Pim1 Kinase Cooperates with Hormone Treatment to Promote Bladder and Ureteral Urothelial Hyperplasia

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Abstract

The Pim serine/threonine kinases have been shown to be overexpressed in cancer. Elevated levels of Pim1 kinase were demonstrated in human leukemia and lymphomas, as well as in solid tumors such as pancreatic, prostate and bladder cancers, and have been proposed as a prognostic marker. Although the Pim kinases have been identified as oncogenes in transgenic mouse models, they have only weak transforming abilities on their own. However, they have been shown to greatly enhance the ability of other genes or chemical carcinogens to induce tumors. To explore the role of Pim1 in bladder and ureteral urothelial cancer, we generated a conditional Pim1 transgenic mouse model and found that prostate specific antigen-(PSA)-driven Cre expression lead to transgene expression in the bladder upon (testosterone/estrogen) hormone treatment. We then explored the effect of Pim1 overexpression on hormone treatment, either alone or in combination with Pten haplosufficiency. We found that Pim1 overexpression increased the severity of bladder and ureteral urothelial hyperplasias in both backgrounds, leading to pyelonephritis in transgenic animals. Our data suggest that Pim1 might contribute to progression, rather than initiation, and that the hyperplasias also contribute to the development of pyelonephritis.

Keywords: Pim1 kinase; Mouse models; Pyelonephritis; Urothelial hyperplasia

Introduction

The Pim proteins (Pim1, Pim2 and Pim3) are a family of short-lived serine/threonine kinases that are highly conserved in multicellular organisms. The different members are highly homologous at the amino acid level [1], but differ in their tissue distributions [2]. However, functional redundancy between the three Pim kinases has been shown in vitro [3,4] and in vivo [5,6].

Pim kinase transcription is rapidly upregulated in response to a wide range of growth factors [7-9], including interleukins and interferons. The majority of these factors transduce their primary signal through the JAK/STAT pathway [5]. Additionally, Pim1 is able to negatively regulate the JAK/STAT pathway by binding to SOCS proteins [10]. Gene expression of any of the 3 Pim kinases is also induced by activation of the NF-kB signaling pathway, hypoxia [11] and DNA damage, thereby protecting cells from apoptosis [12]. Pim kinases are not regulated by post-translational modifications like other kinases but are primarily regulated by transcription, translation, and proteosomal degradation [13-16].

Although the Pim kinases are only weakly transforming oncogenes, they have been shown to greatly enhance the ability of c-myc to induce lymphomas and prostate cancer [17-21], perhaps by counteracting Myc-induced apoptosis [22].

Pim kinases mediate their physiological activities through the phosphorylation of a wide range of cellular substrates, including cell cycle regulators such as p21^[waf1] and p27^[kip1] [23,24], cdc25A [25] and cTAK/MARK3/Par1A; pro-apoptotic proteins such as Bad and ASK1 [26,27]; and transcriptional regulators such as RuNX1 and RuNX3 [28], HP1, NFATc1, c-Myb or p100 [29-33]. More recently, Pim2 has been shown to phosphorylate the ribosomal protein 4E-BP1, affecting protein synthesis [34].

Elevated levels of Pim1 kinase were first reported in human leukemia and lymphomas [8,35,36]. Recently, Pim1 was found to be increased in solid tumors, including pancreatic, prostate and bladder cancers [37-40], as well as squamous cell carcinoma, gastric, colorectal and liver carcinomas [41,42], and liposarcoma [43]. Increased levels of Pim2 kinase have been detected in various lymphomas as well as in prostate cancer [44]. Pim3 kinase has been found to be aberrantly expressed in malignant lesions of endoderm-derived organs, such as the liver and pancreas, and in Ewing’s sarcoma [11].

Bladder cancer (BC) is one of the most common malignancies in the Western world. Approximately 3 out of 4 bladder tumors are diagnosed as non-invasive, with resection being the main therapy. However, the recurrence rate is very high (50-70%), and on average 20% of non-invasive tumors progress to a muscle-invasive disease [45-47]. Therefore, the challenge for clinicians is the identification of novel therapeutic targets for bladder cancer chemotherapy. In this context, Pim1 has been shown to be overexpressed in BC epithelium, and the expression levels were higher in invasive bladder cancer than in non-invasive samples. Furthermore, Pim1 knockdown reduced bladder cancer cell growth and sensitized cells to chemotherapy in vitro [38].

