Over expression of interleukin-1α, interleukin-1β and interleukin-1 receptor antagonist in testicular tissues from sexually immature mice as compared to adult mice

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ABSTRACT. The levels of IL-1α, IL-1β and IL-1Ra were higher in homogenates of testicular tissue from sexually immature than those from mature mice. Immunohistochemical staining of testicular tissues from sexually immature and adult mice show that differentiated germ cells express higher levels of IL-1α compared to Sertoli cells and Leydig cells/interstitial cells. Peritubular cells of sexually immature and adult mice did not express IL-1α. Testicular tissue cells of adult mice showed high levels of expression of IL-1β, mainly in the cytoplasm and nucleus of the spermatogonia and in spermatocytes. Sertoli cells and Leydig/interstitial cells were also highly stained for IL-1β. However, peritubular cells did not express IL-1β. On the other hand, testicular tissue cells from sexually immature mice, showed high levels of IL-1β, mainly in spermatocytes. Spermatogonia showed low levels of IL-1β expression. Also, high levels of IL-1β expression were detected in Leydig/interstitial cells. Peritubular cells clearly showed IL-1β expression. Testicular tissue cells from adult mice, showed IL-1Ra expression in spermatogonia, Sertoli and Leydig/interstitial cells. IL-1Ra expression was clearly present in the Golgi apparatus of spermatogonia and Sertoli cells. However, peritubular cells did not show IL-1Ra expression. Testicular tissue cells from sexually immature mice, also showed high levels of IL-1Ra expression mainly in the cytoplasm and nucleus of the spermatogonia and Sertoli cells. In addition, Leydig/interstitial cells and peritubular cells also expressed IL-1Ra. Our results demonstrate, for the first time, the expression of IL-1β in germ and Sertoli cells, and IL-1Ra in Leydig/interstitial cells of testicular tissues from adult and sexually immature mice, under *in vivo* conditions. In addition, the relative elevated levels of the IL-1 system in the testis of immature mice compared to mature mice may indicate its involvement in the spermatogenesis.

Keywords: interleukin-1, interleukin-1 receptor antagonist, Sertoli cells, Leydig cells, germ cells, testis

INTRODUCTION

Interleukin-1 (IL-1), like other pro-inflammatory cytokines, is an immunoregulatory polypeptide growth factor produced mainly by macrophages in response to foreign antigens, pathogens (infection challenge), and also in chronic inflammation (immunological activation) [1, 2]. Cells of non-immune cell origin, such as dendritic cells, fibroblasts, endothelial cells, smooth muscle cells, mesangial cells, astrocytes and epithelial cells, produce IL-1 [1, 2]. Molecular studies have demonstrated three IL-1 types, namely; IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra) [1, 2]. Both IL-1α and IL-1β types are produced as 31 kDa precursors and secreted as 17 kDa molecules. They bind to the same receptor, show low amino acid homology and share a spectrum of activities involved in physiological and pathophysiological functions [1, 2]. A unique feature of the IL-1 system is the naturally occurring IL-1Ra. Its genetic structure is homologous with the IL-1α and IL-1β genes to a certain degree [3, 4]. It binds to the same receptors without transmitting any signal; thus it inherits an equally important role in the regulation of IL-1 action [5, 6].

IL-1s exert growth-inducing, growth-inhibitory and differentiation-inducing activities, and are autocrine/paracrine factors with pleiotropic activity [1, 2]. In mammals, the process of spermatogenesis occurs within the seminiferous tubules that release spermatozoa into the rete testis. The seminiferous tubules contain germ cells (GC) and Sertoli cells (SC). Peritubular myoid cells (PC) surround the tubules, and are in contact with the basal surface of the Sertoli cells and spermatogonia. Leydig cells (LC) are located in the interstitium of the testis, between the tubules [7].

Spermatogenesis is a highly controlled process of proliferation, meiosis, and differentiation, which occurs in a

variable number of sets of spermatogenic cell associations or stages [7, 8]. There are probably a number of cytokines that are involved in the regulation of the various differentiation steps in this process [9, 10].

