

Constitutive expression of IL-18 binding protein in murine testicular tissues and cells

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Accepted for publication January 24, 2008

ABSTRACT. In the present study, we examined the cellular origin and the expression levels of interleukin-18 binding protein (IL-18 BP), during normal maturation of murine testis. Immunohistochemical staining of testicular tissues from sexually mature mice, shows that testicular germ cells, at different stages of differentiation, express IL-18 BP. Leydig cells/interstitial cells and peritubular cells express higher levels of IL-18 BP, as compared to other testicular cells. The levels of IL-18 BP were similar in testicular tissues and homogenates from sexually immature and mature mice, as examined by western blot, ELISA and real time PCR analysis. Our results demonstrate, for the first time, the expression of IL-18 BP in murine testicular tissues and cells. Together with our previous studies, which showed over-expression of IL-18 in testicular tissues of immature mice as compared with mature mice, these results may indicate a role for IL-18 in testicular development, and also in the regulation of testicular functions under physiological conditions.

Keywords: interleukin-18 binding protein, IL-18, testis, development, spermatogenesis

Spermatogenesis is a highly controlled process of proliferation, meiosis, and differentiation, which occurs in a number of sets of spermatogenic cell associations and stages [1, 2]. In addition to the regulatory effect of gonadotropins and androgens in the initiation and maintenance of spermatogenesis, a number of cytokines are also involved in the regulation of various differentiation steps in this process [3-5].

Cytokines such as the interleukin-1 (IL-1) family, tumor necrosis factor alpha (TNF- α) and IL-6 have been detected in testicular lysates and cells [5-14].

IL-18 is a multifunctional, pro-inflammatory cytokine, which was formally called interferon- γ (IFN- γ)-inducing factor. It is a potent inducer of IFN- γ production and it participates in the regulation of both Th1 and Th2 responses, and in innate and acquired immunity [15-18]. Recently, we have shown that active IL-18, IL-1 β -converting enzyme (ICE) and IL-18R α are over-expressed in testicular homogenates from sexually immature as compared to mature mice [19, 20]. In addition, we have demonstrated that Leydig cells and testicular germ cells express higher levels of IL-18 as compared to other testicular cells [19, 20]. In addition, Strand M-L *et al.* [21] have recently shown IL-18 expression in rat testicular tissues and demonstrated its involvement in germ cell growth. IL-18 binding protein (IL-18 BP) is a novel, naturally occurring and constitutively expressed and secreted pro-

tein. It has high affinity binding to IL-18 and neutralizes its biological activity [22]. IL-18 BP is not the soluble receptor for IL-18. It resembles the extracellular domain of Ig-like receptors, but has no transmembrane form. In the mouse, there are two IL-18 BP isoforms c and d, where d is able to neutralize both human and murine IL-18 [23-25]. IL-18 BP is constitutively expressed in human spleen, colon, small intestine, and prostate [23]. IL-18 BP mRNA has been shown to be induced by IFN- γ in several cell lines, such as keratinocytes, colon carcinoma/epithelial cells, and renal mesangial cells and by endothelial cells and macrophages [24, 26, 27].

Since IL-18 was demonstrated in the testicular cells and found to be over-expressed in testicular tissues from sexually immature as compared to mature mice, the aim of the present study was to examine the expression levels and cellular origin of IL-18 binding protein (the endogenous inhibitor of IL-18) in testicular tissue and cells from sexually immature and mature mice, under physiological conditions. This may deepen our understanding of the possible functions of IL-18 in the testes of sexually immature and mature mice.

METHODS AND MATERIALS

Reagents

Interleukin-18 BP levels in testicular homogenates were measured using a murine IL-18 BP ELISA kit.

Recombinant-mouse IL-18 binding protein (CytoLab Ltd. Rehovot, Israel), and monoclonal anti mouse- and biotinylated anti-mouse IL-18 BP antibodies for ELISA were purchased from MBL (International Corporation, Woburn, MA, USA) (specific for mouse only). The range of the standard curve was 2-500 pg/mL, and the sensitivity of the kit was < 16 pg/mL.

Casein, proteinase K, Tween 20, diamino-benzidine tetrahydrochloride (DAB) were purchased from Sigma (St. Louis, MO, USA). Urea (ANALAR; BDH). Biotinylated antibodies, streptavidin-peroxidase conjugate and normal goat serum (Zymed, San Francisco, CA, USA). Eukitt (GmbH). All other chemicals (analytical grade) were purchased from commercial sources.

The investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals Promulgated by the Society for the Study of Reproduction. Sexually mature (adults; 8-10 week old) and sexually immature (2-week-old) BALB/c mice (Harlan Laboratories, Jerusalem, Israel) were used. At the age of 2 weeks (14 days old), although Sertoli cells are almost completely differentiated, the spermatogenic process is incomplete and mice are not producing spermatozoa [28].

Preparation of testicular homogenates

Testicular homogenates were prepared from sexually immature and mature mice. Single testis from each mouse was prepared and examined separately. The tunica albuginea was removed and the remaining testicular tissue was homogenized in 0.8 mL cold PBS in ice. At the end of the homogenization process, the mixture was centrifuged at 13 000 RPM for 15 min, the supernatant was collected and stored at -70°C. Total protein was examined using Biorad reagent according to the manufacturer's instructions (BIO-RAD Laboratories GmbH, Munchen, Germany). The Bio-Rad Protein Assay is a dye-binding assay based on the differential color change of a dye in response to various concentrations of protein [29].

Extraction of total RNA, reverse transcriptase (RT)-PCR and real time PCR analysis

Total RNA was extracted from murine testis, using the EZ RNA Reagent protocol (Biological Industries, Beit Hamek, Israel). First-strand complementary DNAs (cDNAs) were synthesized from 2.5 µg total RNA with 0.5 µg random oligonucleotide primers (Roche Molecular Biochemicals, Mannheim, Germany) and 200 U of Moloney-Murine Leukemia Virus-Reverse Transcriptase (M-MLV-RT; Life Technologies, Inc., Paisley, Scotland, UK) in a total volume of 20 µL Tris-HCl-MgCl₂ reaction buffer, supplemented with DTT, dNTPs (0.5 mmol/liter; Roche Molecular Biochemicals) and RNase inhibitor (40U; Roche Molecular Biochemicals). The reverse transcriptase (RT) reaction was performed for 1 h at 37°C and stopped for 10 min at 75°C. The volume of 20 µL was subsequently made up to 60 µL with water. Negative controls for the reverse transcriptase reaction (RT-) were prepared in parallel, using the same reaction preparations with the same samples but without M-MLV-RT.

RT-PCR analysis

The PCR, performed subsequently, contained cDNA samples in a final dilution of 1:15 with two pairs of oligonucleotide primers (0.9pmol/µl; 5'AGAGGGAAATCGT-GCGTGAC3'; and 3'GCCGGACTCATCGTACTCCT5' for the murine β-Actin cDNA sequence, 5'CCTGGAGGTTCTCAATGA3'; and 3'GAAAGAAGTGC-CACTGAATGGA5' for the mouse IL-18 BP cDNA sequence (Sigma). To assess the absence of genomic DNA contamination in RNA preparations and RT-PCR reactions, PCR was performed with negative controls of the RT reaction (RT-) and without cDNA (cDNA-). The PCR reactions were carried out on a Cycler II System Thermal Cycler (Ericomp, San Diego, CA, USA). For β-actin we used 25 cycles and for IL-18 BP we used 40 cycles. Twenty microliters of each PCR product were run on 2% agarose gel, containing ethidium bromide, and photographed under UV light. The protein levels were quantified from the different samples of the western blot analysis using TINA software (version 2.10 g) (raytest Isotopenmessgeraets, GmbH, Straubenhardt, Germany).

Real time PCR analysis

Real-time quantitative PCR amplification of total cDNA (500ng/sample) used specific primers of IL-18BP, forward primer, 5'- GAAAGAAGTGCCACTGAATGGAA-3'; reverse primer, 5'-CCTGGGAGGTTCTCAATGA-3', β-actin forward, 5'-AGAGGGAAATCGTGCCTGAC-3'; reverse primer, 5'-CAATAGTGACCTGGCCGT-3' (Sigma). The reactions were conducted following the protocol for the Absolute qPCR SYBR Green mix (ABgene House, Blenheim Road, Epsom, UK) containing modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5mM MgCl₂, dNTP mix, and dUTP. The PCR reaction was performed using a real-time PCR machine (MyIQ, Bio-Rad Laboratories, USA) according to the manufacturer's instructions. The following PCR protocol repeated 45-50 times was used: denaturation (95°C for 10 min), amplification and quantification (94°C for 10 s, 60°C (IL-18BP and β-actin), 72°C for 30 s with a single fluorescence measurement, melting curve (60-95°C with a heating rate of 0.5°C per 30 s and a continuous fluorescence measurement), and a cooling step to 4°C. PCR products were identified and distinguished by the melting curve generated. The "threshold cycle" (C_t) values, representing the cycle number at which sample fluorescence rises statistically above background, and crossing points (CP) for each transcript, were defined. The relative quantity of gene expression was analyzed by the 2-ΔΔC_t method. The quantities of the IL-18BP mRNA was normalized to the endogenous control, β-actin.

