Jade-1, a candidate renal tumor suppressor that promotes apoptosis

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Medical therapies are lacking for advanced renal cancer, so there is a great need to understand its pathogenesis. Most renal cancers have defects in the von Hippel–Lindau tumor suppressor pVHL. The mechanism by which pVHL protein functions in renal tumor suppression remains unclear. Jade-1 is a short-lived, kidney-enriched transcription factor that is stabilized by direct interaction with pVHL. Loss of Jade-1 stabilization by pVHL correlates with renal cancer risk, making the relationship between Jade-1 and renal cancer compelling. We report that Jade-1 expression was barely detectable in all tested renal cancer cell lines, regardless of VHL status. Strikingly, proteasome inhibitor treatment increased endogenous Jade-1 expression up to 10-fold. Jade-1 inhibited renal cancer cell growth, colony formation, and tumor formation in nude mice. Intriguingly, Jade-1 also affected the pattern of cell growth in monolayer culture and 3D culture. Jade-1 increased apoptosis by 40–50% and decreased levels of antiapoptotic Bcl-2. Antisense Jade-1-expressing cells confirmed these results. Therefore, Jade-1 may suppress renal cancer cell growth in part by increasing apoptosis. Jade-1 may represent a proapoptotic barrier to proliferation that must be overcome generally in renal cancer, perhaps initially by pVHL inactivation and subsequently by increased proteasomal activity. Therefore, Jade-1 may be a renal tumor suppressor.

pVHL | renal cancer | von Hippel–Lindau | proteasome

Identification of the von Hippel–Lindau disease gene VHL provided a key starting point to understand the molecular basis of sporadic renal cancer (1). VHL loss is thought to occur early in renal tumorigenesis (2). The molecular and genetic events subsequent to VHL inactivation as well as the mechanism of pVHL-mediated renal tumor suppression remain largely unknown.

pVHL facilitates protein processing. Ubiquitinated proteins accumulate in the absence of pVHL (3), which suggests that pVHL participates in their disposal. Furthermore, pVHL is the receptor for a ubiquitin ligase that targets the hypoxia-inducible factor (HIF) alpha transcription factors (4), as well as other proteins, for destruction. Thus, pVHL plays a critical role in controlling proteasome-dependent protein fate.

pVHL inhibits apoptosis, slows cell growth, and alters patterns of tissue morphogenesis. In comparison with pVHL-deficient renal cancer cells, pVHL-transfected cells exit the cell cycle with serum depletion (5), produce an altered extracellular matrix (6), and are less invasive (7). pVHL-transfected cells are protected from endoplasmic reticulum stress (3) and UV-mediated apoptosis (8, 9). Also, they form cobblestone epithelial monolayers (10). How pVHL accomplishes these biologic activities remains unclear.

Jade-1 encodes a short-lived candidate transcription factor with PEST (proline, glutamate, serine, and threonine) protein degradation and plant homeodomain motifs (11, 12). Jade-1 is the first member of a small family of proteins (13, 14). Jade-1 is a strong pVHL interactor that is also stabilized by pVHL (11). Moreover, stabilization of Jade-1 is pVHL-mutation-dependent and may correlate with renal cancer risk (14), which raises the question of how Jade-1 might participate in renal cancer pathogenesis. Therefore, we examined the effects of Jade-1 on renal cancer cell growth and apoptosis. We propose that Jade-1 represents a proapoptotic candidate renal tumor suppressor.

Methods

Cell Culture and Reagents. The cell lines RCC4, UMRC6, A498, SW620, and 293T17 were generously provided by C. Buys (University of Groningen, Groningen, The Netherlands), I. Kuzmin (National Cancer Institute, Frederick, MD), W. Krek and J. Lisztwan (Friedrich Miescher Institut, Basel), C. Tseng (Boston University, Boston), and Z. Luo (Boston University, Boston), respectively. Other renal cancer lines were obtained from the American Type Culture Collection. The 293 tet-off cells (Clontech) were grown on type I collagen (BD Biosciences)-coated plates and maintained with 2 μg/ml doxycycline (Dox). Inhibitors in DMSO were provided at 10 μM, except epoxomicin (1 μM), MG262 (1 μM), and chloroquine (25 μM).

Plasmids and Antibodies. Jade-1 constructs have been described (11, 14). For antisense experiments, the complete Jade-1 coding sequence was cloned in reverse orientation into pTRE2 (Clontech). Western blotting was performed as described in refs. 11 and 14. Antibodies included unpurified Jade-1 antisera (11), poly(ADP-ribose) polymerase (PARP) antisera (Boehringer), and Bel-2 antibody (clone 100, Calbiochem).