It has been shown that IL-1 is present in lysates of testicular tissue [11, 12]. Further investigations have identified several cellular sources of IL-1 in the testis. The interstitium, Leydig cells [13, 14] and testicular macrophages [15] were found to produce and secrete IL-1, preferentially the beta form. IL-1 alpha production can be detected in tubular sources, namely Sertoli cells [14, 16-18] and germ cells [19]. Residual bodies/cytoplasts from elongated spermatids were found to dramatically stimulate IL-1α production of Sertoli cells [18]. Recently, we have demonstrated that mature human sperm cells express and secrete on IL-1-like molecule under *in vitro* conditions [20-22]. We have also shown that Sertoli cells and germ cells could produce IL-1Ra under *in vitro* conditions [23, 24]. The levels of IL-1Ra in Sertoli cells were induced by LPS, IL-1 and FSH [23].

IL-1 receptors have been identified, characterized and localized in mouse testis [25, 26]. The involvement of IL-1 in the development of spermatogenesis is not yet clear.

In the present study, we examined the cellular origin and the levels of expression of IL-1α, IL-1β and IL-Ra in the testis of sexually immature and adult mice.

MATERIALS AND METHODS

Materials

Recombinant-human IL-1 alpha (92.5 U/ng) and IL-1 beta (280 U/ng) were obtained from Genzyme (Cambridge, MA, USA). Recombinant human IL-1Ra, polyclonal goat anti-mouse IL-1Ra, anti-mouse IL-1α and anti-mouse IL-1β were purchased from R&D Systems (Minneapolis, MN, USA). Casein, proteinase K, Tween 20, diamino-benzidine tetrahydrochloride (DAB) were purchased from Sigma (MO, St. Louis, 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.

Immunohistochemical staining of mouse testicular tissues

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 weeks old) and sexually immature (2 weeks old) Balb/c mice were used. At the age of 2 weeks, even though Sertoli cells are almost completely differentiated, the spermatogenic process is not complete and mice are not producing spermatozoa (27). Three mice were examined in each experiment and each experiment was repeated more than 6 times.

Mice were killed (by CO_2 asphyxiation), and testicular tissues were immediately fixed in neutral formalin solution for immunohistochemical studies.

Four micron-thick sections from formalin-fixed, paraffin-embedded testicular tissue blocks from adult and immature 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 [28]. Thereafter, polyclonal rabbit anti-mouse IL-1α antibodies (20 μg/ml), or polyclonal rabbit anti-mouse IL-1β antibodies (10 μg/ml), polyclonal rabbit anti-mouse IL-1Ra antibodies (20 μg/ml) 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% H_2O_2 in 80% methanol for 15 min. before the streptavidin-peroxidase conjugate was applied. Development was done with 0.06% DAB, and Mayers haematoxylin was used for counter staining. The sections were mounted in Eukitt. Preabsorption with the relevant recombinant peptide showed a significant decrease in positive staining for each primary antibody. Negative controls were included for each specimen using normal rabbit serum or PBS/casein instead of the primary antibodies.

Pre-absorption of the first antibodies

The antibodies anti-IL-1α (20 μg/ml), anti-IL-1β (10 μg/ml) or anti-IL-1Ra (20 μg/ml) were incubated with various concentrations (1-30 μg/ml) of the relevant recombinant cytokines. After overnight incubation at 4°C, the mixture was used as the first antibody to stain the testicular tissues. An example is depicted in Figure 2E for IL-1β staining after pre-absorption.

Preparation of testicular homogenates

Testicular homogenates were prepared from 22 immature mice and 26 mature mice. Testies from each mouse were prepared and examined separately. The tunica albugina was removed from the testies and the remaining testicular tissue was homogenized in 0.8 ml of PBS in ice. At the end of the homogenization process, the mixture was centrifuged at 13000 RPM for 15 min. and the supernatant was collected and stored at – 70°C. Total protein was determined using Biorad reagent. IL-1α, IL-1β and IL-1Ra levels were examined using specific ELISA kits.

