acidic milieu [3,4]. It is currently unclear, whether this cell type-specific expression of CA II is maintained during the malignant transformation of the exocrine duct cell. Furthermore, although CA II plays a critical role in duct cell specific function, very little is currently known regarding the regulation of CA II gene expression in human pancreatic tumor cells. To address this problem we have recently developed an in vitro system using human pancreatic ductal tumor cell lines [5]. Using this in vitro model, we previously found that the vitamin A derivative *all-trans*-retinoic acid (ATRA) exerts profound effects on the cell biology of ductal tumor cells, which is mainly characterized by a profound inhibition of anchorage-dependent and -independent growth [5]. Because this observation bears important therapeutical implications, we were therefore interested to further investigate the effects of ATRA on ductal specific cell function, using CA II gene expression as a representative model. We now present evidence that CA II is expressed in human malignant pancreatic ductal cells in vivo and in vitro; furthermore, CA II is transcriptionally downregulated by ATRA in the human pancreatic carcinoma cell line DANG.

2. Materials and methods

2.1. Cell culture

DANG cells were grown in DMEM medium supplemented with 10% charcoal stripped fetal calf serum. Cells were kept under 95% air and 5% CO_2 at 37°C. ATRA was added from stock solutions, prepared under subdued light. Control vehicles received ethanol, and the final concentration of ethanol in the medium did not exceed 0.1%.

2.2. Northern blots

RNA was isolated by the guanidinium isothyanate procedure [6]. RNA was denatured by formaldehyde, subjected to elctrophoresis through a 1% agarose gel in the presence of formaldehyde and then transferred onto nitrocellulose. Prehybridisation, hybridisation and washing procedures were carried out exactly as previously described [7], using a random primed cDNA probe for human CA II [8]. All filters were then sequentially hybridised with a cDNA encoding human β-actin. Repeated quantitative analysis was performed by slot-blotting [7]. The hybridisation signal was quantitated by laser densitometry, normalised to β-actin and then expressed as % of control.

2.3. Nuclear run on analysis

Nuclei were isolated by sucrose gradient centrifugation. RNA products were purified using deoxyribonuclease, proteinase K, and salt precipitation according to the procedure of Nelson and Groudine [9]. Care was taken that each experimental condition contained the same amount of nuclei and radioactivity. Prehybridisation, hybridisation and washing procedures were carried out exactly as previously described [7].

2.4. Western blots

Cell lysates were prepared by freeze-thawing cells in phosphate-buffered saline and pelleting the debris. 50 μg protein per lane were run on SDS gels, transferred to a nylon membrane [10] and probed with rabbit anti-human CA II antibody followed by goat anti-rabbit IgG conjugated to alkaline phosphatase. Blots were developed with nitroblue tetrazolium and bromochloroindoyl phosphate [11]. The detected signal was then quantitated by laser densitometry.

2.5. In situ hybridisation

Surgical samples of human pancreatic adenocarcinomas were fixed in 10% neutral formalin and embedded in paraffin. Sections of 4 μm were cut from specimen blocks using a microtome. The human CA II cDNA was subcloned into pRC/CMV vector in either orientation with respect to the T7 promotor site in order to transcribe antisense and sense RNA probes. Digoxigenin-labeled cRNA probes were generated exactly as previously described [12], using identical hybridisation conditions. The immunodetection was carried out using a sheep anti-digoxigenin antibody followed by a colour reaction in a chromogen solution [12].

2.6. Statistics

Statistical analyses were performed using the Student's t-test. Differences were considered to be significant at $P < 0.05$. All data are given as mean ± S.E.M.

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3. Results

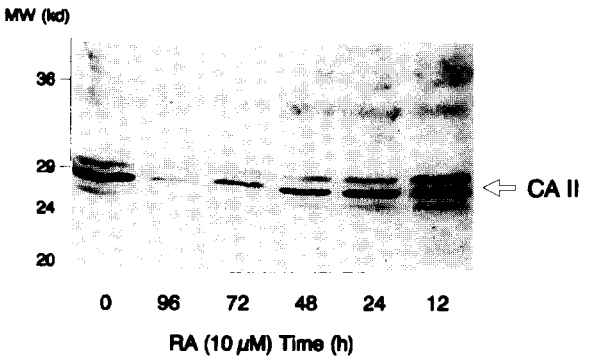
3.1. CA II expression in malignant pancreatic ductal cells

To verify that CA II gene expression remains preserved during malignant transformation we performed non radioactive in situ hybridisation on 5 independent surgical resected specimens of human pancreatic adenocarcinomas (Fig. 1). In all tumors examined, we observed a homogenous expression of CA II mRNA molecules restricted to the malignant transformed ductal cells. In contrast, no specific hybridisation signal was observed either in the surrounding connective tissue nor in the negative (sense) control (Fig. 1).

