Promoter methylation is associated with the age-dependent loss of N-cadherin in the rat kidney

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Aging is associated with alterations in organ structure and function, with the human kidney vulnerable to significant structural and functional deficits during aging (9). The normal kidney loses about 25% of its mass during aging, with both glomeruli and tubules in the cortex affected (24). There is also an enhanced susceptibility of the aged kidney to physiological and xenobiotic insult (2), which may explain the increased incidence of acute renal failure in older patients (23, 41). It becomes important therefore to identify and understand critical changes that occur in the kidney during aging that lead to reduced function, as well as an increased susceptibility to insult.

Cadherins are a large family of transmembrane molecules that mediate calcium-dependent intercellular adhesion between cells of all solid tissues (34). Intercellular adhesion is required for membrane integrity, cell polarity, and vectorial transport, all of which are critical for normal renal function. In fact, several human nephropathies are associated with loss of cadherin function (36). Intercellular adhesion between proximal tubule epithelial cells involves several distinct complexes such as zona occludens (tight junction), desmosomes, and zona adherens (adherens junction), with each complex containing several combinations of proteins. The adherens junction, the predominant regulator of intercellular adhesion, is composed of classical cadherins, which include E- and N-cadherin, with N-cadherin being the predominant cadherin expressed in adult human and rat proximal tubular epithelial cells (26). We previously reported a selective loss of N-cadherin in the proximal tubules of aging male Fischer 344 rats (19). This effect was not seen in other organs such as the liver, brain, and testes, where N-cadherin is also expressed. Interestingly, the age-dependent loss of N-cadherin was reversed by caloric restriction, suggesting that the loss of N-cadherin may be important in the development of age-dependent nephropathy. The loss of mRNA parallels that of protein expression, suggesting a transcriptional mechanism underlying the age-dependent loss of N-cadherin.

DNA methylation is an important mechanism for suppressing gene expression. The mechanism is an epigenetic alteration that has been implicated in embryogenesis, development, and silencing of X-linked and imprinted genes (28). The level and pattern of methylation in the adult are cell and tissue specific, suggesting that this is a critical mechanism regulating gene expression (7, 33). In vertebrates, methylation occurs exclusively at cytosine-guanine (CpG) dinucleotide sites, and ~60–90% of CpG in the genome of adult mammals is thought to be methylated (5). However, the CpG sites in promoter regions are essentially unmethylated. DNA methylation of CpG islands (clusters of CpGs) within gene regulatory elements such as promoters generally suppresses their function (7), but the repression of affected genes is reversed upon treatment with methylation inhibitors such as 5-azacytidine and 5′-aza-2′-deoxycytidine (8, 18, 25). There is evidence to suggest that members of the cadherin family are affected by DNA methylation. CpG hypermethylation of the promoter region was found in the human N-cadherin gene and observed basic promoter activity in a 3,992-bp flanking region of the rat N-cadherin gene; a similar CpG profile was found in the human N-cadherin 5′ flanking region. Methylation-specific PCR analysis demonstrated that the promoter region of N-cadherin is heavily methylated in aged, but not young, rat kidney. Interestingly, the promoter is not methylated in age-matched, calorically restricted animals. In contrast, the promoter region is not methylated in either young or aged rat liver; this corresponds to the finding that aging is not associated with decreased N-cadherin expression in the liver. In addition, N-cadherin expression is markedly induced in NRK-52E cells treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine, further suggesting that methylation at CpG in the promoter region may underlie the age-dependent decrease in renal N-cadherin expression.

aging; caloric restriction; adherens junction

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cadherin (cadherin-13) gene expression in cancer was attributed to the hypermethylation of its 5′ guanine-cytosine-rich sequences (17, 30). Sheng et al. (32) also reported the suppression of N-cadherin expression in the versican V1-transfected NIH3T3 fibroblast through methylation of its promoter. Interestingly, aging is linked to changes in methylation status of sequences in or near regulatory elements of specific genes, and the methylation status may increase (hypermethylation) or decrease (hypomethylation), depending on the tissue and the gene (28). Therefore, in the present study, we have investigated the hypothesis that promoter DNA hypermethylation is the mechanism underlying the silencing of N-cadherin gene in aging kidney.

