c-myc-induced Apoptosis in Polycystic Kidney Disease Is Independent of FasL/Fas Interaction

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Abstract

Apoptosis is a critical early cellular event in the development of polycystic kidney disease (PKD) in humans and mice. In the SBM transgenic model of PKD, both apoptosis and proliferation are c-myc driven and are independent of p53 and Bcl-2 pathways. On the basis of recent evidence implicating the FasL/Fas pathway in c-myc-induced apoptosis, we investigated the potential interaction of these pathways in vivo. We first evaluated the expression of FasL in renal tissues of SBM mice. This analysis showed that the level of FasL expression was elevated 3–4-fold in the SBM kidneys, indicating a potential autocrine suicidal mechanism. We next crossed the SBM mice with gld mice mutated in FasL. The progeny had comparable renal epithelial apoptotic and proliferation rates and a cystic phenotype in all SBM genotypes irrespective of the FasL genotype. Our study proves that c-myc-induced apoptosis can be independent of the FasL/Fas pathway in vivo and implicates the existence of a novel c-myc-driven apoptotic pathway.

Introduction

ADPKD is one of the most frequent human genetic disorders, affecting 1 in 1000 individuals, and constitutes a major cause of end stage renal failure worldwide. The PKD1 gene is responsible for 85–90% of ADPKD cases and is believed to encode an integral membrane protein. ADPKD affects both kidneys, and cyst formation begins early during kidney development. This disorder is characterized by the progressive expansion of renal epithelial cysts involving tubules and glomeruli, leading to remodeling of the renal architecture and physiological dysfunction. Interestingly, the cystic epithelium in adult ADPKD kidneys displays several cellular physiological properties of the developing renal epithelium. We and others have shown that the epithelial cell polarity is frequently altered in cystic and in noncystic renal tubules (1, 2), similar to the immature renal epithelium. Moreover, ADPKD kidneys undergo extensive epithelial proliferation (3, 4) and apoptosis (4, 5), processes also prevailing during renal organogenesis. The renal apoptosis in ADPKD occurs in the face of increased expression levels of the antiapoptotic factor Bcl-2, but unaltered p53 expression. Notably, in the kidneys of ADPKD patients, both apoptosis and proliferation are associated with increased levels of c-myc, as they are in normal fetal kidneys. This association led us and others to propose that ADPKD may result from a failure of epithelial cells to fully differentiate and/or switch out of the renal developmental program.

As a parallel to these human studies, we have generated an ADPKD transgenic mouse model, called SBM, by targeted overexpression of c-myc to the renal tubular epithelium in vivo (6). All of our SBM mice in 18 transgenic lines consistently develop severe renal anomalies characteristic of PKD and die of renal failure at 3–4 months of age. Spontaneous mutations occurring in the transgene resulted in rever- sion of the PKD phenotype (7). At E16.5, the SBM transgenic fetuses produce tubular and glomerular renal cysts (8). Overexpression of c-myc in the renal epithelium of SBM mice results in 10–100-fold increases in cellular proliferation and in apoptosis relative to control (8, 9). The relevance of this model to the human disease is further supported by the increased c-myc expression observed in both human ADPKD kidneys and in the model of renal cystic disease induced by targeted disruption of the mouse Pkd1 gene (10).

On the basis of our observations that the kidneys of human ADPKD and murine SBM PKD manifest similar degrees of cellular proliferation and apoptosis, we sought to investigate the signaling pathways whereby c-myc-induced apoptosis modulates the disease progression. Since the initial discovery of a role of c-myc in apoptosis (11), the c-myc apoptotic pathway(s) has been extensively studied (12). Our murine studies have revealed that the two major modulators of the c-myc apoptotic pathway, p53 and Bcl-2, are not involved in renal cystogenesis. Indeed, cross-mating SBM mice to mice with either p53 inactivation or Bcl-2 overexpression did not modulate the rate of c-myc apoptosis (9). More recently, an alternative apoptotic pathway for c-myc has been identified and can proceed through cell surface interaction of Fas ligand with its receptor Fas (APO-1, CD95; Ref. 13). This study proposed that c-myc acts by increasing sensitivity of fibroblasts to the Fas death signal through shared signaling pathways. To this point, there is evidence that c-myc can directly stimulate the regulation of FasL expression, possibly through an autocrine mechanism (13, 14). Although the full molecular apoptotic pathways shared by Fas and c-myc remain to be elucidated, it is known that the Fas death activation signal can bypass the mitochondrial Bcl-2 family members (15) and lead to the activation of caspase 8, a critical inductive event in the caspase executioner pathway (16). Hence, the Fas signaling pathway is an attractive candidate pathway to investigate in the murine SBM model of PKD for several reasons. First, introduction of c-myc into cell culture systems has supported the requirement of an autocrine FasL/Fas-dependent signaling pathway in the induction of apoptosis (13). Second, the FasL/Fas pathway is known to potentially bypass the Bcl-2 family members. Finally, our previous observations in both human and murine PKD of a clustering of apoptotic events in neighboring cystic epithelium suggested a role for local autocrine mechanisms. To explore the hypothesis that FasL/Fas signaling is central to c-myc-induced apoptosis in PKD, we introduced a mutation of the FasL into the SBM murine model. Strikingly, our studies demonstrate that c-myc-induced apoptosis can occur independently of the Bcl-2, p53, or FasL/Fas autocrine pathways in PKD renal epithelial cells. Thus, these results implicate the existence of a yet unidentified myc-apoptotic pathway.
Materials and Methods