We generated a conditional transgenic Pim1 mouse model that

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Carcinogenesis induced by testosterone and estradiol

We used mice with an average age of 8 weeks. The hormones testosterone (Sigma) and β-Estradiol (Sigma) were mixed with colorless silicone (Soudal) and dried for 48 h. Pellets were stamped out using a 5 mm biopsy punch (Stieffel), resulting in a 30 mg hormone/silicone pellet. A 5 mm incision was made on the lower back (after anesthesia with 2% isoflurane), and the pellets were inserted under the fur. The procedure was repeated after 8 weeks. The total doses of the implanted hormones are as follows: Implanted total 1st dose at 8 weeks of age: Testosterone: 12.5 mg; β-Estradiol: 1.25 mg.

Implanted total 2nd dose at 16 weeks of age: Testosterone: 18.75 mg; β-Estradiol: 1.87 mg.

To ensure the health of the animals, the mice were monitored every 24-48 h, depending on the health status of each animal.

Necropsy and pathological analysis

Tissues were fixed in 10% formalin for 24 h, dehydrated at different ethanol concentrations with xyloid and embedded in paraffin at 65°C. Tissue fixation and paraffin embedding were carried out at the Histopathology Unit at the CNIO.

Statistical data analysis

To determine the statistical significance of the lesions and the statistical significance of the differences in the incidence of pyelonephritis, either a one-way ANOVA or a one-tailed Student’s t-test were used as indicated in the figure legends.

Immunohistochemistry

Prepared paraffin tissue blocks were cut into 2 μm sections using an automated microtome and the sections were dyed with H&E or various antibodies. All staining were carried out at the Compared Pathology Unit at the CNIO according to established protocols. We used the following antibodies: anti-p21 from Santa Cruz (sc-397-G) and horseradish peroxidase (goat anti-rabbit) secondary from Dako (P0448).

Results

Generation of transgenic mice carrying the PIM1 transgene

We generated mouse lines that conditionally express the Pim1 transgene by inserting a stop cassette flanked by LoxP sequences. These lines were crossed with a transgenic mouse line expressing Cre recombinase under the control of the PSA promoter, allowing CRE expression primarily in prostate. Upon Cre recombinase expression, the stop cassette would be excised allowing Pim1 transgene expression, the stop cassette would be excised allowing Pim1 transgene expression. These lines were crossed with a transgenic mouse line expressing Cre recombinase under the control of the PSA promoter, allowing CRE expression primarily in prostate. Upon Cre recombinase expression, the stop cassette would be excised allowing Pim1 transgene expression. We identified two Pim1 transgenic founders mice that clearly expressed the Pim1 transgene under PSA-Cre control transcription (Figure 1A). Two Pim1 transgenic founders mice that clearly expressed the Pim1 transgene under PSA-Cre control transcription (Figure 1A).

As Pim1 is regarded a “weak” oncogene, we decided to study the induction of bladder hyperplasia solely by Pim1 overexpression, as well as the effect of Pim1 overexpression in the absence of one Pten allele. To that end, we used conditional knock-out mice bearing a floxed Pten allele. Upon Cre recombinase expression, the Pten allele will be inactivated by excision of exon 5 of the Pten gene [49] in cells in which Pim1 transcription is activated. A summary of the mouse line genotypes used in this study are as follows: tgPim1 [Pim1(Tg/+);PSA-
Figure 1: (A) Scheme of the transgene strategy used. (B) Relative expression of PIM1 in Pim1/PSA-Cre mice after 2 rounds of hormone treatment. RNA was extracted from different tissues of 24 weeks old mice after testosterone and estradiol treatment. Reverse transcriptase PCR was performed to obtain cDNA, which was amplified using specifically designed primers. PCR fragment length was checked on a 1.5% agarose gel. (C) Levels of transgenic PIM1 mRNA determined by quantitative RT-PCR. Graph shows average levels of expression in the bladder of PIM1 mRNA of per genotype performed in triplicate. Data were normalized to the endogenous levels of GADPH in each sample. ND: Not detected. Transgenic PIM1 mRNA was not detected in WT or PTEN-Het mice. (D) Urothelial hyperplasia in hormone treated mice. To determine the development of urothelial hyperplasia due to hormone treatment, 8-week-old untreated mice of each genotype and hormone treated mice of corresponding genotypes, were sacrificed and bladder tissue was taken and distended with fixative (10% formalin). Upper pictures: Representative hyperplasia observed in bladder walls. Bottom pictures: Pictures show representative increases in bladder size over treatment course. H&E staining of bladders from tgPim1 mice before treatment and after 1 or 2 treatment rounds, respectively. All pictures were taken at the same magnification, (Panoramic viewer – ZeissE) Urothelial hyperplasia grades reached. (E) Grading of hyperplasia in the different conditions. Hyperplasia of epithelial cells in bladder, before and after hormone treatment, was graded using the following grading scale: bh-grade 0: normal (2-3 cell layers); bh-grade 1: slight hyperplasia (4 cell layers); bh-grade 2: slight/moderate hyperplasia (5-8 cell layers); bh-grade 3: moderate hyperplasia (9-10 cell layers); bh-grade 4: moderate/severe hyperplasia (11-12 cell layers); bh-grade 5: severe hyperplasia (>12 cell layers). See text for details.
CRE (Tg+/+), Pten-Het [Pten(loxp/+);PSA-CRE (Tg/+)], tgPim1/Pten-Het [Pim1(Tg/+); Pten(loxp/+); PSA-CRE (Tg/+)].