Results evaluation

Each experiment included groups of 3 adult mice and 3 immature mice. Each experiment was repeated at least three times.

Immunohistochemical staining was calculated by intensity from 0 to 3, which included intensity of the staining and percentage of the stained cells in a 10 High Power Field (HPF).

The levels of each cytokine were evaluated as pg/ml/μg protein in the testicular homogenate.

Statistics

Student's *t* test was used for statistical evaluation.

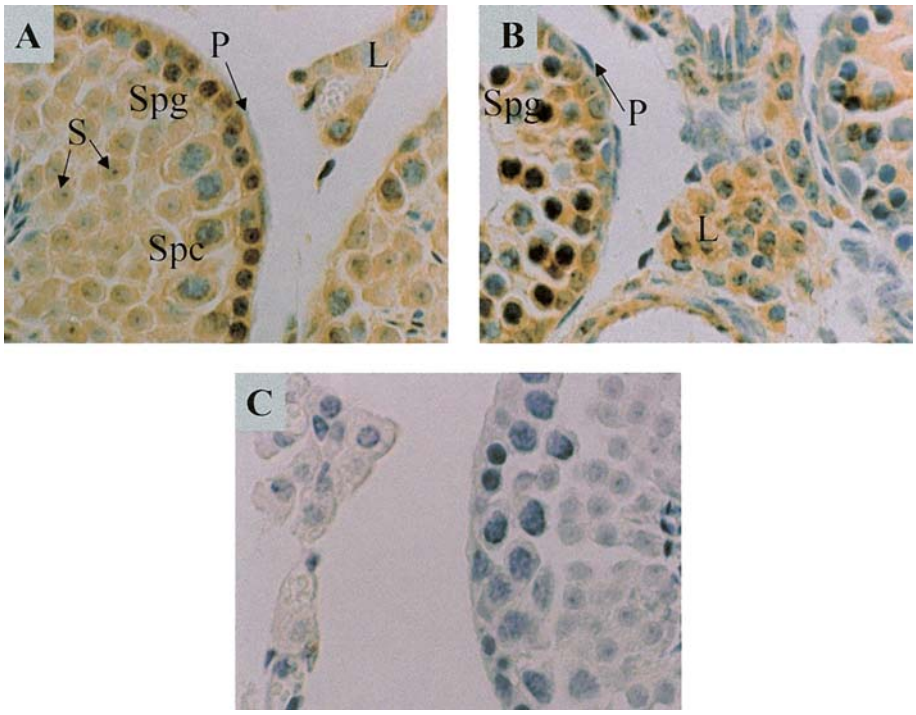

Figure 1
Expression of IL-1α in testicular tissues from adult and sexually immature mice
Immunohistochemical staining of testicular tissues from adult (A) and sexually immature mice (B) with polyclonal rabbit anti-mouse IL-1α antibodies (20 μg/ml) (× 400). As a negative control (C), we used normal rabbit serum. P – peritubular cells; L – Leydig cells (interstitial cells); S – Sertoli cells; Spg – spermatogonia; Spc – spermatocyte.

RESULTS

Expression of IL-1α in testicular tissues from adult and sexually immature mice

Immunohistochemical staining of formalin-fixed, paraffin-embedded testicular tissues of adult mice show that spermatogonia/spermatocytes express higher levels of IL-1α compared to Sertoli cells and interstitial cells (which are composed mainly of Leydig cells) (Figure 1A). In some spermatozoa the tails were also positively stained for IL-1α. Peritubular cells did not express IL-1α. The same pattern of IL-1α expression was found in testicular tissue cells from sexually immature mice (Figure 1B). Negative controls (Figure 1C) did not show IL-1α expression.

