Original Article

Inhibition of the liver expression of arylalkylamine N-acetyltransferase increases the expression of angiogenic factors in cholangiocytes

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Background and aims: Reduction of biliary serotonin N-acetyltransferase (AANAT) expression and melatonin administration/secretion in cholangiocytes increases biliary proliferation and the expression of SR, CFTR and Cl^-/HCO_3^- AE2. The balance between biliary proliferation/damage is regulated by several autocrine neuroendocrine factors including vascular endothelial growth factor-A/C (VEGF-A/C). VEGFs are secreted by several epithelia, where they modulate cell growth by autocrine and paracrine mechanisms. No data exists regarding the effect of AANAT modulation on the expressions of VEGFs by cholangiocytes.

Methods: In this study, we evaluated the effect of local modulation of biliary AANAT expression on the cholangiocytes synthesis of VEGF-A/C.

Results: The decrease in AANAT expression and subsequent lower melatonin secretion by cholangiocytes was associated with increased expression of VEGF-A/C. Overexpression of AANAT in cholangiocyte lines decreased the expression of VEGF-A/C.

Conclusions: Modulation of melatonin synthesis may affect the expression of VEGF-A/C by cholangiocytes and may modulate the hepatic microvascularization through the regulation of VEGF-A/C expression regulating biliary functions.

Keywords: Serotonin N-acetyltransferase (AANAT); vascular endothelial growth factor-A/C (VEGF-A/C); biliary proliferation

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Introduction

The intrahepatic biliary ductal system is lined by simple epithelia, cholangiocytes of different size and functions (1-3). In addition to secrete water and electrolytes (4,5), cholangiocytes are the target cells in a number of human biliary disorders such as primary sclerosing cholangitis and primary biliary cirrhosis, diseases that are characterized by an imbalance between biliary growth and loss (2,6,7). Constitutively, normal rodent cholangiocytes have low proliferative activity (8,9), but proliferate or are damaged in response to experimental maneuverers including ligation of the extrahepatic bile duct ligated (BDL) and acute carbon tetrachloride administration (9).

A number of studies support the concept that biliary development and homeostasis is coordinately regulated by several neuroendocrine autocrine factors including vascular endothelial growth factor-A/C (VEGF-A/C), angiopoietins, serotonin, melatonin and sex hormones (10-17). In fact,

we have previously shown that normal cholangiocytes express the mRNA for VEGF-A/C and secrete VEGF-A/C that are upregulated following BDL (14). We have also shown that VEGFA/C stimulates biliary growth of normal and BDL rats by autocrine/paracrine pathways (14). In support of this concept, we have previously demonstrated that after BDL, the peribiliary plexus (that secrete angiogenic factors such as VEGF) proliferate supporting the increased nutritional needs of the proliferated biliary epithelium (18). However, the proliferation of the peribiliary plexus only occurs after cholangiocytes proliferation (18), supporting the concept that VEGF stimulates biliary growth by an autocrine loop. Another study has shown that cholangiocytes generate a VEGF gradient that is crucial during the migratory stage, when it determines arterial vasculogenesis in their vicinity, whereas angiopoietin-1 signaling from hepatoblasts contributes to the remodeling of the hepatic artery necessary to meet the demands of the developing epithelium (12). VEGF and angiopoietin-1 have autocrine proliferative effect on cholangiocyte growth and paracrine effect on portal vasculature, thus promoting the growth of the cysts and their vascular supply (11).

Serotonin N-acetyltransferase (AANAT), is the key enzyme for the synthesis of melatonin (19), and it is expressed by the pineal gland as well as small intestine and in the liver mostly by cholangiocytes (20). We have recently shown that melatonin secretion inhibits biliary hyperplasia by interaction with type 1 (MT1) receptors (17).

We have recently demonstrated that inhibition of biliary AANAT expression (by administration of Vivo-Morpholino sequences of AANAT) induces an increase in biliary proliferation, ductal mass and increases SR, CFTR, and Cl^-/HCO_3^- AE2 expression (21). We have also shown that *in vitro* overexpression of AANAT in cholangiocyte cell lines decreases the basal proliferative rate and the expression of SR, CFTR, and Cl^-/HCO_3^- AE2 in these cells (21). Thus, based on these findings we propose to demonstrate that *in vivo* and *in vitro* modulation of AANAT biliary expression induces change in the expression of VEGF-A/C, two key trophic factors sustaining biliary proliferation (15,18,22).