To ensure accurate product and the absence of contaminating DNA, all the products of the RT-PCR which were used for real time PCR, for all the examined factors, were also examined in parallel by PCR analysis with all the negative controls, and were found to give a single specific band with the suitable size when run on 2% agarose gel.

Immunohistochemical staining of murine testicular tissues

Four-micron-thick sections from formalin-fixed, paraffin-embedded testicular tissue blocks from adult mice were mounted on saline-coated slides, dried at 37°C for 48 hrs and stored at room temperature. Before the primary antibodies were applied, blocking of the nonspecific background was performed with PBS containing 0.05% casein and/or normal goat serum. This solution was also used to dilute the primary antibodies. Sections were boiled in 6 M urea for 10 min [30]. Thereafter, polyclonal goat anti-mouse IL-18 BP antibodies (4 µg/mL) (Santa Cruz Biotechnology, Inc., CA, USA) were used as primary antibodies. After the primary antibodies had been applied for 1 hour, the PBS/casein solution was used for all further washings. The biotinylated antibody and the streptavidin-peroxidase conjugate were applied according to the suppliers' directions. Endogenous peroxidase was blocked with 3% H₂O₂ in 80% methanol for 15 min. before the streptavidin-peroxidase conjugate was applied. Development was performed with 0.06% DAB and Mayer's haematoxylin was used for counterstaining. The sections were mounted in Eukitt. Negative controls were included for each specimen using normal serum instead of the primary antibodies.

Western blot analysis

Testicular homogenates were incubated in sample buffer (0.02% Bromophenol blue and 2% Dithiothreitol [DTT]). Aliquots (50 µg protein) from the samples were submitted to electrophoresis on a 12% sodium dodecylsulfate (SDS)-polyacrylamide gel. The separated peptides were transferred onto nitrocellulose membranes, and the latter were blocked by incubation with milk at 25°C for 2 hours. The blots were sequentially incubated for 12 hours at 4°C with milk containing primary antibody [polyclonal rabbit anti-mouse IL-18 BP (abcam, Cambridge Science Park, Cambridge, UK) (1 µg/mL)] or anti-mouse β-actin (0.6 µg/mL) (Saint Louis, MO, USA). After three wash cycles with PBS-Tween 20, secondary antibodies (diluted in milk) were added to the nitrocellulose filters and developed in Enhanced Chemiluminescence (ECL) (Biological Industries, Beit Haemek, Israel).

Evaluation of results

Each experiment included three to five adult mice and three to five sexually immature mice, and was repeated at least three to six times.

The levels of IL-18 were evaluated as pg/µg protein of the testicular homogenate. The results are presented as mean of pg/µg protein + SEM.

The sensitivity of the ELISA kit was < 64 pg/mL; intra- and inter-assay precision (CV [%] were 9-12 and 11-13 respectively).

Statistics

Student's *t* test was used for statistical evaluation, and *p* values below 0.05 were considered significant.

RESULTS

Localization of IL-18 BP in murine testes

Immunofluorescence staining of formalin-fixed, paraffin-embedded testicular tissue from 10-week-old mice (adult) (figure 1A) showed that spermatogonia (spg), spermatoocytes (spc) and interstitial cells (which are composed mainly of Leydig cells (LC) and macrophages) stained positively for IL-18 BP. Interstitial cells and peritubular cells expressed higher levels of IL-18 BP as compared to other testicular cells.

Negative control for testicular tissues (figure 1B), using normal serum and the second antibody, did not stain for IL-18 BP.