Expression of IL-1β in testicular tissues from adult and sexually immature mice

Testicular tissues from adult mice showed high levels of expression of IL-1β, mainly in the cytoplasm and nucleus of the spermatogonia and in spermatocytes (Figure 2A). Sertoli cells and interstitial cells (mainly Leydig cells) were also highly stained for IL-1β. The tails of some spermatozoa were also stained for IL-1β. However, peritubular cells did not express IL-1β.

On the other hand, testicular tissue cells from sexually immature mice, showed high levels of IL-1β, mainly in spermatocytes (Figure 2B, C). Spermatogonia showed low levels of IL-1β (Figure 2B, C). These cells also showed nuclear IL-1β staining (Figure 2C). The positive staining of the nucleus was also clear when the tissues stained only for IL-1β without hematoxylin staining (Figure 2D). High levels of IL-1β expression were also detected in interstitial cells (mainly Leydig cells) (Figure 2B). Peritubular cells clearly showed IL-1β expression (Figure 2B). Negative control using normal rabbit serum or PBS instead of the first antibody (Figure 2E) or pre-absorption of the first antibodies (10 μg/ml) with recombinant IL-1β (30 μg/ml) (Figure 2F) showed testicular tissues unstained for IL-1β.

Expression of IL-1Ra in testicular tissues from adult and sexually immature mice

Testicular tissues from adult mice showed IL-1Ra expression mainly in the cytoplasm of the spermatogonia and Sertoli cells (Figure 3A). Also, interstitial cells (mainly Leydig cells) expressed high levels of IL-1Ra. IL-1Ra expression was clearly seen in the Golgi apparatus of spermatogonia and Sertoli cells. Peritubular cells did not show IL-1Ra expression.

Testicular tissues from sexually immature mice showed high levels of IL-1Ra expression mainly in the cytoplasm and nucleus of the spermatogonia and Sertoli cells (Figure 3B). Also, interstitial cells (mainly Leydig cells) and peritubular cells showed IL-1Ra expression. IL-1Ra was also expressed in the Golgi apparatus of Sertoli and spermatogonia cells from both adult and sexually immature mice (Figures 3A and 3C respectively). Negative control (Figure 3D) did not show IL-1Ra expression.

Summary of the expression levels and cellular compartment of IL-1α, IL-1β and IL-1Ra in testicular cells from adult and sexually immature mice

Table 1 summarize, the intensity of expression (–, +, ++) of IL-1α, IL-1β and IL-1Ra and their cellular compart-