Materials and methods

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. The substrate for γ-glutamyltranspeptidase (γ-GT), N (γ-L-glutamyl)-4-methoxy-2-naphthylamide, was purchased from Polysciences (Warrington, PA, USA). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): (I) VEGF-A (JH121) a mouse monoclonal antibody recognizing full length VEGF-A of human origin; and (II) VEGF-C (H-190) a rabbit polyclonal antibody against amino acids 230-419 of VEGF-C of human origin. Commercially available ELISA kits for the measurement of VEGF-A/C levels were obtained from RayBiotech, Inc., Norcross, GA, USA.

The RNeasy Mini Kit for the isolation of total RNA was purchased from Qiagen (Valencia, CA, USA). The Vivo-Morpholino sequences of AANAT (5'-GTTCCCCAGCTTTGGAAGTGGTCCC, to reduce the biliary expression of AANAT) (21) or mismatched Morpholino (5'-GTTCCCGACCTTTGCAACTCGTCCC) were designed and purchased from Gene-tools LCC (Philomath, OR, USA) (21). We have previously used this Vivo-Morpholino approach to reduce the biliary expression of AANAT in the liver (21).

Animal models

Male Fischer 344 rats (150-175 gm) were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed at 22 ℃ with 12:12 hr light/dark cycles. The animals had free access to standard rat chow and drinking water. We used normal and BDL rats that immediately after surgery were treated with Vivo-Morpholino sequences of AANAT or Morpholino mismatched (1 mg/kg BW/day) for one week via an implanted portal vein catheter as described by us (21). To minimize the amount of Vivo-Morpholino that circulates outside of the liver, we used a lower dose (1.0 mg/kg BW/day) (21) of Vivo-Morpholino than that used in a previous study (3.0 mg/kg/day) (23). Animal experiments were performed in accordance with a protocol approved by the Scott & White and Texas A&M Health Science Center IACUC.

Freshly isolated cholangiocytes and immortalized large murine biliary lines

Cholangiocytes (by γ-GT histochemistry) (24) were isolated by immunoaffinity separation (25,26) using an antibody (by Dr. R. Faris, Brown University, Providence, RI, USA) recognizing an unidentified antigen expressed by all intrahepatic cholangiocytes (27). Immortalized large murine cholangiocytes (MCLs, obtained from large bile ducts) (28) display morphological and functional traits similar to that of freshly isolated large cholangiocytes (21,29). MCLs were

cultured as described by us (21,29).

Evaluation of VEGF-A/C levels in liver sections, cholangiocytes and total liver lysates

We first evaluated by semi-quantitative immunohistochemistry (30) the percentage of cholangiocytes positive for VEGF-A/C (key angiogenic factors regulating biliary function) (11,14) in liver sections from the selected groups of rats. Immunohistochemical pictures were taken in a coded fashion by a BX-51 light microscope (Olympus, Tokyo, Japan) with a Videocam (Spot Insight; Diagnostic Instrument, Inc., Sterling Heights, MI, USA) and analyzed with an Image Analysis System (IAS; Delta Sistemi, Rome, Italy). Negative controls were included. The percent of bile ducts positive for VEGF-A/C in liver sections was evaluated as described by us (21,26). When 0-5% of bile ducts were positive we assigned a negative score; a +/- score was assigned when 6-10% of ducts were positive; a + score was assigned when 11-30% of bile ducts were positive (26).

We evaluated by real-time PCR (31) the expression of VEGF-A/C in total RNA of liver tissue and cholangiocytes from rats treated with mismatch or AANAT Vivo-Morpholino for one week. A $\Delta\Delta C_T$ analysis was obtained using normal cholangiocytes or normal total liver as control samples (31). The primers for rat VEGF-A/C (SABiosciences) were designed according to the NCBI GenBank Accession numbers: NM_031836 (VEGF-A); and NM_053653 (VEGF-C). Data were expressed as relative mRNA levels ± SEM of the selected gene to glyceraldehyde-3-phosphate dehydrogenase (GAPDH NCBI GenBank Accession NM_017008) ratio.

We next determined VEGF-A/C levels in cholangiocyte and total liver lysates from the selected groups of animals by ELISA kits according to the instructions provided by the vendor.

Overexpression of AANAT in MCL and measurement of VEGF-A/C expression

MCL were transfected using an AANAT cDNA clone vector from OriGene Technologies, Inc. (Rockville, MD, USA) as described by us (21). Transfected cells were selected by the addition of 10 µL/mL geneticin into the media and the selection process was allowed to continue for 4-7 days (21). Surviving cells (MCL-AANAT) were assessed for the relative expression of AANAT compared to the control transfected cells (MCL-puro) by real-time PCR and FACS analysis as described previously by us (21). The clone with the greatest degree of overexpression was selected as described by us (21). In these cells, we measured mRNA of VEGF-A/C by real-time PCR (31) (see above). A $\Delta\Delta C_T$ analysis was obtained using MCL-pure as control samples (31). Data were expressed as relative mRNA levels ± SEM of the selected gene to GAPDH ratio.