Cell-Growth Assays. Cell growth was assessed by cell counting with a hemacytometer. Triplicate plates of cells were each counted in quadruplicate. We used the clonal 786-O Jade-1 lines J9, J18, and J22, as well as the Jade-1/VHL lines JV21 and JV37. Parental 786-O cells and pooled empty vector lines served as controls. For Jade-1 antisense cells, we used the 293 tet-off lines ASJ2, ASJ3, and ASJ9. Two experiments were performed for each group of cells. Results are reported as ±1 SD.

[3H]Thymidine Incorporation Assay. Clonal Jade-1 786-O lines included J9, J18, J22, and J54, as well as the Jade-1/VHL lines JV21 and JV37. Parental and pooled 786-O hemagglutinin (HA) empty vector cells served as controls. Cells were pulse-labeled with 1 μCi/ml [3H]thymidine (20 Ci/mmol; 1 Ci = 37 GBq) for 4 h, fixed with 10% trichloroacetic acid, and solubilized with 1 N NaOH. Scintillation counting was performed in triplicate. Two experiments were performed. Results are reported as ±1 SD.

Invasion Assay. The 786-O renal cancer cells were plated at 8,000 cells per ml into type I collagen gels (BD Biosciences). We tested the clonal lines J9, J18, J22, J54, JV21, and JV37, as well as parental and pooled HA empty vector cells. Cell growth and tube formation were examined for 2 weeks. Degree of branching and extraneous growth were scored in replicates of six by a

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Abbreviations: Dox, doxycycline; HA, hemagglutinin; PARP, poly(ADP-ribose) polymerase.
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significance was assessed by two-tailed analysis. Abundance was assessed by Jade-1 immunoblotting in nine renal cell lines (Fig. 1a), or in kidney cortex or mouse proximal tubule cells (11), than in any cancer line. Thus, Jade-1 expression is low in renal cancer cells regardless of VHL status.

The effect of proteasome inhibitor treatment on Jade-1 abundance was tested because the protein half-life is short (11). Endogenous Jade-1 protein levels were increased up to 10-fold with proteasome inhibitors in each of 11 cell lines (Fig. 1a and b). The relative increase in Jade-1 expression with proteasome inhibition was greater in pVHL-deficient cells than pVHL stably transfected cells (Fig. 1b). This observation is consistent with the finding that Jade-1 turnover is more rapid in the absence of pVHL (11) and with the hypothesis that pVHL protects Jade-1 from proteasomal degradation. Moreover, proteasome inhibitors of five different classes sharply increased endogenous Jade-1 expression (Fig. 1c). Thus, rapid Jade-1 turnover strongly and specifically depends on the proteasome protein degradation pathway. Jade-1 down-regulation might be a requirement for renal cancer development in general, which might be accounted for partly by pVHL inactivation and partly by increased proteasomal activity.

**Jade-1 Is Growth-Suppressive in Renal Cancer Cells.** To assess the effects of Jade-1 on cell growth, stable, clonal cell lines expressing Jade-1 were derived from parental 786-O renal cancer cells. Fewer drug-resistant colonies arose in the Jade-1 group than in the VHL group. Moreover, 23 of 55 stable lines (42%) expressed Jade-1, in contrast to only 4 of 55 stable lines (7%) that expressed pVHL.

**Immunohistochemical Analysis.** Frozen tumor samples were cut by cryostat (5 μM), fixed in acetone/methanol (1:1), and probed with polyclonal antibody for activated caspase 3 (Biocare Medical, Concord, CA) or MIB-1 monoclonal antibody for Ki-67 (Biologics, San Jose, CA), provided by C. Andry (Boston University, Boston). Nonspecific binding was blocked with 1% BSA in PBS for 30 min. Secondary antibodies were labeled with FITC or Cy5. Positive cells were counted from 10 low-power fields containing a total of >1,000 cells for each point. Five experiments were performed. Data are expressed as ±1 SE. Statistical significance was assessed by two-tailed t test.

**Results**

**Jade-1 Protein Expression Is Low in Renal Cancer Cell Lines and Increases with Proteasome Inhibitor Treatment.** Jade-1 protein abundance was assessed by Jade-1 immunoblotting in nine renal cancer cell lines. Six lines were VHL-deficient, and three lines had at least one wild-type VHL allele. Endogenous Jade-1 expression was low or undetectable in all untreated renal cancer cell lines (Fig. 1a and b). For comparison, Jade-1 levels were substantially higher in 293T17 human embryonic kidney cells (Fig. 1a), or in kidney cortex or mouse proximal tubule cells (11), than in any cancer line. Thus, Jade-1 expression is low in renal cancer cells regardless of VHL status.