We measured the levels of Pim1 transgene expression by quantitative RT-PCR in bladder tissues from all strains and found that the levels of Pim1 transgene expression in the bladder were not as high as in prostate (Figure 1B) but were specific to mice expressing Cre recombinase (Figure 1C) upon hormone treatment.

Hormone treatment induces urothelial hyperplasia in the bladder of mice overexpressing Pim1 alone or with simultaneous loss of one Pten allele.

Hormone treatment protocols, such as those used in this work, have not been reported to cause urothelial hyperplasia or severe bladder pathology. However, we detected an increase in general bladder size (Figure 1D) A large and macroscopically pathological bladder was only evident after hormone treatment and correlated with the expression of the Pim1 transgene or the loss of one Pten allele.

To explore the observed bladder hyperplasia, we decided to analyze

<table>
<thead>
<tr>
<th>Bladder hyperplasia grade (bh-grade)</th>
<th>Effect on bladder</th>
<th>Cell layers</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>2-3</td>
</tr>
<tr>
<td>1</td>
<td>Slight hyperplasia</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Slight/moderate hyperplasia</td>
<td>5-8</td>
</tr>
<tr>
<td>3</td>
<td>Moderate hyperplasia</td>
<td>9-10</td>
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<tr>
<td>4</td>
<td>Moderate/severe hyperplasia</td>
<td>10-12</td>
</tr>
<tr>
<td>5</td>
<td>Severe hyperplasia</td>
<td>&gt;12</td>
</tr>
</tbody>
</table>

Table 1: Classification of bladder hyperplasia in mice after hormone treatment. Bladder hyperplasia grade (bh-grade) was established on bladder tissue that was extended with fixative (10% formalin) at necropsy.

Figure 2: Urothelial hyperplasia developed after hormone treatment. Example for (A) average of hyperplasia grade observed in each genotype (B) maximum of hyperplasia grade in each genotype. To determine the development of urothelial hyperplasia due to hormone treatment, 8-week-old untreated mice of each genotype and hormone treated mice (1 or 2 rounds) of corresponding genotypes were sacrificed and the bladder was taken. H&E staining of bladder tissue was used for grading and statistics as shown in Figure 1D.
the bladder epithelia microscopically (Figure 1E). Hyperplasia of epithelial cells in the bladder before and after hormone treatment was graded using the grading scale shown in Table 1. After one treatment round, wild type mice showed no hyperplasia (bh-grade 0), tgPim1 mice displayed mostly bh-grade 1 (Figures 1E and 2A) reaching the maximum of bh-grade 3 (Figures 1D and 2B), and PTEN-Het mice primarily displayed bh-grade 1 (Figures 1D and 2A) with 3 animals reaching bh-grade 3 (Figure 2B). The increase in urothelial hyperplasia was even more significant in tgPim1/PTEN-Het mice, where several animals displayed bh-grade 4 and 1 animal reached bh-grade 5 (Figures 1D and 2B).

**Hormone treatment induces hyperplasia of the ureter in mice overexpressing Pim1 alone or in combination with loss of one Pten allele.**

Similarly, we analyzed the epithelial layers of the ureter in

**Figure 3:** Ureter hyperplasia in hormone treated mice. To determine the development of ureteral hyperplasia due to hormone treatment, 8 week old untreated mice of each genotype and hormone treated mice of corresponding genotypes were sacrificed and ureters were taken.

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transgenic animals subjected to hormone treatments. As in the bladder, we observed an increase in ureteral hyperplasia (Figure 3). This hyperplasia clearly decreases the light of the ureter, reaching full occlusion after one treatment round in some cases in tgPim1/Pten-Het mice or after 2 hormone treatment rounds in tgPim1 mice (Figure 3B).

Pim1 and Pim2 phosphorylate the cell cycle inhibitor p21(Cip1/WAF1) (p21) on Thr145 in vitro and in vivo [23]. It has been reported that the overexpression of Pim kinases in cells leads to the increased stability of p21 and results in enhanced levels of endogenous p21 proteins [50,51]. Knockdown of Pim expression via siRNA results in reduced expression of endogenous p21, indicating that Pims are legitimate p21 kinases regulating p21 stability. To explore whether Pim1 overexpression triggers the stabilization of p21, we quantified the number of cells showing p21 nuclear staining for all lesion grades in all cohorts. We observed an increased number of umbrella cells with p21 in high-grade lesions (Figures 4A and 4B) (tgPim1, Pten-Het and tgPim1/Pten-Het genotypes) but not in hyperplastic tissues. We did not observe a high number of cells showing nuclear staining for p21 in low-grade hyperplasias in any cohort.