Statistical analysis

All data are expressed as mean ± SEM. Differences between groups were analyzed by Student's unpaired *t*-test when two groups were analyzed and ANOVA when more than two groups were analyzed, followed by an appropriate post hoc test.

Results

Effect of AANAT knockdown on the expression of VEGF-A/C levels in liver sections, cholangiocytes and total liver lysates

We have previously validated our model showing that reduction of biliary AANAT expression (by Vivo-Morpholino) was associated with enhanced biliary proliferation and IBDM (21). Concomitant with increased cholangiocyte proliferation, we have shown that the protein expression of VEGF-A/C increased in intrahepatic bile ducts from normal and BDL rats treated with AANAT Vivo-Morpholino compared to controls (*Figure 1* and *Table 1*). Furthermore, VEGF-A/C levels were higher in the lysate of total liver and isolated cholangiocytes from BDL rats compared to normal rats (*Table 2*). VEGF-A/C levels increased in the lysate of total liver and cholangiocytes from both normal and BDL rats treated with AANAT Vivo-Morpholino compared to mismatch-treated rats (*Table 2*). There was increased expression of the mRNA VEGF-A/C in total liver and cholangiocytes from BDL rats treated with AANAT Vivo-Morpholino compared to control animals (*Figure 2A,B*).

Effect of overexpression of AANAT in MCL on the expression of VEGF-A/C

In cholangiocyte lines that stably overexpress AANAT, we demonstrated that there was reduced mRNA expression for VEGF-A/C compared to control cholangiocytes (*Figure 3*).

Discussion

Our study demonstrates that concomitant with reduced

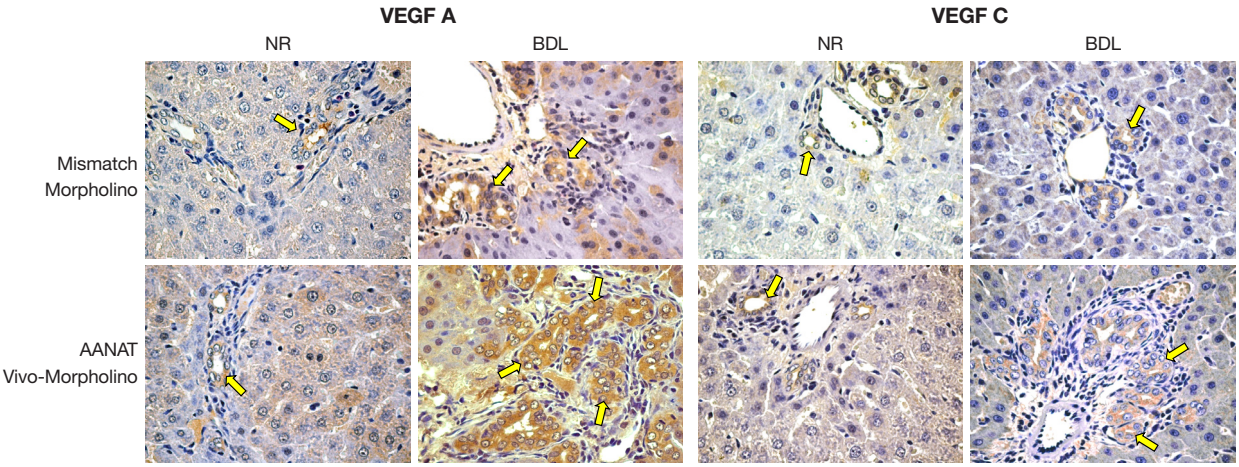

Figure 1 The protein expression of VEGF-A/C increased in bile ducts from normal and BDL rats treated with AANAT Vivo-Morpholino compared to controls (for semiquantitative data see *Table 1*). Analysis was performed in ten randomly selected fields of three slides. Original magnification: ×40. VEGF-A/C, vascular endothelial growth factor-A/C; BDL, bile duct ligated; AANAT, serotonin N-acetyltransferase.

Table 1 Evaluation of the percentage of cholangiocytes positive for VEGF-A/C in liver sections

Groups	VEGF-A (%)	VEGF-C (%)
Normal rats + mismatch Morpholino	+/–	–/+
Normal rats + Vivo-Morpholino	+	+
BDL rats + mismatch Morpholino	++	++
BDL rats + Vivo-Morpholino	+++	+++

When 0-5% of bile ducts were positive we assigned a negative score; a +/– score was assigned when 6-10% of ducts were positive; a + score was assigned when 11-30% of bile ducts were positive. VEGF-A/C, vascular endothelial growth factor-A/C; BDL, bile duct ligated.

