Inhibition of DNA methylation increases follistatin expression and secretion in the human adrenocortical cell line NCI-H295R

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Abstract

Activin affects adrenocortical steroidogenesis and increases apoptosis, while follistatin (FS) acts as an activin antagonist by binding to activin, preventing attachment to its receptors. The regulation of FS expression in the adrenal cortex is poorly understood. Adrenocortical tumors often display aberrant methylation. In the present study, we investigated the effect of DNA methylation on FS mRNA expression and peptide secretion in adrenocortical cells. We treated human NCI-H295R adrenocortical cells with the methylation inhibitor 5-Aza-2'-deoxycytidine (Azad; 0.1–100 μ M for 1, 4 or 7 days) and measured FS mRNA expression by Northern blot and quantitative real time RT-PCR analyses as well as FS secretion by specific ELISA.

Methylation-specific PCR showed decreased methylation in the FS promoter region after Azad treatment. A significant ($P < 0.05$) time- and dose-dependent increase in FS mRNA expression (up to 4.6-fold) and peptide secretion (up to 17.1-fold) was detected after Azad treatment. We conclude that FS gene expression and peptide secretion in NCI-H295R adrenocortical cells are regulated by DNA methylation. Thus, variable methylation in different adrenocortical tumors may influence activin bioactivity and its consequences in steroidogenesis and cell proliferation/apoptosis.

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Introduction

Follistatin (FS) is a single-chain polypeptide originally isolated from ovarian follicular fluid and described as a follicle-stimulating hormone secretion inhibitor. It has, however, far more diverse effects, many of which are due to its ability to bind and thereby neutralize activin and other TGF- β superfamily members (de Winter *et al.* 1996, Phillips & Kretser 1998). Different splicing sites in the FS gene (chromosome 5; 6 kb) give rise to two pre-proteins, which are further cleaved to yield the mature FS peptides of 315 (FS315), 300 (FS300) and 288 (FS288) amino acids. The FS isoforms have different distribution and mechanisms of activin antagonism. After FS288 has bound to activin, the FS-activin complex binds to cell surface proteoglycans thereby preventing activin from attaching to its own receptors (Nakamura *et al.* 1991, Sidis *et al.* 2005). FS315, on the other hand, is a soluble form of FS, that is not capable of binding to the cell surface (Schneyer *et al.* 2004). The core promoter of the FS gene is rich in CG-repeats which can be methylated. The physiological importance of FS is shown in the FS gene knock-out mice dying within hours of birth, and in the FS over-expressing mice being infertile (Matzuk *et al.* 1995, Guo *et al.* 1998).

Little is known about the significance of FS and activins in the human adrenal cortex although they are expressed in human fetal and adult adrenocortical cells (Munro *et al.* 1999, Vanttinen *et al.* 2002). Interestingly, activin A suppressed cortisol and androgen production as well as the expression of steroidogenic enzyme genes in human adrenocortical cells (Vanttinen *et al.* 2003), while in two other recent studies activin increased aldosterone production and the expression of several genes involved in steroidogenesis (Wang *et al.* 2003, Suzuki *et al.* 2004). Furthermore, FS was shown to prevent the activin-induced apoptotic cell death in primary cultures of human fetal adrenal cells and in the human adrenocortical cell line NCI-H295R (Spencer *et al.* 1999, Vanttinen *et al.* 2003).

Hypermethylation has been found in adrenocortical tumors (Szyf *et al.* 1990, Gao *et al.* 2002), and adrenocortical steroidogenesis is modulated by changes in the methylation status of the steroidogenic enzyme genes (Liu *et al.* 2004). Methylated DNA sequences (CpG dinucleotides) are unable to bind to transcription factors, which leads to inhibition of gene transcription (Wade 2001). Malignant adrenocortical tumors have often high insulin-like growth factor II (IGF-II) and low H19 (a putative

tumor suppressor) expression (Ilvesmäki *et al.* 1993, Liu *et al.* 1995) associated with increased methylation of the H19 gene promoter (Gao *et al.* 2002). As a modified deoxynucleotide, 5-Aza-2'-deoxycytidine (Azad) can incorporate into newly synthesized DNA and prevent its methylation. It is thus capable of returning the normal transcriptional activity of the genes silenced by DNA methylation (Liang *et al.* 2002). Our previous studies showed that Azad inhibits methylation of the H19 promoter region and reduces the proliferation of NCI-H295R cells (Gao *et al.* 2002, Liu *et al.* 2004). Aberrant DNA methylation (Szyf *et al.* 1990, Gao *et al.* 2002) and altered activin/inhibin subunit expression (Munro *et al.* 1999, Arola *et al.* 2000) have been detected in adrenocortical tumors. Since FS regulates activin bioactivity, changes in FS expression may modify steroidogenesis and cell proliferation/apoptosis in normal and tumorous adrenals. To clarify the role of FS in adrenocortical function and tumorigenesis, we investigated if the expression of the FS gene is regulated by methylation in human adrenocortical carcinoma cells.

