In vivo analysis of *Pim-1* deficiency

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ABSTRACT

The *Pim-1* proto-oncogene encodes a highly conserved serine/threonine phosphokinase (1–5) which is predominantly expressed in hematopoietic organs and gonads in mammals (6–9). Overexpression of *Pim-1* predisposes to lymphomagenesis in mice (8, 10, 11). To develop a further understanding of *Pim-1* in molecular terms, as well as in terms of its potential role in hematopoietic development, we have generated mice deficient in *Pim-1* function. *Pim-1*-deficient mice are ostensibly normal, healthy and fertile. Detailed comparative analyses of the hematopoietic systems of the mutant mice and their wild-type littermates showed that they are indistinguishable for most of the parameters studied. Our analyses revealed one unexpected phenotype that correlated with the level of *Pim-1* expression: *Pim-1* deficiency correlated with a erythrocyte microcytosis, whereas overexpression of *Pim-1* in *Eμ-Pim-1*-transgenic mice resulted in erythrocyte macrocytosis. In order to confirm that the observed decrease in erythrocyte Mean Cell Volume (MCV) was attributable to the *Pim-1* deficiency, we developed mice transgenic for a *Pim-1* gene construct with its own promoter and showed that this transgene could restore the low erythrocyte Mean Cell Volume observed in the *Pim-1*-deficient mice to near wild-type levels. These results might be relevant to the observed involvement of the *Pim-1* gene in mouse erythroleukemogenesis (12). The surprising lack of a readily observed phenotype in the lymphoid compartment of the *Pim-1*-deficient mice, suggests a heretofore unrecognized degree of *in vivo* functional redundancy of this highly conserved proto-oncogene.

INTRODUCTION

The *Pim-1* gene was discovered as a common insertion site in MoMuLV-induced T-cell lymphomas (5, 13). It has subsequently been found to be involved in B-cell lymphomas (10, 11) and

erythroleukemias (12) as well. The gene encodes a cytoplasmic serine/threonine-specific protein kinase with a remarkably short half-life (4). *Pim-1* is expressed at high levels in mouse embryonic stem cells (ES cells) (P.W.L., unpublished results), in fetal liver in both mouse and man and, to a lesser extent, in spleen, thymus, bone marrow, lymph nodes and gonads (6–9). Overexpression of *Pim-1* in the lymphoid compartment of *Eμ-Pim-1*-transgenic mice leads to a low incidence of T-cell lymphomas (8) and to an approximately 20-fold increase in sensitivity to chemically-induced T-cell lymphomagenesis (14, 15). Many human hematopoietic malignancies show a high expression of *Pim-1* (6, 16). There are no published reports of any biological or transforming effects of *Pim-1* outside the hematopoietic system.

The *Eμ-Pim-1* transgenic mice have been of limited value in furthering our understanding of the normal function of the proto-oncogene *Pim-1*. Aside from their predisposition to lymphomagenesis, they do not show any clear hematopoietic abnormalities such as a preneoplastic expansion of the susceptible hematopoietic compartments (8). However, a critical role of *Pim-1* in the development or regulation of a hematopoietic compartment should be revealed by alterations in that compartment in the absence of *Pim-1* function. Therefore, we have generated mice deficient in *Pim-1* function using the recently developed gene targeting technology (17–19).

MATERIALS AND METHODS

Generation of *Pim-1*-deficient mice

To construct the targeting vector, a 1.3-kb *Cla*I-*Sac*I fragment of *Pim-1* was replaced by a 1.8-kb fragment containing the PGK-NEO-PA cassette (See legend, (18, 20)), destroying the *Cla*I and *Sac*I sites, but introducing *Eco*RI and *Hind*III sites. The *Pim-1*-homologous region (BALB/c derived DNA) extends 1.5 kb on the right to an *Xba*I site and 12.1 kb to the left to an *Xba*I site, destroyed by the linkage to the MC1-TK cassette. The ES cell line E14 was maintained in Buffalo Rat Liver (BRL) conditioned medium as described (21). Approximately 10⁸ ES cells were mixed with 40 μg *Xba*I linearized DNA and

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electroporated as described elsewhere (22). G418 selection was initiated the following day at 200 μ g/ml. After 5 to 7 days ganciclovir (2 μ M) was added. Approximately 200 out of 900 G418-resistant colonies survived counter selection, of which 33 were analyzed on Southern blot. The NEO probe contained the complete protein-coding region of the NeoR gene. The *Pim-1* probe is a 0.9-kb BamHI fragment located outside the pGTP810 construct and is described as probe A elsewhere (13). ES cell injections were performed as described (23) into recipient C57BL/6 blastocysts or into F2(C57BL/6 $\times$ CBA/Ca) blastocysts.