**MATERIALS AND METHODS**

**Cloning of the rat N-cadherin 5′ flanking sequence.** A 3,992-bp of the 5′ flanking region of the rat N-cadherin gene was amplified by PCR from the CH-2309F9 rat BAC library (CHORI) using Takara LA Taq. EcorI I and SnaB I restriction sites were added to the 5′-end of the sense and antisense primers, respectively. The PCR product was TOPO cloned 5′ to the β-galactosidase (β-gal) gene of the pBlue-TOPO reporter vector (Invitrogen). The construct was confirmed by restriction digestion with EcorI I and SnaB I and DNA sequencing in both directions using T7 and gene specific primers. The DNA sequence was analyzed with MatInspector V2-2 professional software both directions using T7 and gene specific primers. The DNA sequence was analyzed with MatInspector V2-2 professional software both directions using T7 and gene specific primers. The DNA sequence was analyzed with MatInspector V2-2 professional software both directions using T7 and gene specific primers.

**Biosystems).** Briefly, cell lysates were centrifuged at high speed for 2 min. Activity was expressed as the relative luminescence units. The difference in N-cadherin expression between young and old kidney and liver samples was analyzed using a t-test for independent samples.

**Western blot analysis.** Kidneys and livers were obtained from male Fischer 344 rats, and tissue was homogenized in 500 μl of lysis buffer (10 mM Tris, pH 7.4, 4% SDS, 1 mM leupeptin, 1 mM PMSF, 1 mM sodium orthovanadate). In certain experiments, NRK-52E cell lysates were prepared using the same lysis buffer. Proteins were quantified by the Bradford assay, and 30 μg of sample protein was separated by 8% SDS-PAGE and transferred to nitrocellulose membrane (Hybond C, Amersham). The membrane was incubated with a monoclonal antibody against N-cadherin (BD Biosciences, 1:1,000) for 2 h at room temperature. Following several washes, the membrane was incubated with secondary antibody (1:20,000) for 1 h at room temperature. Membranes were stripped with the Western Blot Recycling kit (Alpha Diagnostic) and reprobed to determine equal protein loading with a monoclonal anti-β-catenin antibody (BD Biosciences, 1:2,000). **MSP.** Genomic DNA was isolated from the kidneys and livers of male Fischer 344 rats (young, aged ad libitum (AL), and aged caloric restriction (CR)) using the DNAeasy kit (Qiagen). Purified DNA samples (2 μg) were modified with sodium bisulfite by use of the EZ DNA Methylation kit (Zymo Research). Primers for methylation-specific PCR (MSP) were designed with the software program MethPrimer (22). The MSP primers used were 5′-tattttcattatgtagtatc-3′ and 5′-attttctctacataaccccctgcg-3′ (methylated forward and reverse, respectively); 5′-tattttcattatgtataggatac-3′ and 5′-attttctctctaataacaaaaa-3′ (unmethylated forward and reverse). MSP was performed in a PTC 200 thermal cycler (MJ Research) using the following conditions: 95°C for 5 min; 35 cycles of 95°C for 45 s, 55°C for 45 s, 72°C for 45 s; and a 10-min final extension time at 72°C. All amplifications utilized JumpStart Taq DNA polymerase (Sigma). PCR products were analyzed on 2% agarose gels. The expected products sizes are 182 and 181 bp for methylated and unmethylated, respectively. Methylated and unmethylated statuses were determined by the presence of expected products in samples amplified with the methylated and unmethylated primers, respectively.