Materials and Methods

Cell cultures

NCI-H295R human adrenocortical cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). DMEM-F12 medium containing 2% Ultrosor (Life technologies, Inc.), 1% ITS+1liquid media supplement (Sigma, St Louis, MO, USA), penicillin (100 IU/ml), streptomycin sulfate (100 µg/ml) (Gibco, Invitrogen) and glutamine (0.5 mM) (Gibco, Invitrogen) was used. Cell cultures were maintained at 37 °C in a 95% air/5% CO₂ humidified environment. The media were refreshed every second or third day, and the cells reseeded once a week.

Treatment with Azad

One day after plating 1×10^6 NCI-H295R cells/well on 35-mm plastic culture dishes, the actively dividing cells were refreshed with either plain medium or one with 0.1–100 µM Azad (R&D Systems, Minneapolis, MN, USA; reconstituted according to the manufacturer's protocol). The cells were then cultured for 7 days, and the conditioned medium was collected and total RNA of the cultured cells isolated. This experiment was repeated three times with duplicate wells.

Secondly, at the same time point (1 day after plating 1×10^6 cells/well), the cultured cells were refreshed with either plain medium or with medium containing 10 µM Azad. Total RNA of the cells was isolated and the conditioned medium collected after 1, 4 and 7 days of treatment to yield the medium from 0 to 24 h, 24 h to

4 days and 4 to 7 days of treatment, respectively. The cells not harvested were refreshed after 1 and 4 days of treatment to assure the availability of Azad in the medium. The experiment was performed in triplicate wells for each time point, and it was repeated three times.

RNA preparation and reverse transcription

The total RNA of the cells on the culture plates was extracted with TriZol Reagent (Life Technologies). Trace amounts of genomic DNA were removed from the total RNA samples with DNase treatment (DNA-free, Ambion, Austin, TX, USA) according to the manufacturer's instructions. The concentration and purity of the RNA samples were analyzed spectrophotometrically and the integrity was confirmed with standard agarose gel electrophoresis. cDNA was synthesized using High-Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Reverse transcription reactions were performed in the total volume of 20 µl containing 2 µg DNase treated total RNA, $1 \times$ reaction buffer, dNTP mixture, random primers and 50 U MultiScribe reverse transcriptase. Reaction mixtures were incubated at 25 °C for 10 min followed by incubation at 37 °C for 2 h.

Quantitative real-time PCR

Quantitative real-time PCR was carried out in the Applied Biosystems 7500 Real Time PCR System using TaqMan gene expression assays for the two major FS splice variants encoding FS288 and FS315 (assay ID Hs00246260_m1 and Hs01121164_m1, respectively). β-Glucuronidase (assay ID Hs99999908_m1) was chosen for endogenous control as it showed least variation after Azad treatments in TaqMan human endogenous control plate (Applied Biosystems). Standard series of five dilutions containing 96, 24, 12, 3 and 1 ng template cDNA were prepared from pooled sample cDNAs. Sample dilutions comprised of 12 ng template cDNA. All standards and samples were run in the total volume of 20 µl in triplicate.

Northern blot

The extracted RNA was transferred onto a nylon membrane by the Northern blot technique (Liu *et al.* 1994) and hybridized for FS mRNA. Shortly, 20 µg total RNA, $10 \times$ MOPS, deionized formamide, 37% formaldehyde and ethidium bromide in H₂O were used. The RNA samples were size-fractionated in denaturing 1.0% agarose-gel (Pronadisa, Alcobendas, Spain). After the electrophoresis, RNA was transferred onto Hybond-N+ nylon filters (Amersham Pharmacia Biotech), and fixed by u.v. cross-linking. The prehybridization and hybridization techniques were used as previously described (Liu *et al.*

1996). The relative intensities of autoradiographic signals were quantified by densitometric scanning. An antisense oligonucleotide probe for FS (5'-GCC CCC GTT GAA AAT CAT CCA CTT GAA GAG-3') recognizing the different FS mRNA splice variants was used, and the RNA data were normalized with the respective 28S ribosomal RNA signals.