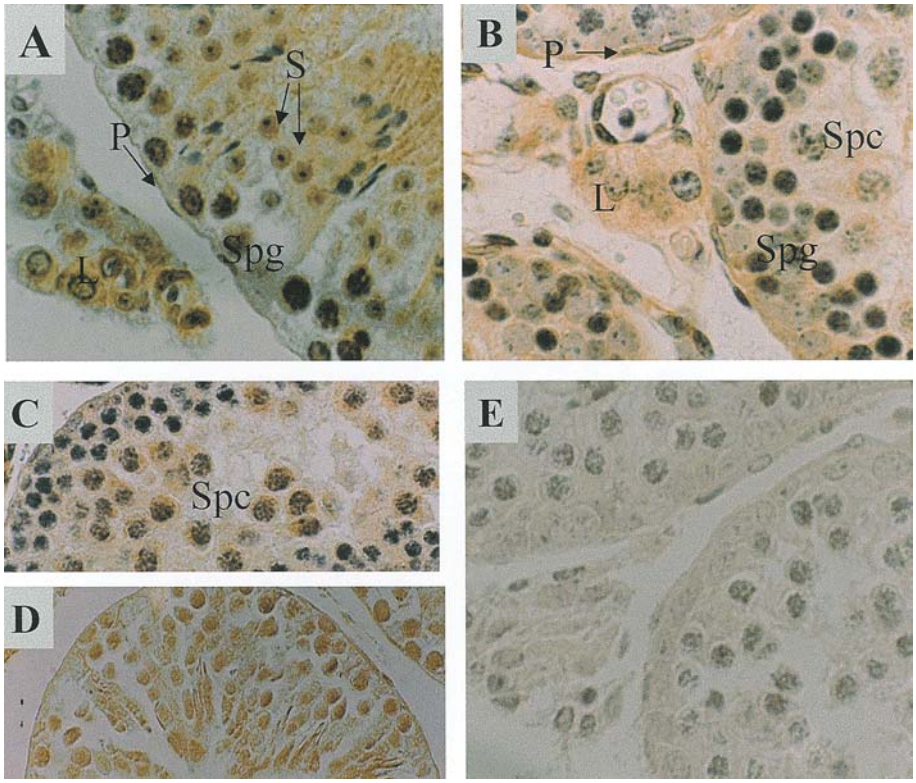

Figure 2
Expression of IL-1β in testicular tissues from adult and sexually immature mice
Immunohistochemical staining of testicular tissues from adult (A) and sexually immature mice (B,C) with polyclonal rabbit anti-mouse IL-1β antibodies (10 μg/ml) (× 400). Nuclear staining of IL-1β was clear when the tissues stained only with IL-1β without hematoxylin staining (D). As a negative control (E), we used normal rabbit serum instead or pre-absorption of the IL-1β antibodies with recombinant IL-1β (F).
P – peritubular cells; L – Leydig cells (interstitial cells); S – Sertoli cells; Spg – spermatogonia; Spc – spermatocyte.

ment (cytoplasm, nucleus and Golgi apparatus) in testicular cells of adult and sexually immature mice.

IL-1α, IL-1β and IL-1Ra levels in homogenates of testicular tissue from immature and mature mice

As shown in Table 2, homogenates of testicular tissue from immature mice contain significantly high levels of IL-1α, IL-1β and IL-1Ra as compared to mature mice ($p = 0.0002$, $p = 0.0000006$ and $p = 0.00000001$ respec tively). IL-1α was significantly lower than IL-1β or IL-1Ra in homogenates of testicular tissue from both immature and mature mice.

DISCUSSION

Using immunohistochemical studies we could show for the first time that IL-1α, IL-1β and IL-1Ra are expressed in germ cells and/or somatic cells of both sexually immature and mature mice. Although both IL-1α and IL-1β were expressed (IHC) in germ cells of sexually immature mice, IL-1α is highly expressed compared to IL-1β. IL-1Ra was expressed in a similar manner as IL-1α. On the other hand, we demonstrated higher levels of IL-1α, IL-1β and IL-1Ra, in the homogenates of testicular tissue from sexually immature mice than those from in the homogenates of mature mice. The levels of IL-1β and IL-1Ra were higher than IL-1α. The involvement of all testicular cells (germ cells and somatic cells) in the production of the IL-1 system suggests that IL-1s could be involved in the regulation of crucial physiological functions in the testis. In addition, the significantly higher levels of IL-1α, IL-1β and IL-1Ra in the testis of sexually immature mice than mature mice may indicate their involvement in the regulation of testicular cells/tissue functions related to spermatogenesis, such as proliferation, apoptosis and differentiation of germ cells. It is also possible that IL-1 may affect Sertoli and Leydig cell functions related to spermatogenesis. Indeed, IL-1 has been demonstrated to induce immature Sertoli cell proliferation, production of transferrin, production of IL-1α and IL-1Ra and inhibition of FSH-induced aromatase activity [23, 24, 29-32]. IL-1α was also able to elicit a transient but significant increase in Sertoli cell sertolin expression, which increases with testicular development, and is likely to be associated with the onset of spermatogenesis [33]. IL-1 was shown to promote DNA synthesis, and differentiation of spermatogonia and preleptotene spermatocytes [34, 35]. IL-1α has been suggested to play a critical role in postmeiotic germ cell development through the control of glucose metabolism [36]. The expression of IL-1α, but not IL-1β, was developmentally regulated in rat testis. It was expressed in a stage-dependent pattern during the cycle of the seminiferous epithelium. This expression in Sertoli cells was suggested to be dependent upon interaction with germ cells [35-38]. Moreover, there is increasing evidence confirming the involvement of IL-1 in testicular control of spermatogenesis [10]. In the interstitial compartment of the testis, IL-1 has been shown to affect immature Leydig cell prolifera-