**RESULTS**

**Cloning of the rat N-cadherin promoter: analysis and activity.** We isolated 3,992-bp (from ATG) of the 5′-flanking region of the rat N-cadherin gene (GenBank Accession No. NC_005117) from the rat BAC library (clone CH-2309F9). The 1,672-bp sequence from the ATG start site is shown in
Fig. 1A. The sequence does not contain a TATA box but consists of a GC-rich region. Computer-based analysis of the sequence using MatInspector V2-2 professional software (core similarity 1, matrix similarity 0.95) using Transfac 4.0 matrices showed that the sequence contains several putative transcriptional factor binding sequences for Sp1, MZF1, and AP1 (Fig. 1A) that have been implicated in the regulation of N-cadherin gene in the chicken (21) and human (20). In the characterization
of N-cadherin promoter region in the human, Le Mee et al. (20) identified a minimal 318-bp region with strong promoter activity in human osteoblasts. By use of GenSeqAlignView on Ensembl, the minimal promoter region of human N-cadherin shows 87% identity with the 318-bp 5’ from ATG of rat N-cadherin (Fig. 1B). The 3,992-bp was amplified by PCR and then cloned into pBlue-TOPO β-galactosidase reporter vector. The construct (pBlue-TOPO/3.9-kb) was confirmed to contain the correct insert size and sequence by using added EcoR I and SnaB I restriction sites and by direct sequencing in both orientations. Functional analysis of the 3,992-bp rat N-cadherin promoter was then conducted to confirm the presence of a promoter activity in this region. As shown in Fig. 1C, transfection of NRK-52E cells with pBlue-TOPO/3.9kb construct showed a threefold increase in activity compared with promoterless pBlue-TOPO vector and mock transfection. The results suggest that the 3,992-bp clone fragment contained the promoter of rat N-cadherin.

Identification of a CpG island in the promoter region of N-cadherin. We next examined the 3,992-bp promoter region of rat N-cadherin for CpG sites using Methprimer software and identified clustered CpG dinucleotides characteristic of a CpG island from nucleotides −1,158 to −55 relative to ATG with an observed-to-expected CpG ratio > 0.60. (Fig. 2). Interestingly, a similar CpG island pattern was observed when the 5’ region of the human N-cadherin was analyzed (not shown). Thus promoter methylation may be a mechanism regulating N-cadherin gene expression.

Expression of N-cadherin in aging correlates with promoter methylation. Analysis of mRNA and protein expression by quantitative RT-PCR (Fig. 3A) and Western blot analysis (Fig. 3B) demonstrated that the expression of renal N-cadherin was reduced markedly in the aged (24 mo) rat compared with the young (4 mo). We designed two sets of primers, utilizing Methprimer program, to amplify a fragment within the CpG island of the rat N-cadherin promoter: one primer set specific for the methylated fragment of the CpG island on the promoter and a second one for unmethylated promoter fragment. We then conducted an MSP analysis of the renal N-cadherin promoter in the young and aged-AL rats, using sodium bisulfite-modified DNA as the template. MSP demonstrated that the N-cadherin promoter is heavily methylated in aged rat kidney, but the promoter is unmethylated in young rat kidney (Fig. 3C). Importantly, the promoter was also unmethylated in aged-matched, calorically restricted rats (Fig. 3D). We next carried out similar analyses on rat liver where, N-cadherin is also expressed but not affected by aging (19). As hypothesized, the N-cadherin mRNA and protein expression did not decrease in aged liver as shown in Fig. 4, A and B, respectively; this correlates with MSP analysis, which revealed that the CpG islands in the liver N-cadherin promoter are heavily methylated.

Fig. 1. Cloning of the rat N-cadherin promoter. A: 3,992-bp upstream from ATG was analyzed using MatInspector V2-2 software and the Transfac 4.0 database. The 3,992-bp putative promoter region does not contain a TATA box but showed a high overall GC content, as well as several consensus sequences for transcriptional factors Sp1, MZF1, and AP1 (highlighted) that have been implicated in the regulation of N-cadherin gene in the chicken and human (20, 21). The 1,672-bp sequence upstream from the ATG start site is shown. B: alignment of the 318-bp promoter region of the human N-cadherin promoter with the corresponding rat sequence reveals 87% homology. C: the rat N-cadherin promoter coupled to a reporter construct (β-galactosidase) was transfected into NRK-52E cells and incubated for 48 h. β-Galactosidase activity was measured in the lysates via the Galacto-Star System (Applied Biosystems). Assays were carried out in triplicate using lysates from mock-transfected cells, promoterless pBlue-TOPO, and cells transfected with pBlue-TOPO containing the promoter region of rat N-cadherin (pBlue-TOPO/3.9kb). Each data point represents the mean + SD of 3 replicates; *significant difference from vector control (P < 0.001).
unmethylated in both young and aged rats (Fig. 4C). The results demonstrate that the N-cadherin 5’ CpG island in the promoter region is hypermethylated in aged rat kidney, but not liver, and corresponds with decreased expression.