Resulting male chimeras were test bred with BALB/c females to score for germ-line contribution of the male 129/Ola-derived ES cells as monitored by transmission of the 129/Ola-derived *c^{ch}*, *p* and *A^w* alleles, which lighten the coat color (23). The resulting outbred (129/Ola $\times$ BALB/c) mice carrying the targeted allele were backcrossed with 129/Ola mice. The resulting mice were used for the experiments described. The genotype was monitored as described (24).

Protein analysis

Immunoprecipitation with polyclonal anti-PIM-1 c-terminal peptide serum and *in vitro* kinase assay were performed as described previously (4), with the exception that freeze-thaw lysates of equal numbers of cells were made in PIPES pH=7.0, 30 mM NaCl, 5 mM MgCl₂, 14 mM β -mercaptoethanol, 1% Aprotinin, 1 mM phenylmethylsulfonylfluoride, 1 mM Leupeptin and 1 μ g soybean trypsin inhibitor per ml. Furthermore, 5 mM CHAPS was used instead of 1% Nonidet P40.

Flow cytometry

Bone marrow cells obtained from femurs of two-month-old *Pim-1*-deficient mice and control littermates were simultaneously stained with phycoerythrin-S7 (anti-CD43), biotin-6B2 (anti-B220), fluorescein-30F1 (anti-HSA) (Fig.2b) or with phycoerythrin-S7, fluorescein-6B2 and biotin-anti-BP1 (Fig.2a and 2c). Biotin reagents were revealed by cytochrome-streptavidin. Thymocytes were stained simultaneously with fluorescein-MT4 (anti-CD4) and biotin-Ly2 (anti-CD8). Biotin was revealed with phycoerythrin-streptavidin. Data shown are representative of several analyses. Cells were analyzed on a FACSScan (Becton Dickinson).

Proliferation assays

Splenocytes derived from two-month-old *Pim-1*-deficient mice and control littermates were plated at various seeding densities in 96-well plates in 100 μ l complete Iscove's medium, supplemented with 2.5 μ g ConA/ml or 30 μ g LPS/ml. After either one (left) or two (right) days of culture, ³H-Thymidine was added to a final concentration of 4 μ Ci/ml. The cultures were incubated for an additional 4 hours at 37°C. The cells were collected on filters and incorporation was determined by scintillation counting.

Red blood cell scans

Blood cell scans were performed with an automated cell counter (Sysmex Toa F800). Experimental mice were compared with wild-type littermate controls and the results expressed as a percentage of the wild-type values.

Transgenic mice

Transgenic mice were generated essentially as described (8), but in FVB/NA mice. The injected construct was the 10.9-kb EcoRI

fragment of the wild-type *Pim-1* gene as shown in Fig. 1a. This fragment encompasses the entire *Pim-1* gene along with 5 kb of upstream sequences.

RESULTS

Generation of *Pim-1*-deficient mice

Pim-1-deficient mice were generated by standard gene-targeting techniques using homologous recombination in ES cells (17–19, 23). The design of the targeting vector (Fig. 1a) was guided by the principles of the positive-negative selection (PNS) strategy (19) and the desire to generate a true null allele for *Pim-1*. The latter point was addressed by the deletion of promoter sequences, transcription and translation initiation sites and a large segment of the coding region containing the conserved lysine residue of the ATP binding site of the protein kinase domain (25). Homologous recombination with this vector would yield a *Pim-1* allele incapable of producing a functional PIM-1 protein.

ES cells were electroporated with the targeting vector pGTP810 and subjected to the positive-negative selection procedure. Doubly resistant clones were screened by Southern blot analysis. Fig. 1b shows representative examples of a homologous recombinant clone and of a random integrant clone. Homologous recombination at the *Pim-1* locus with this replacement-type targeting vector occurred at a frequency of 5 out of 33 counterselected clones.

Two homologous recombinant clones were chosen on the basis of their morphology and karyotype for injection into blastocysts. The resulting chimeras were test bred by mating to females of strain 129/Ola, the strain of origin of the ES cells. These crosses bred very slowly and so chimeras were also mated to female BALB/c mice. Both ES clones yielded germ-line chimeras. Heterozygous offspring of test crosses between male germ line chimeras and female BALB/c mice were interbred to obtain mice homozygous for the mutated *Pim-1* allele. The Southern blot analysis of a random sampling of such offspring in Fig. 1c shows that homozygous *Pim-1*-deficient mice are viable. The breeding results of more than 300 offspring derived from crosses between heterozygotes did not show a statistically significant deviation from the expected ratios of either heterozygotes or homozygotes. This holds true when the data are subdivided by gender or strain background.