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Jade-1 stably (Table 1). Jade-1 expression in 786-O renal cancer cell lines was only weak or moderate, as shown in Fig. 2a. Thus, stable increases in Jade-1 expression proved to be disadvantageous to the growth of renal cancer cell colonies in culture.

Cell proliferation was examined in growth curves and DNA synthesis studies with the Jade-1 stable renal cancer cell lines. The growth rates were consistently lower and similar in the Jade-1 and Jade-1/H2O862 VHL lines than in the vector lines (Fig. 2b). The Jade-1 and Jade-1/VHL lines showed similar and substantially reduced rates of thymidine incorporation than the vector control lines (Fig. 2c). Thus, stable Jade-1 overexpression slows cell proliferation and reduces the rate of DNA synthesis in renal cancer cells.

Jade-1 Alters Branching Morphogenesis and Solid Tumor Formation. Growth in 3D culture was tested also by using the Jade-1 stable 786-O renal cancer cell lines. 786-O renal cancer cells have been shown to form highly branched tube structures when grown in 3D culture (7). Growth of the Jade-1 stable lines was slower than the vector lines. Moreover, the Jade-1-expressing lines exhibited less branching and less extraneous growth than vector control cells (a). Extent of branching (gray bars) and extraneous growth (black bars) were scored as follows: 1, negligible; 2, moderate; or 3, extensive. *P < 0.05, versus controls. (Error bars, SE.) (d) Jade-1 inhibits growth of renal cancer cells in nude mice. *P < 0.01, versus control. (Error bars, SE.)

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**Table 1. Success rates generating stable renal cancer cell lines**

<table>
<thead>
<tr>
<th>Constructs</th>
<th>No. of stable colonies, expressing/tested</th>
<th>Expressing colonies, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-Jade-1</td>
<td>8/125</td>
<td>6.4</td>
</tr>
<tr>
<td>HA-VHL</td>
<td>23/55</td>
<td>42</td>
</tr>
<tr>
<td>HA-Jade-1 and HA-VHL</td>
<td>4/70</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Comparison of the number of colonies expressing the transgene(s) from the total number of drug-resistant colonies tested for transgene expression.
presses cell growth. The Jade-1 0.8/H11006 evidence of piling with Jade-1 at baseline levels (Fig. 5). In addition, growth suppressive effects, Jade-1 may modulate tissue morphogenesis.

In vivo growth of the Jade-1 stable renal cancer cell lines was also tested in athymic nu/nu male mice. As shown in Fig. 3d, the Jade-1 cell lines produced tumors (26 ± 5 mg) ~1/10 the mass of the parental or vector lines (292 ± 46 mg). For comparison, the Jade-1/VHL and VHL lines formed similar-sized masses of 0.8 ± 0.5 and 1 ± 1 mg, respectively. Therefore, Jade-1 suppresses cell growth.

**Jade-1 Antisense Increases Cell Growth Rates.** Effects of Jade-1 on cell growth were also examined by using an antisense approach. Human embryonic kidney 293 cells were used because their baseline Jade-1 expression is high. In the Jade-1 antisense lines ASJ2, ASJ3, and ASJ9 (data not shown), endogenous Jade-1 expression was reduced ~80% with transgene induction (Fig. 4a). In confirmation of the 786-O renal cancer cell experiments, Jade-1 antisense expression led to nearly doubled numbers of 293 cells in culture (Fig. 4b). The pattern of cell growth in culture also reflected the cell-counting experiments. With Jade-1 antisense active, the cells grew to superconfluence and were heaped on top of each other with no unfilled space on the monolayer (Fig. 5a and c). In contrast, cells were sparse and showed less evidence of piling with Jade-1 at baseline levels (Fig. 5b and d).

Cell attachment of the 293 antisense Jade-1 lines was also different when cells were grown in and out of Dox. Cells grown in the absence of Dox consistently adhered well to the collagen substrate. In contrast, cells with baseline Jade-1 levels tended to lift off of the collagen surface (data not shown). This phenomenon was not observed with the renal cancer cells in monolayer culture, but loss of cell attachment might be a partial explanation of why fewer 293 cells were found under baseline Jade-1 conditions. Jade-1 may therefore promote cell detachment or anoikis.

**Jade-1 Promotes Apoptosis.** Apoptosis could account for part of the effects of Jade-1 on cell growth. FITC–annexin V binding and FACS analysis were used to assess early apoptosis in the stable renal cancer lines (Fig. 6a). The Jade-1 lines exhibited 42% more annexin V binding than the parental or vector cells. In contrast, pVHL alone or pVHL with Jade-1 reduced baseline apoptosis by 24% and 20%, respectively. Thus, antia apoptotic pVHL (8, 9) protects against Jade-1-induced apoptosis. Examination of PARP cleavage, which is indicative of caspase 3 activity, confirmed that Jade-1 promotes apoptosis that can be blocked by pVHL (Fig. 6b). Moreover, levels of antia apoptotic Bcl-2 protein were reduced in the Jade-1 cell lines (Fig. 6c). Thus, the renal cancer cell lines overexpressing Jade-1 have a substantial increase in baseline apoptosis and decreased levels of Bcl-2.