3.2. ATRA inhibits CA II gene expression in human DANG cells

Having confirmed the expression of CA II in human pancreatic carcinoma, we next investigated the effects of ATRA on CA II expression in the human pancreatic carcinoma cell line DANG. Western blotting revealed the expression of a 29 kDa CA II protein which is in good agreement to what has previously been described [13]. Incubation of DANG cells with ATRA (10 μM) resulted in a time-dependent decrease of CA II protein concentration with a maximal inhibition occuring after 96 h of ATRA treatment (18 ± 6% of control, $n = 4$, $P < 0.05$) (Fig. 2). To further investigate the underlying mechanisms responsible for CA II inhibition by *all-trans*-retinoic acid, we next analysed the effects of ATRA on CA II mRNA concentrations. Using a cloned human cDNA for CA II in Northern blotting we detected a single mRNA species of 2.0 kb in DANG cells (Fig. 3a). Incubation of DANG cells with ATRA resulted in a time-dependent decrease of CA II mRNA concentrations, while β-actin mRNA levels which served as an internal control, did not change under any experimental condition (Fig. 3a). Again, maximal inhibition was observed after 96 h of ATRA incubation (20 ± 5% of control, $n = 3$, $P < 0.05$) (Fig. 3b). Extending the ATRA incubation beyond 96 h did not result in a further decrease of CA II mRNA concentrations (data not shown). The effects of ATRA on CA II gene expression were dose-dependent with half-maximal effects observed at 50 nM and maximal effects observed at 10 μM of ATRA (21 ± 4% of control, $n = 4$, $P < 0.05$) (Fig. 4). Testing a variety of synthetic and natural retinoids in terms of their biological potency to inhibit CA II gene expression we observed the following gradient: *all-trans*-RA = 13-*cis*-RA > 9-*cis*-RA > AM80 > AM 580 (data not shown).

Fig. 1. In situ hybridisation of CA II in human pancreatic carcinoma. Shown is the intense staining of the centrally located ductular tumor cells, while the surrounding connective tissue as well as the negative control (sense) show no specific staining. Shown is a representative of 5 independent tumors analysed.



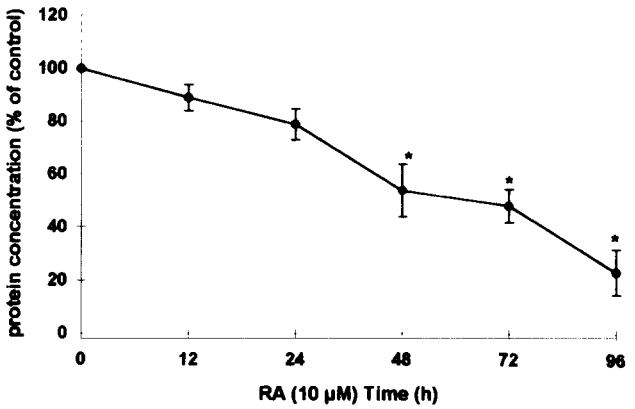


Fig. 2. Effects of ATRA on CA II expression in DANG cells. Shown is a representative Western blot (a) and the statistical analysis of 4 independent experiments (b) (*$P < 0.05$).