Table 2 Evaluation of VEGF-A/C levels in lysate from total liver samples and isolated cholangiocytes

Parameters (pg/mL)	Normal rats + mismatch Morpholino	Normal rats + AANAT Vivo-Morpholino	BDL rats + mismatch Morpholino	BDL rats + AANAT Vivo-Morpholino
Total liver VEGF-A levels	101.7±25.2 (n=6)	115.8±25.5[a] (n=6)	125.8±25.5[b] (n=6)	156.2±19.6[c] (n=6)
Cholangiocyte VEGF-A levels	36.9±1.8 (n=3)	45.1±2.8[a] (n=3)	48.6±1.8[b] (n=3)	56.2±0.4[c] (n=3)
Total liver VEGF-C levels	323.2±68 (n=3)	567.9±23[a] (n=3)	701.5±22.8[b] (n=3)	1,157±142.4[c] (n=3)
Cholangiocyte VEGF-C levels	1,142.1±67 (n=3)	1,323.2±81.5[a] (n=3)	1,668.3±58[b] (n=3)	1,841±23.5[c] (n=3)

Data are mean ± SEM. [a], P<0.05 *vs*. the corresponding value of normal rats treated with mismatch Morpholino; [b], P<0.05 *vs*. the corresponding value of normal rats treated with mismatch Morpholino AANAT Vivo-Morpholino; [c], P<0.05 *vs*. all the other groups. Abbreviations: VEGF-A/C, vascular endothelial growth factor-A/C; AANAT, serotonin N-acetyltransferase; BDL, bile duct ligated.

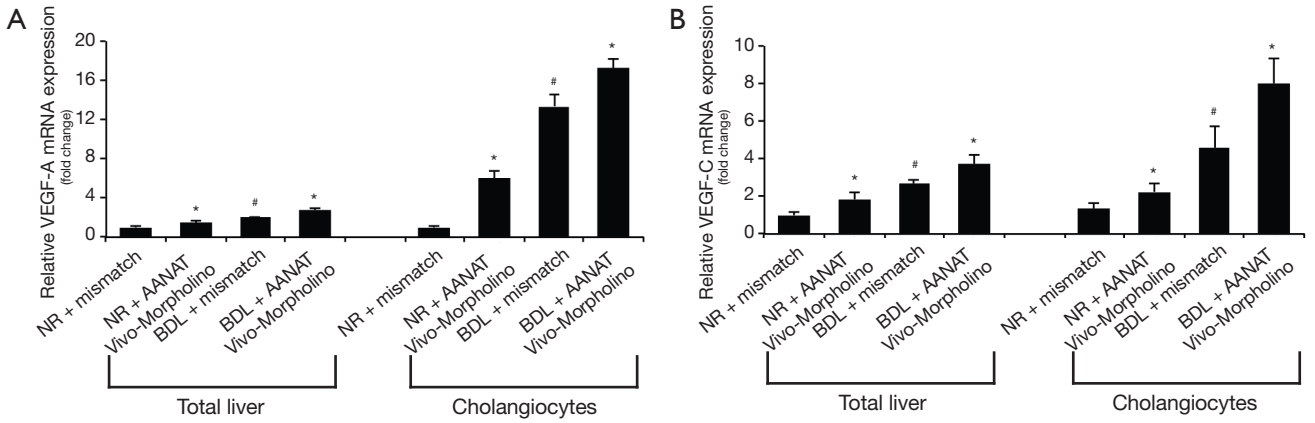

Figure 2 Effect of AANAT knock-down on the mRNA of VEGF-A/C in lysate from total liver samples and isolated cholangiocytes. There was increased expression of VEGF-A/C in total liver and cholangiocytes from normal and BDL rats treated with AANAT Vivo-Morpholino compared to controls. Data are mean ± SEM of six experiments. #, P<0.05 *vs.* the corresponding value of normal rats treated with mismatch Morpholino. *, P<0.05 *vs.* the corresponding value of normal and BDL rats treated with mismatch Morpholino. AANAT, serotonin N-acetyltransferase; VEGF-A/C, vascular endothelial growth factor-A/C; BDL, bile duct ligated.