Immunoassay for human FS

The FS concentrations were measured from conditioned culture media samples with ELISA (Quantikine DFN00; R&D Systems, Inc., Minneapolis, MN, USA); the assay was performed according to the protocol provided with the kit. The assay is a solid-phase ELISA based on the quantitative sandwich immunoassay technique with monoclonal antibodies specific for FS (recognizing the FS288, FS300 and FS315 isoforms).

PCR-based methylation analysis of the FS gene promoter

To analyze the methylation status of the FS gene promoter area, a PCR analysis based on the inability of HpaII to cut methylated CCGG sequences was used as previously described (Singer-Sam *et al.* 1990, Gao *et al.* 2002). Shortly, genomic DNA was isolated (Liu *et al.* 1997) and DNA was then digested for 6 h with HpaII/MspI enzymes (Roche Molecular Biochemicals) according to the manufacturer's recommendations. The PCR reactions were performed in 20 µl containing 0.2 µM of each dNTP, 1× reaction buffer, 1.75 mM MgCl₂, 1.3% DMSO, 0.5 µM forward and reverse primers, 1.5 U Taq DNA polymerase (Fermentas, Tamro Corporation, Vantaa, Finland) and 50 ng of template DNA. Optimal PCR conditions (95 °C for 1 min, 58 °C for 30 s and 72 °C for 1 min; 40 cycles and final extension at 72 °C for 10 min) with FS promoter primers, 5'-GCA GGA CTT GAA GTG GGT GT-3' (sense) and 5'-CGA TTC AAT GGA CGT CAG AA-3' (antisense), produced a clean 367-bp PCR product from undigested genomic DNA, but no product from DNA digested with MspI (a methylation-insensitive isoschizomer of HpaII). The PCR primers were chosen to amplify the promoter region where CpG dinucleotides comprise 8% of the sequence. IGF-II exon 9 was used as an internal control for DNA amount; this region has no HpaII/MspI cutting site (Gao *et al.* 2002). The PCR products were resolved on 2% agarose gels. The PCR-based analyses were performed at least twice to ensure reproducibility of the results.

Statistical analyses

To test the overall effect of Azad on FS expression and FS peptide secretion, the non-parametric Kruskal-Wallis test was used. The independent samples non-parametric

Mann-Whitney test was used to determine whether FS mRNA expression or FS peptide secretion differed significantly between the control and Azad treated cells at any time point or between the different Azad concentrations after 7 days of treatment. The same test was used for the Northern blot data. Differences were regarded significant if the *P*-value was <0.05.

Results

The basal FS secretion depended expectedly on the cell number and duration of culturing. The unconditioned cell culture medium contained no detectable amounts of FS peptide as measured by the FS ELISA. Since Azad has previously been shown to reduce proliferation in this cell line (Liu *et al.* 2004) and because a lower number of cells was seen in the Azad treated than in the control wells under phase contrast microscopy, all FS peptide results were normalized by the total RNA amount. The RNA amount was chosen for a marker of cell number since RNA extractions and measurements are more quantitative and reliable than those of DNA. The mean total RNA amounts in the Azad treated (10 µM) wells were 81%, 46% and 11% of the control after 1, 4 and 7 days of treatment, respectively. Basal FS secretion from the cells ranged 339–600 pg/µg total RNA/day (mean 459). Since the absolute concentrations of FS in these conditioned media are not of special interest and since they are highly dependent on the culture environment, we express the FS results in relation to the respective controls.

Our initial studies with Northern blot hybridizations showed an increase in FS mRNA accumulation after 10 µM Azad treatment for 7 days (mean increase 2.3-fold, *n*=4, *P*<0.05; Fig. 1). To further clarify the effect of Azad on FS mRNA expression, we used quantitative real time RT-PCR for measuring the two major splice variants (encoding FS288 and FS315). We found that Azad treatment (0.1–100 µM for 7 days) increased FS mRNA expression dose-dependently. 10 µM Azad increased FS288 and FS315 mRNA 4.6 and 3.8-fold respectively, but higher Azad concentrations resulted in a lower increase in FS mRNA expression (Fig. 2A–B). FS peptide secretion into the culture media was also up-regulated dose-dependently in the same treatments (up to 10.4-fold, Fig. 2C). In contrast to the FS mRNA expression, the maximal effect on FS peptide secretion was seen with the highest Azad dose (100 µM). In time course experiments, 10 µM Azad increased both FS288 and FS315 mRNA accumulation in a time-dependent manner (1.3 and 1.05–; 2.5 and 2.7–; 3.2 and 3.1-fold increase after 1, 4 and 7 days, respectively; Fig. 3A–B). FS secretion increased at a slightly slower rate, reaching maximum (17.1-fold increase) at the last 7-day time point (Fig. 3C).