3.3. Effects of ATRA on CA II gene transcription

We then examined whether the effects of ATRA on CA II

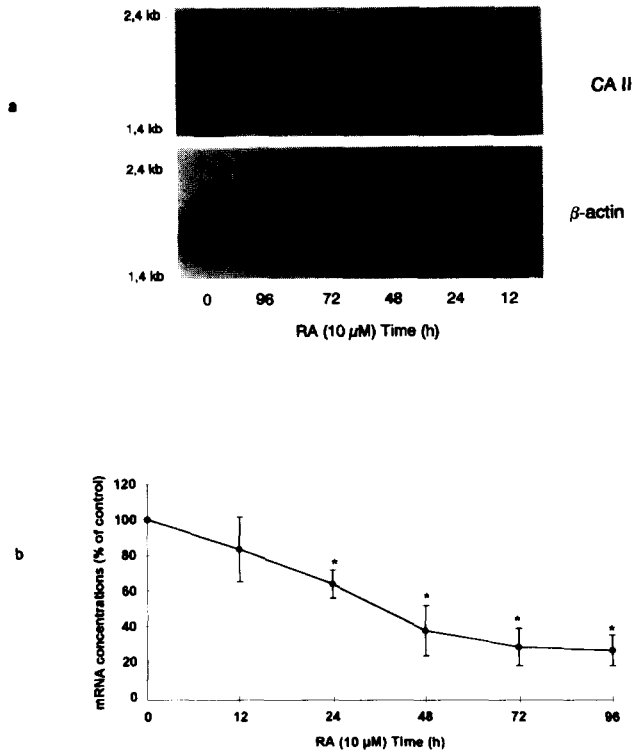


Fig. 3. Effects of ATRA on CA II mRNA concentrations in DANG cells. Shown is a representative Northern blot experiment (a) and the statistical analysis of 3 independent experiments (b) (*$P < 0.05$).

gene expression were due to a decreased rate of CA II gene transcription. Using the nuclear run on technique in control nuclei and nuclei which had been pretreated with ATRA for 48 h we repeatedly observed a decreased hybridisation signal for CA II in the ATRA pretreated cells (Fig. 5). Densitometric analysis revealed a CA II transcription rate of 23 ± 8% of control ($n = 3$) after ATRA preincubation for 48 h. These inhibitory effects were specific for CA II because β-actin gene transcription was not altered by ATRA under any experimental condition (Fig. 5). The specificity of the hybridisation signal was confirmed by including the RNA polymerase II inhibitor α-amanitin in one aliquot of nuclei, which completely abolished the hybridisation signal (data not shown).

4. Discussion

We have previously shown that *all-trans*-retinoic acid inhibits growth in the human pancreatic carcinoma cell line DANG [5]. This observation might bear important clinical implications in terms of developing new experimental strategies for the treatment of pancreatic carcinoma. Based on these observations we were interested to gain further insights into the effects of ATRA on duct cell specific functions. CA II, by catalyzing the hydration of carbon dioxide, is essential for the bicarbonate production by pancreatic duct cells and therefore represents a central duct cell specific functional molecule. The duct cell restricted expression of CA II has been demonstrated in the nontransformed pancreas of the mouse, guinea pig and human [14–16]. Given that pancreatic carcinomas very often display a highly dedifferentiated phenotype with a frequent loss of duct cell characteristics [17], there is however currently no information available whether the expression of CA II is maintained during malignant transformation of the human pancreatic duct cell. By using a non radioactive in situ hybridisation technique we found a homogenous and duct cell restricted expression of CA II mRNA transcripts in all human pancreatic carcinomas examined. In accordance to the nontransformed pancreas, CA II was not expressed in acinar, endocrine or mesenchymal cells. This observation suggests, that although highly dedifferentiated, human pancreatic carcinomas maintain the expression of the duct cell specific protein CA II during malignant transformation. In analogy to the in vivo data, we also demonstrated the expression of CA II mRNA and protein in the human pancreatic carcinoma cell line DANG; this cell line should therefore serve as a useful model to study the effects of ATRA on malignant duct cell biology. The pleiotropic effects of retinoids are mediated by two families of nuclear receptors, designated retinoic acid receptors (RAR) and retinoid X receptors (RXR) each consisting of three receptor subtypes, named α, β and γ (reviewed in [18,19]). Both, RAR and RXR act as ligand-activated transcription factors, controlling gene transcription initiated from promotors of retinoid regulated genes by interacting with *cis*-acting elements, the so-called RAREs (retinoic acid-responsive elements) [18,19]. We have previously shown by