Inhibition of methyltransferase increases N-cadherin expression in a renal cell line. To confirm whether promoter hypermethylation silences N-cadherin expression, we treated NRK-52E cells with 5′-aza-2′-deoxycytidine. The reagent is a potent inhibitor of DNA methylation with the ability to reactivate the expression of genes silenced by methylation following S-phase dependent incorporation into the cellular DNA (6). As shown in Fig. 5, N-cadherin protein expression is markedly induced in NRK-52E cells treated with 10 μM 5-aza-2′-deoxycytidine. This result provides further evidence for the role of methylation in the regulation of renal N-cadherin, and it supports our hypothesis that hypermethylation of N-cadherin promoter is responsible for the age-dependent decrease in renal N-cadherin expression.

DISCUSSION

Our laboratory reported previously the loss of N-cadherin in the proximal tubules of aging male Fischer rats (19). This loss of N-cadherin was kidney specific and was associated with a loss of polarity in the proximal tubular epithelium; it was attenuated by caloric restriction. These findings suggest that the decreased expression of N-cadherin may represent a critical change that is related to functional deficits associated with aging and end-stage renal disease. In this study, we examined whether promoter hypermethylation is the mechanism that underlies the loss of N-cadherin expression in aging rat kidney given that DNA promoter methylation is a critical mechanism...
for suppressing gene expression in aging (3, 15, 28, 35). The promoter region of N-cadherin had been characterized in the chicken (21) and in human (20), but not in the rat. We provided evidence for the presence of promoter activity in the 3,992-bp (from ATG) of the 5’ flanking region of the rat N-cadherin gene. This region contain multiple binding sites for the Sp1, MZF1, and AP1 transcription factors that are implicated by Li et al. (21) and Le Mee et al. (20) in the regulation of N-cadherin in chicken and human, respectively. Secondly, consistent with a role for DNA methylation in gene regulation, we identified a classical 5’ CpG island in the rat N-cadherin promoter that spans 1,214 bp (from ATG). A similar CpG profile was also located in the human N-cadherin promoter (profile not shown). The rat N-cadherin promoter is extensively methylated in aged but not in young rat kidney, whereas the promoter region remains unmethylated in both young and aged rat liver where N-cadherin is also expressed. Finally, treatment of the NRK-52E cell line with 5-aza-2’-deoxycytidine, an inhibitor of DNA methylation, markedly induced the expression of N-cadherin. Taken together, these data implicate promoter DNA methylation as a likely mechanism that underlies the loss of renal N-cadherin in the aging rat.

Aging is associated with a significant decrease in renal functional reserve (16) and there is an increased susceptibility of the aging kidney to nephrotoxic insults, including ischemia (2). The challenge is to identify critical changes that occur during aging that makes the kidney more susceptible to insults and nephropathies, one of which is acute renal failure. Interestingly, N-cadherin is the predominant cadherin expressed in the proximal segment of adult rat and human kidneys (26). Therefore the loss of N-cadherin may represent a critical change that may be partly responsible for the decreased function of the aging kidney. The finding that the N-cadherin promoter was not methylated in aged-CR rats supports this hypothesis. In addition, N-cadherin, as well as R-cadherin and K-cadherin, is significantly downregulated in hydropneumorotic renal samples (12). Although not much is known about the impact of aging on the cadherin-catenin complex, Goomer et al. (10) reported age-related changes in the expression of mesenchyme specific cadherin-11 (OB-cadherin). They proposed that the age-related changes in the expression of cadherin-11 may be associated with bone remodeling, leading to reduced bone density and high fracture risk in older subjects.