The *Pim-1* deficiency in the homozygous mice was confirmed at the DNA level by showing that the segment deleted in the mutated *Pim-1* allele was indeed lacking in these mice (not shown). The complete absence of the wild-type band in the Southern analysis in Fig. 1c rules out artefacts such as the third class of recombination event described by Adair *et al.* (26). We confirmed that our mutation completely abolished *Pim-1* function by analyzing PIM-1 expression at the protein level (Fig. 2). We used a very sensitive *in vitro* autophosphorylation assay (4) to rule out residual PIM-1 function. As shown in Fig. 2, no PIM-1 phospho-kinase activity could be detected in spleen derived from *Pim-1*-deficient mice. We conclude that our mutation results in a *Pim-1* null allele.

Analysis of *Pim-1*-deficient mice

The *Pim-1*-deficient mice did not display any overt abnormalities. Behavior of the mice seemed normal and their body weights were in the range of their heterozygous and wild-type littermates. A morphological and histological analysis of all major organs did not reveal any abnormalities. The only organs aside from

Figure 1. The generation of *Pim-1*-deficient mice. **a**, *Pim-1* gene targeting strategy. The structures of the mouse *Pim-1* gene (5), the targeting vector pGTP810 and of the predicted disrupted *Pim-1* allele are shown approximately to scale. Dotted lines indicate borders of homologous DNA segments between the maps. Arrows indicate gene orientation. Double-headed arrows indicate restriction fragments used in the Southern blot analysis. Filled boxes denote *Pim-1* protein-coding regions, shaded boxes non-translated exon segments. The PGK-NEO-PA cassette (heavily hatched box) is an MC1-derived Neo gene (18) controlled by the mouse phosphoglycerate kinase (PGK) promoter and polyadenylation signals (20). The MC1-TK gene is a Herpes Simplex Virus thymidine kinase (tk) gene controlled by the MC1 promoter (18) and its own polyadenylation signal. Restriction sites indicated are: H, HindIII; X, XbaI; S, SacI; E, EcoRI; C, ClaI. **b**, Southern blot analysis of ES cell clones. A representative example of a pGTP810 random integrant (RI) and homologous recombinant (HR) clone are shown. The numbers refer to the sizes of the bands corresponding to the double-headed arrows in figure 1a. 'R' refers to the random integrant fragment of variable length. **c**, Genotype analysis by Southern blotting of a random sampling of progeny mice from (+/-) × (+/-) crosses. WT, wild-type; KO, targeted band. Genotypes are shown above the lanes.

hematopoietic tissues with significant *Pim-1* expression in the mouse are the gonads (7, 9). Nevertheless, both male and female *Pim-1*-deficient mice showed normal fertility.

Analysis of the hematopoietic system with a set of standard lymphoid and myeloid surface markers did not reveal differences in cell suspensions derived from spleen, lymph nodes, and peripheral blood of *Pim-1*-deficient mice and their control littermates. The cell populations positive for MHC class I and II, CD45R/B220, CD3, CD4, CD8, ab T-cell receptor, Thy-1, Pgp-1, and Mac-1 showed the expected tissue distributions. Serum immunoglobulin levels were indistinguishable from wild-type controls (data not shown).

Analysis of the more immature lymphoid compartment by flow cytometry also failed to reveal differences. The population sizes of CD4/CD8 (double) positive and negative (Fig. 3d) or CD3+ (data not shown) thymocytes did not differ between *Pim-1*-deficient and control mice. Immature B-cell populations in bone-marrow, quantitated by monoclonal antibodies against CD45R/B220, CD43, heat stable antigen (HSA), and BP-1, as published (27), were also within normal range (Fig. 3a-c).

A role for *Pim-1* in the regulation of the development of a particular hematopoietic compartment may not be apparent in the pool sizes once steady state has been reached, but might be revealed in a proliferative assay following a stimulatory signal or a disturbance of the equilibrium. Splenocytes derived from *Pim-1*-deficient mice showed a stimulatory response to Concanavalin A (ConA) and Lipopolysaccharide (LPS) similar to that of the control splenocytes (Fig. 4). Preliminary results of short-term repopulation experiments by bone marrow cells derived from *Pim-1*-deficient mice as assayed by day 12 spleen colony formation (28) in lethally irradiated syngeneic mice did not show any effect of *Pim-1* deficiency (data not shown). We are currently setting up long-term repopulation assays to test whether perhaps more immature cell populations are affected by *Pim-1* deficiency.