Genotype Analysis

The SBM transgenic mice were previously produced by use of exons 2 and 3 of the c-myc gene linked to the β-globin promoter and SV40 enhancer on a (C57BL/6J × CBA/J)F2 background (6). SBM mice were crossed with the heterozygous Fasl<sup>+/−</sup> mutant (C57BL/6J) mice obtained from The Jackson Laboratory (Bar Harbor, ME). Double heterozygous Fasl<sup>+/−</sup> SBM mice were then subsequently mated to the Fasl<sup>+/−</sup> mutant mice. Six genotypes were generated, including Fasl<sup>+/−</sup>/SBM, Fasl<sup>+/−</sup>/SBM, SBM, Fasl<sup>+/−</sup>/SBM, Fasl<sup>+/−</sup>/SBM, and wild type. The SBM genotype was revealed by Southern blot as described previously (7). Screening for the Fasl<sup>+/−</sup> genotype was performed by sequencing a PCR-amplified fragment containing the mutated Fasl region. Genomic DNA (~100 ng) was amplified in PCR buffer [10 mM Tris (pH 7.2), 50 mM KCl, 1.5 mm MgCl<sub>2</sub>] containing 0.2 mM each dNTP, 0.5 μM both primers (forward, 5′-CCTCAGGCTTACAAGGCCTCCCTCCTGT-3′; reverse, 5′-ATATTTCTGCTGGTCCCGATGAT-3′), and 0.5 unit of Taq polymerase. PCR conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final elongation of 7 min at 72°C. The 442-bp amplified products were then sequenced using a kit (T7 Sequenase v2.0; Amersham Life Science) with α-<sup>35</sup>S-dATP and an internal primer, 5′-TGTTCTTGGATGCTGTCA-3′. The wild-type sequence for Fasl was 5′-GAATCTTACAGCCTTTTGCTAC-3′, whereas the Fasl<sup>+/−</sup> sequence contained a point mutation (indicated by bold italics) replacing a T for a C as follows: 5′-GAATCTTACAGCCTTTTGCTAC-3′. This point mutation in the Fasl gene prevents recognition of the Fas receptor.

Expression Analysis

RNA Extraction. Adult kidney samples (4 months) were obtained from the SBM75 transgenic line and control mice (C57BL/6J × CBA/J)F1. Adult testis and liver samples from control F1 mice were used as positive tissues for Fasl and Fas expression. RNA was extracted by the guanidinium thiocyanate method as described previously (6). Finally, the RNAs were resuspended in RNase-free diethylpyrocarbonate-treated water. The integrity of RNA was monitored on 0.8% agarose formaldehyde gels.

RT-PCR. All RNA samples (3 μg) were simultaneously reverse transcribed in Bethesda Research Laboratories reverse transcription buffer containing 0.5 mM each dNTP (Pharmacia), 1.2 units/μl RNasin (Boehringer Mannheim), 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), and 0.5 μg of poly(dN)<sub>6</sub> random primers (Pharmacia). Reverse transcription was performed at 37°C for 1 h in a total reaction volume of 20 μl.

For all of the amplification reactions, initial control experiments were carried out using various quantities of reverse transcription aliquots to ensure that the conditions were within the linear range. The reverse transcription aliquots were subsequently amplified in PCR buffer [20 mM Tris (pH 8.8), 50 mM KCl, 3.0 mM MgCl<sub>2</sub>] containing 0.2 mM each dNTP, 20 pmol of each primer, [α-<sup>32</sup>P]CTP, and 0.5 unit of Taq polymerase in a total volume of 20 μl. Parallel control reactions were carried out without DNA template. The primers used in these analysis were as follows: for Fasl, 5′-CAACCTTTCACCAGCAAGG-3′ and 5′-CAGGGAATGACCGCTGAG-3′; for Fas, 5′-ACAGCACCACGCAATAAC-3′ and 5′-GTGTCTTGGATGCTGTCA-3′; and for internal control S16 ribosomal protein gene product, 5′-AGGACGATTTTGCACCGAT-G-3′ (forward nucleotides 1451–1472; exon 3) and 5′-GCTACCCGGTTTGAGATGA-3′ (reverse nucleotides 1620–1641; exon 4; Ref. 9). Each pair of sense and antisense primers was designed such that only spliced mRNA would produce the predicted amplification product. Conditions for Fasl and S16 amplification were 94°C for 5 min, followed by 25 cycles of 94°C for 15 s and 66°C for 30 s. Conditions for Fas and S16 amplification were 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s, with a final elongation for 7 min at 72°C. Samples were separated on 6% polyacrylamide/Tris-borate-EDTA gels and quantified by phosphorimager screen. The expected amplification fragments for the Fasl, Fas, and S16 gene products were 172, 323, and 104 bp, respectively.