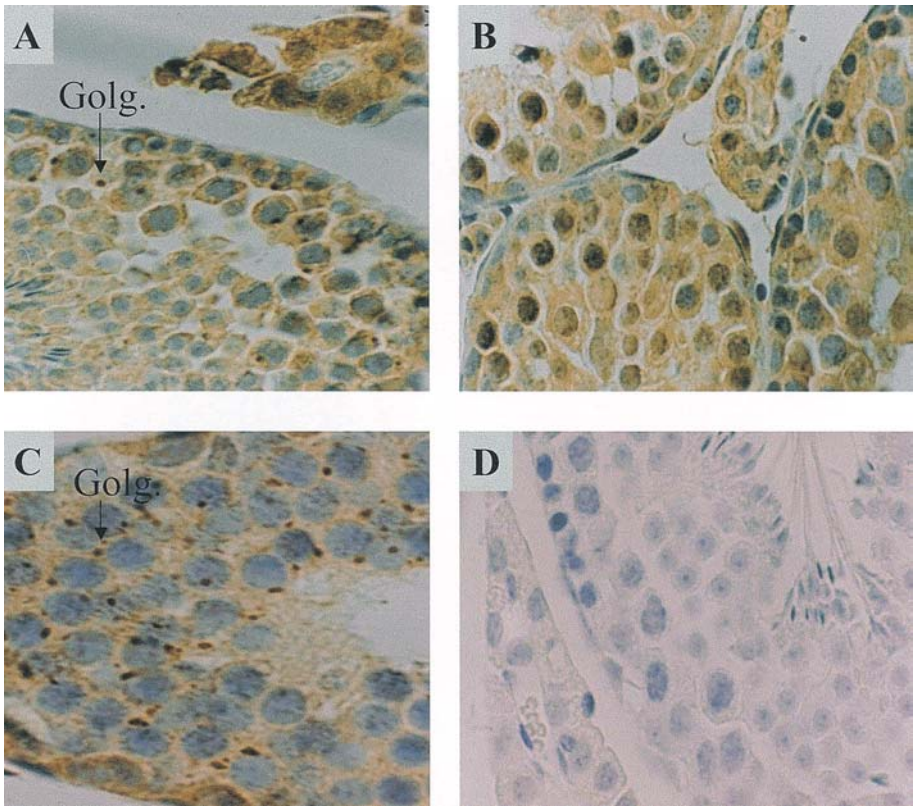

Figure 3
Expression of IL-1Ra in testicular tissues of adult and sexually immature mice
Immunohistochemical staining of testicular tissues from adult (A) and sexually immature mice (B,C) with polyclonal rabbit anti-mouse IL-1Ra antibodies (20 μg/ml) (× 400). As a negative control (D), we used normal rabbit serum instead.
P – peritubular cells; L – Leydig cells (interstitial cells); Spg – spermatogonia; Spc – spermatocyte. Golg. – Golgi apparatus.

Table 1
Summary of the expression of IL-1α, IL-1β and IL-1Ra in testicular cells of adult and sexually immature mice.

Cell type	Adult mice			Sexually immature mice		
	IL-1α	IL-1β	IL-1Ra	IL-1α	IL-1β	IL-1Ra
Leydig (interstitial cells)	+ Cyto	+ Cyto	+ Cyto	+ Cyto	+ Cyto	+ Cyto
Sertoli	+ Cyto	+ Cyto/Nucl	+ Cyto	+ + Cyto	+ Cyto	+ Cyto/Nucl
Peritubular	–	–	–	–	+	+
Spermatogonia	+	+ + Cyto/Nucl	+ Cyto/Gol	+ + Cyto	+ + Cyto/Nucl	+ + Cyto/Nucl/Gol
Spermatids	+ Tails	+ Tails	–	ND	ND	ND

–, +, ++, Indicate the intensity of the staining; Cyto – cytoplasm; Nucl – nucleus; Gol – Golgi apparatus; ND – not determined.