Our survey of the hematopoietic system of *Pim-1*-deficient mice did reveal an unexpected, but subtle effect of the lack of the PIM-1 gene product. The mean cell volume (MCV) of erythrocytes is significantly and reproducibly smaller in *Pim-1*-deficient mice compared to their wild-type littermates

Figure 2. Analysis of PIM-1 protein expression assayed by *in vitro* autophosphorylation of the PIM-1 proteins. Splenocytes were isolated from two *Pim-1*-deficient mice (-/-) and one wild-type littermate (+/+). Immunoprecipitation with polyclonal anti-PIM-1 c-terminal peptide serum and were performed either in the presence (B) or absence (I) of blocking c-terminal peptide, followed by *in vitro* kinase assays. The two PIM-1 protein species are indicated (34 and 44 kD).

Figure 3. Triple color flow cytometry of early lymphocytes in the bone marrow (a–c) and thymus (d). a, Contour plots of bone marrow cells stained simultaneously with anti-B220 and anti-CD43 antibodies. The B220 $^{+}$, CD43 $^{+}$ cell population, corresponding to approximately 2.5% of total bone marrow, is boxed (R1) and does not differ between the two genotypes. b, Heat Stable Antigen (HSA) staining profile of B220 $^{+}$, CD43 $^{+}$ cells gated in box R1 (a). c, As b, BP1 staining is shown. d, CD4, CD8 double staining of thymocytes.

(experiment 1), whereas the erythrocytes are larger in *E μ -Pim-1*-transgenic mice compared to their wild-type littermates (experiment 2; see Table 1). It should be noted that erythrocyte size varies among mouse strains, which is of particular relevance given the different mouse strains used in our experiments. We therefore restricted our analyses to comparisons between littermates and expressed the values obtained for the test mice

Figure 4. Proliferative responses of *Pim-1*-deficient and wild-type cells. Proliferative stimulation of splenocytes by concanavalin A (ConA) or lipopolysaccharide (LPS) in vitro after one day (a) and two days (b).

as percentages of the mean values obtained for their wild-type littermates, which were set at 100% for comparison. The peripheral blood concentration of erythrocytes (RBC) is not elevated in *Pim-1*-deficient mice to compensate for the microcytosis, which means that hemoglobin levels are reduced. The *E μ -Pim-1*-transgenic mice do seem to show a compensatory decrease in the erythrocyte concentration, resulting in normal hematocrit and hemoglobin levels (see Table 1).

To prove that the *Pim-1* deficiency was responsible for the observed erythrocyte microcytosis, we performed rescue experiments with a *Pim-1* transgene with expression similar to that of the endogenous *Pim-1* gene. Transgenic mice were generated with the 10.9-kb EcoRI fragment of the wild-type *Pim-1* gene as depicted in Fig. 1a. This fragment encompasses the entire *Pim-1* gene along with 5 kb of upstream sequences and is referred to as ppWT. Resulting transgenic mice were bred with mice carrying the *Pim-1* mutant allele and the resulting offspring were interbred to generate *Pim-1*-deficient mice with and without the transgene. The analysis of the red blood cell mean volume of the progeny of such crosses (experiment 3) is shown in Figure 5 and compared to the values obtained in the earlier experiments 1 and 2. Once again, the values for the homozygous *Pim-1*-deficient mice were considerably depressed as compared to their wild-type controls. Introduction of the ppWT transgene into the *Pim-1*-deficient background restored the MCV values to just below wild-type levels (see Figure 5). We conclude that the *Pim-1* gene is responsible for the observed differences in erythrocyte size.

DISCUSSION

The lack of major perturbations of the hematopoietic system or of any of the major organs in the *Pim-1*-deficient mice seems surprising in view of the strong evolutionary conservation of *Pim-1*. This high degree of conservation implies that loss of the open reading frame should yield a phenotype that is counterselected in an evolutionary context.

Whether that is the case for the observed erythrocyte microcytosis is difficult to assess. The absolute erythrocyte sizes of the *Pim-1*-deficient mice fall within the range of some inbred