Histological Analysis

Five-μm-thick paraffin sections of formalin-fixed renal tissue from 3-month-old mice from the six different genotypes were deparaffinized and

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The renal phenotype was observed in mice that did not carry the SBM transgene. Cysts were present in SBM transgenic mice irrespective of the Fasl genotypes. In addition, no difference in cyst severity or hyperplasia was observed in the three different SBM genotypes.
hydrated in graded alcohols. Tissue sections were stained with H&E. Polycystic kidney disease features were evaluated semiquantitatively by analysis of the percentage of renal parenchyma occupied by cysts and displaying epithelial hyperplasia: none (−), 1–30% (1+), 30–60% (2+), and 60–100% (3+). The numbers of mice analyzed per genotype were as follows: FasL$^{gld/}$SBM ($n = 3$), FasL$^{gld/}$SBM ($n = 4$), SBM ($n = 4$), FasL$^{gld/}$SBM ($n = 5$), FasL$^{gld/}$ ($n = 3$), and wild type ($n = 2$).

**Apoptotic Index: TUNEL assay**

For each genotype, 3-μm-thick sections of formalin-fixed renal tissue from 3-month-old mice were deparaffinized and hydrated in graded alcohols. After digestion in proteinase K and immersion in 2% H$_2$O$_2$, sections were incubated with 10 μM biotin-16-dUTP and with 0.2 units/μl TdT in TdT buffer [30 mM Tris-HCl (pH 7.2), 140 mM sodium cacodylate, 1 mM cobalt chloride]. The enzymatic reaction was stopped by immersion in TB buffer (300 mM sodium chloride-30 mM sodium citrate). After incubation with 2% BSA, labeled nucleotides were reacted with avidin/biotin complex (Vectastain; Vector Eltie, Burlingame, CA) followed by visualization with 3,3′-diaminobenzidine and periodic acid-Schiff counterstain. The proliferation index was calculated separately for cystic and noncystic tubules. The proliferation index was expressed as the number of MIB-1-positive cells per tubule, calculated from a minimum of 100 tubules/kidney section. The number of mice analyzed was $n = 4$ for each SBM-positive genotype and $n = 2$ for each SBM-negative genotype.

**Proliferation Index: MIB-1 Immunostaining**

For each genotype, formalin-fixed renal tissues from 3-month-old mice were sectioned at 3 μm, deparaffinized, and hydrated in graded alcohols. After antigen retrieval by microwaving in citric buffer for 25 min, slides were blocked for endogenous peroxidase activity. After blocking with 10% natural goat serum, sections were incubated overnight at 4°C with rabbit polyclonal antibody MIB-1 to Ki67 (Novacastra Laboratories, Ltd., Newcastle upon Tyne, United Kingdom) at a 1:100 dilution. Incubation with biotinylated secondary goat antirabbit antibody was followed by avidin/biotin complex (Vectastain), 3,3′-diaminobenzidine, and periodic acid-Schiff counterstain. The proliferation index was calculated separately for cystic and noncystic tubules. The proliferation index was expressed as the mean number of MIB-1-positive cells per tubule, calculated from a minimum of 100 tubules/kidney section. The number of mice analyzed was $n = 4$ for each SBM-positive genotype and $n = 2$ for each SBM-negative genotype.