High incidence of pyelonephritis

When correlating the genotypes with the treatments in which we observed increased urothelial hyperplasia (especially in tgPim1/Pten-Het mice after one treatment round or tgPim1 mice after 2 hormone treatment rounds), which lead to ureter occlusion, we observed a high incidence of pyelonephritis (Figure 5), with up to 50% of the animals with this specific genotype developing this disease. As pyelonephritis is quite painful and leads to death within 12-24 hours, the animals were sacrificed at the earliest sign of kidney and/or urination problems; no further rounds of hormone treatment were performed due to the high rate of affected animals.

Due to the significantly increased incidence of pyelonephritis in tgPim1/Pten-Het mice during the first round of hormone treatment, a second round of treatment was not administered and humane euthanasia was performed. However, we were able to administer a second round of hormone treatment to the WT and tgPim1 mice. It is interesting to note that after one round of treatment, PTEN-het mice did not develop pyelonephritis while WT mice did not develop disease after 2 rounds. This clearly indicates a role for Pim1 in the secondary development of this disease, most likely due to induced bladder and ureter hyperplasia in these transgenic mice.

Discussion

The hormone treatment classically used to induce prostatic lesions is not known to induce urothelial hyperplasia. Nevertheless, we detected moderate to severe urothelial hyperplasia in 30% of tgPIM1/Pten-Het and Pten-Het mice and light to moderate urothelial hyperplasia in 30% of tgPIM1 mice after one round of hormone treatment. There was no observed urothelial hyperplasia in untreated 10-month-old mice of any genotype.

The PSA/Cre mouse model used in this study has been demonstrated to express the induced transgene not only in prostate tissue but also in bladder after hormone treatment. We did not detect expression of Pim1 in the bladder of untreated 10-week-old mice of any genotype, but there was detectable expression Pim1 after 1 or 2 rounds of hormone treatment in 24-week-old tgPIM1 mice. We do not know the reasons for this unspecific expression of transgene in bladder upon hormone treatment. It is possible that as testosterone activates PSA transcription, the levels of testosterone generated by hormone treatment might be sufficient to induce PSA promoter transcription in bladder, and therefore CRE expression, and might thus activate Pim1.
showed that increased expression of Pim1, alone or in combination, induced hyperplasias do not progress to malignant state due to the same settings as this work, upon hormone treatment, during aging, loss and Pim1 overexpression in hormone-induced hyperplasia.

Accordingly, we explored the effect of PIM1 overexpression in the mice expressing Pim1 in prostate epithelium, and analyzed the contribution of PIM1 to neoplastic initiation and progression [48].

In parallel to this work, we generated conditional Pim1 transgenic mice expressing Pim1 in prostate epithelium, and analyzed the contribution of PIM1 to neoplastic initiation and progression [48]. Accordingly, we explored the effect of PIM1 overexpression in the same settings as this work, upon hormone treatment, during aging, and in combination with the absence of one Pten allele. Consistent with published data indicating that PTEN inactivation has a role in promoting bladder cancer [55], Pten-Het mice show a significant increase in urothelial hyperplasia compared to wild type mice. TgPIM1/Pten-Het mice show an increased severity of hyperplasia in the bladder, confirming the cooperation between Pten loss and Pim1 overexpression in hormone-induced hyperplasia.

Finally, Pim1 overexpression-induced hyperplasia in bladder and ureters may lead to light occlusion, inducing pyelonephritis in transgenic animals. This severe phylonephritis effect may be due to a combination of hyperplasia in bladder and ureters and the hormone-induced prostatic inflammation and hyperplasia observed in PIM1 transgenic mice [48] that could partly obstruct the urinary tract causing urinary reflux, thus contributing to pyelonephritis. This is also supported by works reporting that hormone treatment can lead to bladder outlet obstruction and voiding dysfunction in male mice [64].

The overexpression of PIM1 transgene in prostate leads to an impaired immune response in hormone-treated mice which seems to be related to pyelonephritis and to the absence of senescence markers in prostate neoplasia [48]. However, in ureter and bladder, the immunoresponse was not found significantly increased in any of the genotypes.

Summarying, our data suggest that Pim1 might contribute to progression rather than initiation of urothelial neoplasia and that urothelial hyperplasia is an important factor in the development of pyelonephritis.

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