Table 1. Analysis of the peripheral red blood cell population

	Experiment 1			Experiment 2		
	Wild-type mice ^{a,c}	Pim-1 deficient mice ^{b,c}	p-value ^d	Wild-type mice ^{a,c}	E μ -Pim-1 transgenic mice ^{b,c}	p-value ^d
RBC ^e	100 $\pm$ 6.9 (n=33)	104.6 $\pm$ 11.4 (n=23)	p=0.04	100 $\pm$ 7.8 (n=8)	86.4 $\pm$ 8.0 (n=10)	p=0.002
HGB ^e	100 $\pm$ 7.3 (n=16)	89.1 $\pm$ 12.1 (n=12)	p=0.005	100 $\pm$ 3.5 (n=2)	102.0 $\pm$ 10.9 (n=4)	p=0.425
HCT ^e	100 $\pm$ 6.4 (n=33)	91.7 $\pm$ 13.6 (n=23)	p=0.003	100 $\pm$ 8.0 (n=8)	103.1 $\pm$ 10.4 (n=10)	p=0.278
MCV ^e	100 $\pm$ 3.8 (n=33)	87.3 $\pm$ 5.6 (n=23)	p<0.0001	100 $\pm$ 3.7 (n=8)	119.5 $\pm$ 6.2 (n=10)	p=0.0001
RDW-CV ^e	100 $\pm$ 4.7 (n=13)	99.4 $\pm$ 2.6 (n=11)	p=0.539	100 $\pm$ 2.7 (n=8)	100.8 $\pm$ 6.7 (n=10)	p=0.616

^aThe mean of the values for the wild-type littermate controls in each experiment was arbitrarily set at 100%.

^bThe values of the test mice are given as percentages of these wild-type mean values obtained in each experiment.

^cThe values are given $\pm$ the standard deviations with the number of mice between parentheses.

^dThe results were statistically analyzed (mutant versus wild type) with the Student's t test and p-values are given.

^eAbbreviations: RBC, red blood cell count; HGB, haemoglobin concentration; HCT, haematocrit; MCV, mean red blood cell volume; RDW-CV, red blood cell distribution width.

mouse strains. Variations in erythrocyte size have been described for pathological conditions and as a feature of normal development in mammals.

One well-documented variation in erythrocyte size is the decrease in size during mouse development from midgestation to adulthood (29). Little is understood of the regulatory controls in this process, though the timing of enucleation relative to other erythrocyte differentiation parameters may be one of the determining factors. In addition to this developmental variation in the size of newly formed erythrocytes, there is a gradual size decrease during the life span of the erythrocyte (30). It remains to be seen whether *Pim-1* exerts its effect on erythrocyte size through either of these two normal mechanisms of size variation.

Variations in erythrocyte size have also been described for a variety of pathological conditions. The mild microcytosis seen in *Pim-1*-deficient mice is not reminiscent of any of the known mutations affecting hematopoiesis in mice (31) or of the erythropoietic defect seen in *Rb*-deficient mice, which were generated by the targeted gene disruption of the *Rb* gene (32–34).

The most common cause of microcytic anemia in humans is iron deficiency (35). We plan to investigate whether iron metabolism is disturbed in *Pim-1*-deficient mice. One interesting point in this regard is that excess iron does not raise the MCV above normal, whereas overexpression of *Pim-1* apparently does. This suggests that *Pim-1* may be more directly involved in the regulation of erythropoiesis. It will be interesting to see whether the observed involvement of the *Pim-1* gene in mouse erythroleukemogenesis (12) is relevant in this regard. We hope to achieve a better understanding of the role of *Pim-1* in erythropoiesis by testing whether *Pim-1*-deficiency affects erythropoiesis *in vitro*.

Additional *Pim-1*-deficient phenotypes could be conditional, depending either on environmental factors (e.g. not present or too subtle in a laboratory situation) or on genetic background. It is conceivable that complex organisms such as vertebrates have an extensive network of overlapping regulatory pathways. Such a network would allow intricate regulatory control and provide evolutionary flexibility in the developmental program. However, such a system with a level of built-in redundancy could obscure the effects of the lack of one of the components, e.g. PIM-1.

Figure 5. Comparison of the mean cell volumes of erythrocytes as a function of *Pim-1* genotype. The values for all experimental groups are shown as closed bars as percentages of their wild-type controls with the standard deviations depicted by the open bars. The wild-type level of experiment 1 is shown for illustrative purposes. The numbers of mice used to calculate the averages are as in Table 1 for experiments 1 and 2 and for experiment 3: 6 wild-type mice; 3 homozygous mutant mice; 6 homozygous mutant mice with the ppWT transgene.

If such is the case, then removal of *Pim-1*-deficient cells from the abundance of parallel stimuli in the richly diverse *in vivo* environment may unmask defects resulting from the lack of *Pim-1* function. We are currently testing this concept.

We shall also investigate whether *Pim-1*-deficient mice have an altered susceptibility to MoMuLV-induced lymphomagenesis or whether the pattern of activations of other oncogenes is affected. The latter would provide clues to the cooperative

capability of *Pim-1* with various other oncogenes or the requirement of *Pim-1* for their activity.

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