**Results**

**c-myc Overexpression Induces FasL Expression in Vivo.** Because c-myc-induced apoptosis is Bcl-2 and p53 independent, we investigated the potential involvement of the third known pathway of c-myc-induced apoptosis, the FasL/Fas pathway. We first evaluated the expression level of FasL in normal (C57BL/6J × CBA/J)F1 and SBM mouse kidneys, using RT-PCR. Fig. 1A shows that FasL is expressed in normal mouse kidney and in SBM cystic kidney. However, expression levels of FasL appeared to be 3–4-fold greater in SBM kidneys than in control kidneys. Testicular tissue, which is known to express FasL at very high levels in adult mice, was chosen as the positive tissue control. The levels observed in SBM kidneys also appeared greater than those in the normal testis control.
Similarly, the presence of Fas receptor was investigated by RT-PCR in control and SBM mouse kidneys. As illustrated in Fig. 1B, control and SBM kidneys demonstrated similar expression levels. The levels of expression were comparable to those of normal liver, which served as the positive control (17).

c-myc-induced Cystogenesis Is Unaffected by FasL Mutation. On the basis of increased levels of FasL in SBM mice, we mated the SBM transgenic mice to the FasL gld/gld homozygous mutant mice to investigate the role of FasL in the c-myc-induced apoptotic pathway. For each of the six genotypes (FasL gld/gld, FasL gld/+ , FasL +/+ , FasL gld/gld+SBM, FasL gld/+ SBM, and SBM), the renal cystic phenotype was evaluated semiquantitatively with respect to cyst number and size and the degree of epithelial hyperplasia. As shown in Table 1, no renal cysts or epithelial hyperplasia was identified in the three genotypes (FasL gld/gld , FasL gld/+ , and FasL +/+ ) that did not express SBM. Similar degrees of cyst formation and renal epithelial hyperplasia were observed in the FasL gld/gld+SBM and FasL gld/+ SBM genotypes as in the SBM genotype, indicating that a nonfunctional FasL/Fas pathway does not abrogate the cystic phenotype.

Inactivation of FasL Signaling Does Not Modulate c-myc Apoptosis. The involvement of the FasL/Fas pathway in renal epithelial apoptosis was then quantified by TUNEL assay (Fig. 2). No apoptosis was observed in any of the FasL genotypes lacking the SBM transgene. In contrast, similar increased rates of apoptosis were observed in all three SBM genotypes, regardless of the FasL genotypes (FasL gld/gld+SBM, FasL gld/+ SBM, and SBM). In general, 4–5-fold higher levels of apoptosis were seen in cystic than in noncystic tubular epithelium. Interestingly, apoptotic cells frequently occurred as clusters of 3–10 neighboring cells, as described previously (4, 8). Thus, the inactivated FasL did not interfere with apoptosis, nor did it prevent clusters of apoptotic cells.

c-myc Proliferation Is Unaltered by FasL Mutation in Epithelial Cells. Because of the high frequency of epithelial hyperplasia in SBM kidneys, we then measured the proliferation rate in renal tissues of mice from the FasL gld/gld and SBM mating. All genotypes lacking SBM had low proliferation indices in the range of 0.2–0.4 cells/tubule. Proliferation indices were 2.4–29-fold higher in all three genotypes containing SBM, irrespective of the FasL genotype (Fig. 3). As shown and illustrated in Fig. 3, proliferation indices in SBM kidneys were consistently higher in cystic than in noncystic epithelium, by 5–6-fold. Again, proliferative cells were often observed in clusters of 3–10 adjacent epithelial cells, suggesting a zonal effect.

Discussion

Studies in human and murine models have underscored the importance of c-myc in the pathogenesis of PKD. The dual functionality of c-myc in cell proliferation and cell death appears to be tightly linked
and central to the development of this disorder. Consequently, we sought to determine which molecular pathway(s) underlies the c-myc-dependent apoptotic phenotype in PKD. On the basis of our previous work, the cellular pathways are independent of both key regulators of apoptosis, Bcl-2 and p53. Because an alternative pathway for c-myc-induced apoptosis in fibroblasts was shown to act through the FasL/Fas system, we investigated whether this pathway has a potential role in the SBM model. Whereas our study shows that c-myc overexpression can stimulate FasL expression in vivo, our data also demonstrate for the first time in vivo that disruption of the Fas signaling pathway does not rescue the c-myc apoptotic or cystic phenotype. Both renal proliferation rates and apoptotic rates were unaffected by inactivation of the FasL/Fas interaction in c-myc-induced PKD.

The potential interaction of the FasL and c-myc pathways has never been investigated in epithelial cells, either in vitro or in vivo. This study demonstrates FasL expression in normal renal epithelium in vivo and most importantly, that this expression is up-regulated in c-myc-induced PKD. This up-regulation validates and most importantly, that this expression is up-regulated in vivo, our data also demonstrate for the first time in vivo that disruption of the Fas signaling pathway does not rescue the c-myc apoptotic or cystic phenotype. Both renal proliferation rates and apoptotic rates were unaffected by inactivation of the FasL/Fas interaction in c-myc-induced PKD.

In summary, our study demonstrates that blocking the FasL/Fas pathway in PKD is uniquely independent of all three previously identified central regulators. Future studies will be directed to the elucidation of this novel c-myc-dependent apoptotic pathway with possible therapeutic implications for prevention of cystogenesis in PKD.

References
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