MSP is a PCR-based technique that utilizes a bisulfite-conversion strategy for rapid assessment of the methylation status of CpG sites (13). The technique is extremely sensitive (to 0.1% methylated alleles of a given CpG island focus), and it requires only small quantities of extracted DNA (22). Herman et al. (13) demonstrated the use of the technique to identify promoter region hypermethylation changes associated with transcriptional inactivation of tumor suppressor genes p16, p15, E-cadherin, and von Hippel-Lindau in human cancer. The technique is now widely used for methylation profiling (31). Using the same approach, we evaluated the methylation status of the CpG island in the promoter region of N-cadherin gene from DNA samples extracted from rat kidney and liver (young and old). Our data suggest that methylation at the 5’ CpG island of N-cadherin is responsible for its loss in aging rat kidney. Studies by others have shown that hypermethylation of normally unmethylated CpG islands in the promoter region of members of cadherin family are involved in transcriptional silencing, many of which have been linked to various types of cancer. Yoshiura et al. (39) first implicated hypermethylation as the mechanism of E-cadherin inactivation in human carcinomas. Similarly, the extensive CpG methylation in the promoter region underlies the inactivation of the expression of the E-cadherin in human bladder cancer (27) and in human oral squamous cell carcinoma cell lines HOC-313 and HA-376 (4). Hypermethylation of the 5’ GC-rich sequences of H-cadherin (cadherin-13) is responsible for its reduced expression and loss of activity in human nasopharyngeal epidermoid carcinoma KB cells (17). There are also reports to support the hypothesis that N-cadherin can be regulated by methylation. N-cadherin was among the 13 genes in which 5’ CpG islands were methylated leading to silencing of the genes in primary pancreatic cancers (11); it is also dysregulated in thyroid carcinoma cell lines (14). Yamashita et al. (38) found N-cadherin to be among the methylation-silenced genes in a gastric cancer cell line AGS. Likewise, the suppression of N-cadherin expression in the versican V1-transfected NIH3T3 fibroblast can be attributed to methylation of its promoter region (32). In all of these reports, with the exception of Yoshiura et al. (39), which used a methylation-sensitive restriction enzyme method, the methylation status of the CpG island flanking the genes was revealed by MSP analysis, and the expression of the methylated-silenced genes was reversed upon treatment with methylation inhibitors, consistent with our observations.

Methylation-induced gene repression can occur by direct interference through steric hindrance of transcription factor binding, by alteration of local topology due to attached methyl moieties, or by indirect interference through recruitment of histone deacetylases by methyl-CpG-binding proteins to methylated-CpG dinucleotides leading to transcriptional inactivation (1, 7, 35, 42). An age-related gene silencing mechanism has been proposed in which methylation spreads through the regulatory sequences of key genes, leading to a progressive reduction of gene transcription and ultimately silencing of the gene (3, 35). Furthermore, gene silencing is often mediated by CpG methylation of key protein-binding sites within the regulatory region (35). The 1.214-CpG island that flanks the N-cadherin gene contains multiple putative binding sites for the transcription factors Sp1 and MZF1, both of which have been implicated in the regulation of N-cadherin. It is possible that the methylation of the CpG island prevents these transcription factors from binding, thereby causing repression of N-cadherin in the aged rat kidney. Zhu et al. (42) demonstrated that the methylation of adjacent CpG sites could affect Sp1/Sp3
binding and activity in the p21Cp1 promoter. Thus it is possible that interference with Sp1 and/or MZF1 binding sites by methylation, which increases with aging, is responsible for the loss of N-cadherin in aging rat kidney.

The loss of N-cadherin expression, which increases with age progressively (19), may be associated with loss of normal renal function and may contribute to renal failure in older subjects. Promoter hypermethylation has been demonstrated as the mechanism regulating N-cadherin expression in aging kidney. These results have implications as to our understanding of the regulation of N-cadherin expression, as well as the potential role of methylation in the aging process.

REFERENCES