Table 2
IL-1α, IL-1β and IL-1Ra levels* in homogenates of testicular tissues from sexually immature and mature mice.

	Immature	Mature	Immature/mature (ratio)	*P***
IL-1α	0.677 + 0.591	0.124 + 0.11	5.463	0.0002
IL-1β	4.273 + 2.492	0.641 + 0.412	6.666	0.0000006
IL-1Ra	5.492 + 2.490	0.790 + 0.306	6.95	0.00000001
(IL-1α + IL-1β)/IL-1Ra	0.901	0.968	–	NS

*IL-1 levels are expressed as pg/ml/μg protein of the testis + SD; ** $p < 0.05$ is considered as statistically significant (t-test); NS – not significant.

tion and adult Leydig cell functions, and to induce acute inflammation-like changes in testicular microcirculation [39-41]. IL-1 is considered a potent inhibitor of Leydig cell function. It blocks hCG-induced cAMP and testosterone formation as well as cytochrome p450 side-chain cleavage messenger RNA expression in Leydig cells, which may contribute to the inhibitory effects of IL-1β on Leydig cell steroidogenesis [42].

IL-1, produced by germ cells and somatic cells including Sertoli cells and the interstitial cells, which are composed mainly of Leydig cells in addition to macrophages, may also act as a paracrine and an autocrine factor. Also, IL-1 could act as an inducer of testicular tissue repair (under normal and pathological conditions i.e. inflammatory sites).

IL-1Ra could be involved in the regulation of spermatogenesis and other functions (directly or through the regulation of IL-1 activity), since it is expressed differently in seminiferous tubule cells of sexually immature and mature testicular tissues. Recently, we have demonstrated constitutive expression of intracellular (not secreted) IL-1Ra and IL-1α, but not IL-1β, in Sertoli and germ cells from immature mice [23, 24]. IL-1α and IL-1Ra levels were increased following stimulation with LPS and IL-1; however FSH increased only the levels of IL-1Ra [23]. In the present study, we have shown expression of IL-1α, IL-1β and IL-1Ra in testicular cells from both sexually immature and mature mice. In addition, IL-1Ra was found in the Golgi apparatus, which indicates that this protein could also be secreted. These results may support the possibility of the involvement of endocrine factors (gonadotrophins) in the regulation of the IL-1 system under *in vivo* conditions. This possibility is currently under investigation in our laboratory. Recently, we have demonstrated the production of this cytokine by Sertoli and Leydig cells, *in vitro*, to be under the regulation of FSH and LH [43-45].

The involvement of IL-1 in testicular function is also emphasized by demonstrating the expression of both types of IL-1 receptors (type I/II) in isolated rat, mouse, and human somatic testicular cells (macrophages, Leydig cells, Sertoli cells and peritubular cells). While also present in rat and mouse isolated pachytene spermatocytes and early spermatids, the only germ cell types that were found not to express IL-1RI mRNA were the elongating spermatids [26]. These results may suggest the different and/or selective effect of IL-1 on germ cells during the differentiation stages (during the spermatogenesis).

Our results differ from those obtained in the rat system [46-48], where constitutive expression of IL-1β and IL-1Ra was not demonstrated in the intact rat testis cells of young and adults, and IL-1α was detected only in Sertoli cells. Our suggestion is that this difference could be related to the species used, since our results were also confirmed using human testicular biopsy tissue [49].

Thus, different expression levels of IL-1α, IL-1β and IL-1Ra in testicular tissues from sexually immature and adult mice may suggest the possible involvement of gonadotrophins in the regulation of IL-1 system expression during the spermatogenesis/spermiogenesis processes. Therefore, it is possible to suggest the involvement IL-1 system in the regulation of testicular cell functions during sexual maturation and in the control of spermatogenesis.

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