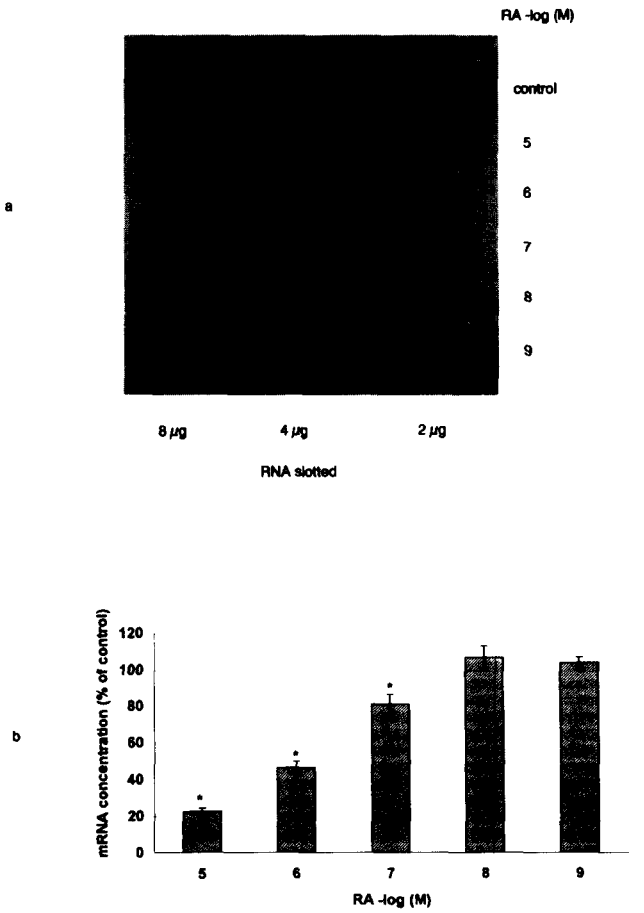


Fig. 4. Dose-dependent effects of ATRA on CA II gene expression. For these experiments DANG cells were incubated with the indicated doses of ATRA for 96 h. Shown is a representative slot-blot experiment (a) and the statistical analyses of 4 independent experiments (b) (*$P < 0.05$).

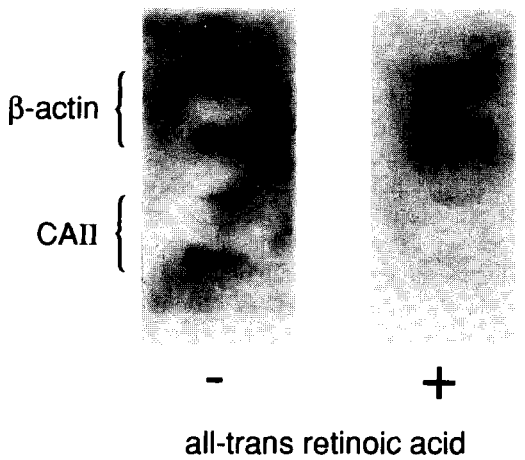


Fig. 5. Effects of ATRA on CA II gene transcription in DANG cells. DANG cells were incubated with 10 μM ATRA or vehicle for 48 h and CA II and β-actin transcription were assessed by nuclear run on assays. Duplicate slots for each cDNA were hybridised to ensure the reproducibility. Shown is a representative of 3 independent experiments yielding nearly identical results.

reverse-transcriptase PCR that the human DANG cell line does express all RAR and RXR subtypes except for the RXRg [5]. It was therefore of great interest how ATRA, besides the inhibition of growth, would interfere with tumor duct cell specific function. We observed a specific, time- and dose-dependent decrease of CA II expression in DANG cells as a consequence of ATRA incubation. This decrease of CA II protein was quantitatively completely explained by a time-dependent decrease of CA II mRNA concentrations, indicating that the effects of ATRA are mainly located at a pretranslational level. Furthermore, the time course of CA II inhibition is in excellent agreement with the previously observed antiproliferative effects of ATRA on DANG cells [5]. Although most of the biological effects of retinoids are believed to occur via transcriptional gene regulation, stabilization of mRNA transcripts and posttranslational stabilization of the protein product by retinoids have previously been described [20,21]. To address this question we performed nuclear run on experiments; CA II gene transcription after 48 h ATRA treatment was reduced to 23 ± 8% of control, which was nearly identical to the CA II mRNA concentrations observed at the same time (27 ± 11% of control). Therefore a decrease in CA II gene transcription is predominantely if not exclusively, responsible for the inhibition of CA II expression in DANG cells. Although the promotor region of the human CA II gene has been cloned and sequenced [22], very few physiological or pharmacological substances have been identified that regulate CA II gene expression. In this context it is of interest, that another member of the steroid hormone superfamily, 1,25-dihydroxyvitamin D_3, exerts opposite effects on CA II gene expression in an osteoclast cell line, characterised by an increase of CA II gene expression [13]. The availability of the promotor region of CA II will now enable us to directly characterize the regulatory sequences responsible for the retinoid mediated inhibition of CA II gene expression in DANG cells.

In summary, we have shown that CA II expression is maintained during malignant transformation of human pancreatic duct cells. Furthermore, we have established the DANG cell line as a valid in vitro model to investigate the effects of retinoids on tumor duct cell function. The observed inhibition of CA II gene transcription during ATRA mediated inhibition of growth needs to be considered when evaluating ATRA as a therapeutical approach for the treatment of human pancreatic cancer.

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