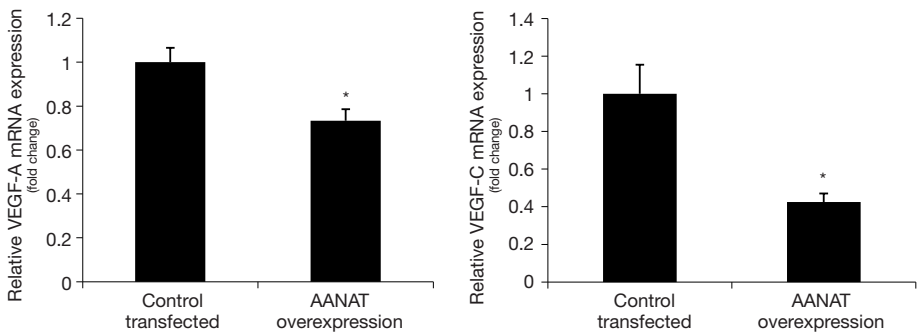

Figure 3 In cholangiocytes overexpressing AANAT, there was decreased mRNA expression for VEGF-A/C compared to control cholangiocytes. Data are mean ± SEM of six experiments. *P<0.05 *vs.* the corresponding value of cholangiocytes transfected with control vector. Abbreviations: VEGF-A/C, vascular endothelial growth factor-A/C; AANAT, serotonin N-acetyltransferase.

AANAT biliary expression (by *in vivo* administration of Vivo-Morpholino), there was enhanced immunoreactivity for VEGF-A/C in liver sections from AANAT Vivo-Morpholino treated rats compared to controls. There was increased mRNA expression for VEGF-A/C in total liver samples and cholangiocytes from normal rats treated with AANAT Vivo-Morpholino compared to controls. *In vitro* overexpression of AANAT in cholangiocytes decreased the biliary expression of VEGF-A/C.

There is growing information regarding the autocrine/paracrine regulation of cholangiocyte proliferation by neuroendocrine factors (e.g., serotonin, VEGF-A/C, secretin and gastrin) which down- and/or up-regulate the growth of the biliary epithelium (10,16,25,32). In a previous study, we found a cross-talk mechanism between cholangiocytes and endothelial cells that mediates the adaptive changes of these cells during liver damage that involves VEGFs (33). The blockage of VEGF secretion decreases cholangiocyte proliferation revealing an autocrine loop, wherein cholangiocytes secrete VEGF interacting with VEGF-R2/R3 to increase biliary proliferation (14). Furthermore, in cholangiocytes from polycystic liver disease samples, VEGF expression is upregulated sustaining cholangiocyte proliferation via autocrine mechanisms (11).

Recently we have also showed the importance of the autocrine role of melatonin (secreted by cholangiocytes) in the regulation of biliary hyperplasia (17,21). In fact, we found that the reduction of biliary AANAT expression and melatonin secretion in cholangiocytes (following AANAT Vivo-Morpholino administration) increases biliary

proliferation and increases the expression of SR, CFTR and Cl^-/HCO_3^- AE2, confirming that the AANAT expression and melatonin secretion axis is an important autocrine loop in the local regulation of biliary proliferation (21).

As known, after BDL, the intrahepatic biliary epithelium undergoes cholangiocyte proliferation (4,8,25,34), which leads the expansion of the bile duct mass, which is followed by an adaptive proliferation of the peribiliary plexus (PBP) (15). The PBP origin from the hepatic artery and nourishes the biliary tree (15). Changes in intrahepatic bile duct mass are always associated with changes of the PBP architecture (15,22). After BDL, the increase in intrahepatic bile duct mass is followed by a parallel growth of the PBP (and its circulating factors including VEGF) (15), which is fundamental in sustaining the enhanced nutritional and functional demands of proliferating cholangiocytes (4,8,15,35).

Since proliferation of the PBP follows, in order of time, the proliferation of bile ducts (15), it is reasonable to suppose that proliferating cholangiocytes modulate the adaptive response of the vascular bed. Consistently, proliferating cholangiocytes express VEGF-A/C and secrete VEGFs (14), which modulates cholangiocyte proliferation by autocrine mechanisms. However, we have demonstrated that cholangiocytes VEGFs secretion increases in BDL AANAT-Vivo Morpholino treated rats. The regulation of biliary VEGF-A/C expression by AANAT levels is of particular importance since these two angiogenic factors sustain biliary growth by autocrine mechanisms (14,36). In support of the current findings, the anti-angiogenic activity of melatonin has been demonstrated in advanced cancer patients (37). Also, melatonin suppresses tumor angiogenesis by inhibiting HIF-1alpha stabilization under hypoxia (38). Since melatonin may affect the angiogenesis of the hepatic microvascularization sustaining biliary functions (33), pharmacological targeting of AANAT may be beneficial for the modulation of biliary disorders.

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