Close observation of 293 cell transient transfections suggested that Jade-1 promoted cell rounding and detachment, a phenotype similar to apoptosis. By using the DNA-intercalating dye Hoechst 33342, levels of apoptosis were 4.0% with vector and 6.1% with Jade-1. Therefore, Jade-1 increased apoptosis by 53% in comparison with vector alone (P < 0.01; two-tailed t test) in six experiments (Fig. 6d).

Apoptosis was examined also in the Jade-1 antisense 293 cell lines. We found that 9% of cells were FITC–annexin V-positive without Dox, compared with 16% with Dox. Thus, reducing Jade-1 decreased apoptosis by 44%. Light side scatter versus light forward scatter was compared by FACS analysis. In this assay, necrotic or apoptotic cells appear in the lower left quadrant (Fig. 6e). Mean values of small, fragmented cells in three experiments using Jade-1 antisense lines ASJ2 and ASJ3 were 4.8 ± 2% with the transgene on versus 13.5 ± 3% with baseline Jade-1 levels, with P < 0.01 (two-tailed t test). Therefore, Jade-1 antisense reduces cell fragmentation by 64%. Moreover, Jade-1 antisense increased endogenous Bcl-2 levels in these cells (Fig. 6f). Thus, Jade-1 promotes apoptosis, and reducing Jade-1 levels decreases apoptosis.

Apoptosis and proliferation were also tested in tissue sections of the 786-O cell tumors grown in nude mice. By immunofluorescence, apoptosis was determined with an antibody to activated caspase 3, whereas proliferation was compared by using a monoclonal antibody to Ki-67. Activated caspase 3 was detected in 2.1 ± 0.8 cells per 100 cells counted with Jade-1 stably transfected cells versus 0.38 ± 0.30 for vector transfected cells.
Ki-67 was detected in 12.2 ± 3.1 cells of 100 in vector transfected cells versus 0.83 ± 0.76 in Jade-1 stably transfected cells (P < 0.001). Therefore, consistent with results in tissue culture, Jade-1 substantially promotes apoptosis and also inhibits proliferation of renal cancer cell xenografts.

**Discussion**

Jade-1 is a short-lived, kidney-enriched transcription factor that is stabilized through direct interaction with tumor suppressor pVHL (11, 12). Loss of Jade-1 stabilization by pVHL may correlate with renal cancer risk (14). Jade-1 expression is low to undetectable in all tested renal cancer cell lines, regardless of VHL status. Expression is high in noncancerous renal cells. Strikingly, Jade-1 expression was strongly induced up to 10-fold by proteasome inhibitors. Jade-1 suppressed renal cell growth in culture, in 3D collagen gels, and in nude mice. Jade-1 also markedly altered patterns of cell growth. Moreover, Jade-1 suppresses growth in part by increasing apoptosis, which may be due to reduction in Bcl-2 levels. Thus, Jade-1 may represent a proapoptotic barrier to proliferation that must be overcome generally in renal cancer, perhaps initially by pVHL inactivation and subsequently by increased proteasomal activity.

The reduced amount of Jade-1 in renal cancer cells, but not in noncancerous renal cell lines, strongly suggests that Jade-1 down-regulation may be a prerequisite for renal cancer development. Events subsequent to pVHL loss in renal cancer may include Jade-1 down-regulation through increased proteasome
activity, which may even be specific for Jade-1. In addition, loss of one Jade-1 allele may occur in renal cancer. The Jade-1 gene resides at chromosome 4q26–27, and 4q loss may occur in as many as 50% of clear-cell renal cancers (15). Therefore, Jade-1 may be considered a haplo-insufficient renal tumor suppressor (16).

Jade-1 also strongly affects cell growth characteristics. Jade-1 antisense alters adhesion of cells to a collagen-coated surface. Jade-1 may directly alter the epithelial nature of renal cells in monolayer culture or in 3D culture. pVHL also affects these characteristics of epithelial cell growth (7, 10, 17–20). pVHL may up-regulate characteristics of epithelial cell growth (7, 10, 17–20). pVHL also affects these characteristics of epithelial cell growth (7, 10, 17–20). pVHL may also up-regulates Bcl-2-independent apoptotic mechanisms may also be involved (21). The interplay between pVHL, Jade-1 and renal cancer cell death serves as a haplo-insufficient tumor suppressor that must be overcome in the process of renal oncogenesis.

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