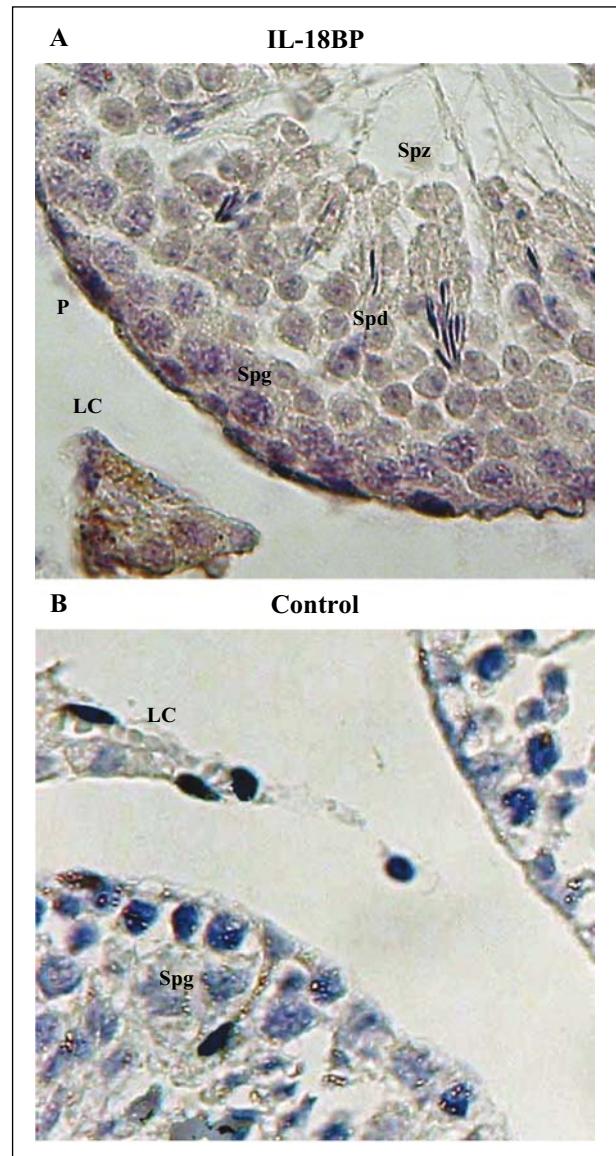


Figure 1
Localization of IL-18 BP in testicular cells from sexually mature murine. Immunohistochemical staining of testes from sexually mature murine (A) with polyclonal rabbit anti-mouse IL-18 BP antibodies (4 µg/mL) (x 400) were performed. As negative control testicular tissues were stained with normal serum (instead of the primary antibodies) and the second antibodies (B). P: peritubular cells; L: LC (Leydig cells/interstitial cells); Spc: spermatoocytes; Spz: spermatozoa.

Constitutive expression of IL-18 BP in testicular homogenates from sexually immature as compared to mature mice

As depicted in *figure 2A*, testicular homogenates from sexually immature and mature mice contain a 40 kDa IL-18 BP peptide. The levels of the 40 kDa form of IL-18 BP were similar in testicular homogenates from sexually immature as compared to mature mice, as examined by western blot analysis (*figure 2A*; a representative experiment is presented) and quantitated by densitometry (*figure 2B*; a summary of three different and independent experiments).

Testicular homogenates from sexually immature and mature mice also contain similar levels of IL-18 BP as examined by specific ELISA (*figure 3A*).

In addition, the expression levels of mRNA for IL-18 BP, in testicular homogenates from sexually immature and mature mice, were also similar as evaluated by real time PCR analysis (*figure 3B*).

DISCUSSION

We are the first to demonstrate the expression of IL-18 BP in murine testicular tissue and cells under physiological conditions. We also show similar expression levels of IL-18 BP in testicular tissues from sexually immature and mature mice. In our previous studies, we have demonstrated the expression of IL-18, ICE and IL-18 Ra (IL-18 family) in testicular cells of murine testis [19, 20]. The expression of IL-18 BP by testicular cells may indicate its possible involvement in the regulation of IL-18 functions in these cells and/or in their microenvironment.