To verify that the effect of Azad on FS mRNA expression and peptide secretion associated with a change

Figure 1 The effect of 10 μ M 5-Aza-2'-deoxycytidine (Azad) on total FS mRNA expression in adrenal NCI-H295R cells after a 7-day treatment. (A) A representative Northern blot autoradiograph. 28S ribosomal RNA was used as a loading control. (B) The graph shows the pooled data (mean $\pm$ S.E.M.) derived from four independent experiments with the control values adjusted to 1. * $P < 0.05$ compared with the control (c).

in the methylation status of the FS gene promoter, we performed a methylation specific PCR analysis for both Azad treated (10 μ M; one week) and control cells. The analysis revealed that the methylation of the FS gene promoter region was indeed reduced after Azad treatment (Fig. 4).

Discussion

Treatment of NCI-H295R adrenocortical cells with the DNA methylation inhibitor Azad increased FS mRNA expression. To our knowledge, this is the first report showing that FS gene expression is influenced by DNA methylation. The increase in FS gene expression was seen only when Azad treatment was started while the cells were dividing. Confluent cell cultures had only a minimal increase in FS mRNA levels after Azad treatment (data not shown). This supports the assumption that Azad affects mainly the (new) replicating DNA. The increase in FS mRNA expression was followed by enhanced

Figure 2 The effect of varying concentrations (0–1 to 100 μ M) of Azad on FS288 (A) and FS315 (B) mRNA expression and FS peptide secretion (C) in adrenal NCI-H295R cells in a 7-day incubation. The graphs show the pooled data (mean $\pm$ S.E.M.) from three independent experiments with the control (Azad concentration 0 μ M) adjusted to 1. * $P < 0.05$ compared with the control.

FS peptide secretion. The mechanism through which Azad up-regulates FS gene expression is probably the inhibition of methylation in the promoter area of the FS

Figure 3 The effect of Azad (10 μ M) on FS288 (A) and FS315 (B) mRNA expression, as well as FS peptide secretion (C) after 1, 4 and 7 days in culture. FS peptide secretion at 1, 4 and 7 d respect the secretion from 0 to 24 h, 1 to 4 and 4 to 7 days of Azad treatment, respectively. The graphs show the pooled data (mean $\pm$ S.E.M.) from three independent experiments with the control (c) for each time point adjusted to 1. * $P < 0.05$ compared with the control at the same time point.

Figure 4 The effect of Azad (10 μ M for 7 days) on the degree of methylation of the FS gene promoter, as analyzed by methylation specific PCR. HpaII treatment followed by FS promoter specific PCR produced a less intact PCR product from the Azad treated than from the non-treated control (c) cells.

gene as suggested by our methylation specific PCR analysis. FS promoter region contains plenty of CpG dinucleotides (about 8% of all nucleotides) and several restriction sites for the methylation sensitive enzyme HpaII. Nevertheless, since Azad affects the expression of several other genes as well, secondary pathways leading to increased FS expression cannot be excluded.

Hypomethylation of the FS promoter associated with a 2- to 5-fold increase in FS mRNA expression after one week of Azad treatment. Furthermore, FS peptide secretion from the Azad treated cells between the 4th and 7th day of treatment was up to 17-fold compared with that from the control cells. It is of interest that after 7 days of treatment FS peptide accumulation was highest with the Azad dose of 100 μ M, but the FS mRNA expression was highest with 10 μ M Azad. Since the real time RT-PCR results were normalized with the endogenous control, the cell number should not affect the mRNA expression data. The smaller increase in FS mRNA levels with the highest Azad doses (compared with 10 μ M) might be explained by the sensitivity of FS mRNA to the possible toxic effect of Azad.

According to our preliminary findings, activin A secretion remains unchanged after Azad treatment. Thus, hypomethylation of the FS gene promoter could lead to relative abundance of FS, which can inhibit the effects of activin (and other TGF- β family peptides) on steroidogenesis and apoptosis in adrenocortical cells (Spencer *et al.* 1999, Vanttinen *et al.* 2003). If, on the other hand, FS gene promoter is hypermethylated, FS gene transcription and translation will be suppressed leaving more activin available. It is of interest whether the methylation status

of FS gene differs between normal and malignant adrenal cells. If that is the case, the FS/activin balance could have a role in adrenal tumor pathogenesis or progression. This remains to be studied further.

To conclude, our data indicate that DNA methylation affects FS gene expression and peptide secretion in adrenocortical cells, which may have a role in the regulation of adrenal steroidogenesis and cell proliferation/apoptosis via modulating the bioactivity of activin and other members of the TGF- β peptide family.

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