In our previous study [19, 20], we demonstrated over-expression of the IL-18 family in testicular homogenates

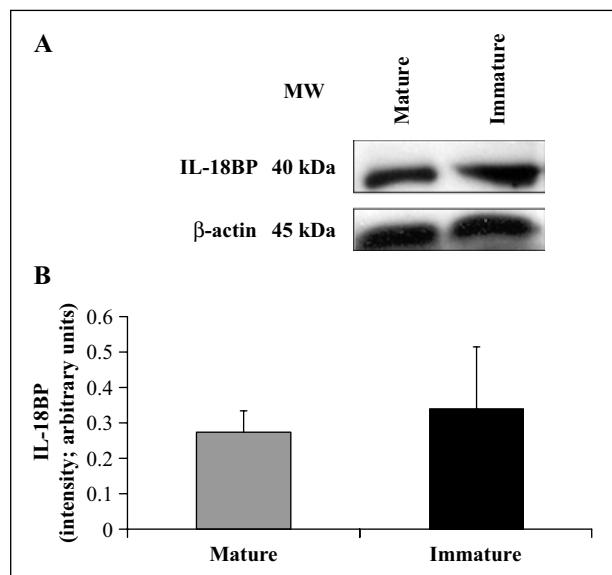


Figure 2

Constitutive expression levels of IL-18 BP in testicular homogenates of sexually immature as compared to mature murine. IL-18 BP levels and molecular weight were evaluated by western blot analysis (A. a representative experiment is presented). Quantitative evaluation of the western products was performed by video densitometry (B. a summary of 3 different and independent experiments; 3 mice were used in each experiment). MW: molecular weight (kDa).

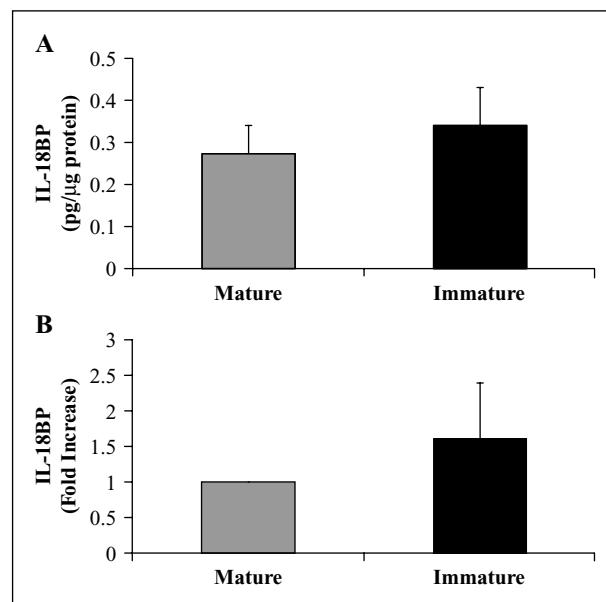


Figure 3

Expression levels of IL-18 BP in testicular homogenates of sexually immature as compared to mature murine. Testes from sexually immature ($n = 23$) and mature ($n = 23$) mice were collected and homogenized. IL-18 BP levels were evaluated by specific ELISA kit. IL-18 BP levels are expressed as pg/μg protein \pm SEM (A). The expression levels of IL-18 mRNA ($n = 9$) were evaluated by real time PCR using specific primer (B).

from sexually immature as compared to mature mice. However, in the present study we show similar expression levels of IL-18 BP (the natural inhibitor of IL-18) in testicular tissues from sexually immature as compared to mature mice. This may indicate that the testes of sexually immature mice contain more functional IL-18 as compared to testes from mature mice. It is possible to suggest that higher levels of functional IL-18 in the testes of sexually immature mice may function as a growth factor for germ cells [21] and other testicular cells, in addition to other functions. These results may suggest different functions for IL-18 in the testes of sexually immature as compared to mature mice.

The expression of similar levels of IL-18 BP in testicular tissues from sexually immature as compared to mature mice may also indicate that IL-18 BP is not regulated by gonadotropins and testosterone, or that high level of these endocrine factors (in sexually mature mice) induces testicular, IL-18 BP expression. Thus, our results may indicate that IL-18 BP and IL-18 are differently regulated by gonadotropins and testosterone. This issue is currently under investigation in our laboratory.

Our hypothesis is that the IL-18 family (IL-18 and its natural inhibitor – IL-18 BP) is involved in the regulation of testicular cells/tissue development/growth, differentiation and function under physiological conditions. These regulatory functions could include different functions of IL-18 in the testis, such as the regulation of innate immunity in the testis and the regulation of testicular cell proliferation and functions.

Acknowledgments. This work was partially supported by a grant (No. 1-760-205.2/2002) from the German-Israel Foundation (GIF) and a grant (No. 4467) from the Ministry of Health, Jerusalem.

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