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Immunolocalization of Steroidogenic Enzymes P450scc, 3 β HSD, P450c17, and P450arom in Göttingen Miniature Pig Testes

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Abstract. The objective of this study was to investigate immunolocalization of steroidogenic enzymes in Göttingen miniature (GM) pig testes. Testes of 6 adult GM pigs were obtained in September 1996 (n=2), February (n=2) and June (n=2), 1997. Steroidogenic enzymes were immunolocalized using polyclonal antisera raised against bovine adrenal cholesterol side-chain cleavage cytochrome P450 (P450scc), human placental 3 β -hydroxysteroid dehydrogenase (3 β HSD), porcine testicular 17 α -hydroxylase cytochrome P450 (P450c17), and human placental aromatase cytochrome P450 (P450arom). Histologically, all types of spermatogenic cells including mature-phase spermatozoa in seminiferous tubules were observed in all testes throughout the year. Moreover, P450scc, 3 β HSD, P450c17 and P450arom were identified in Leydig cells but not in Sertoli cells of all testes. These results suggested that adult GM pig testes have the ability to produce germ cells throughout the year, and the synthesis of progesterone, androgen and estrogen occurs in the Leydig cells of GM pig testes.

Key words: Göttingen miniature pigs, Immunohistochemistry, Leydig cells, Spermatogenesis, Steroidogenic enzymes

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The mammalian testis is a complex organ characterised by two main functions: synthesis of testicular hormones and production of spermatozoa [1]. The synthesis of steroid hormones requires a substrate, that is, a source of the steroid ring system, a series of enzymes and cofactors that together constitute the biosynthetic

pathway and a source of energy [2]. Steroid biosynthesis is accomplished by the actions of two major families of enzymes. The first are the hydroxylase enzymes, encoded by genes belonging to the cytochrome P450 superfamily. The second family, the steroid dehydrogenase enzymes, belongs to one of two distinct groups, the shortchain alcohol dehydrogenase/reductase family or the aldo-keto reductase superfamily. Together, the P450 and dehydrogenase enzymes

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are necessary for steroidogenesis in the gonads, placenta, and adrenals [3]. The precise location of steroidogenic enzymes inside the testes has been studied during recent decades. Immunolocalization of steroidogenic enzymes in the testes has been reported in several species, including brown bears [4, 5], black bears [6, 7] and raccoon dogs [8]. However, similar studies have not been conducted in GM pigs.

The GM pig plays a significant role as a laboratory animal in biomedical research and organ transplantation [9]. Although many observations about reproduction in the GM pig have been reported recently [10–13], there are still many gaps in the understanding of the mechanisms of its reproduction. The aim of the present study was to investigate the localization of steroidogenic enzymes and mechanisms that regulate spermatogenesis and steroidogenesis in the testicular tissues of the GM pig.

Materials and Methods

Animals

Six adult GM pigs (nine months old) maintained for experimental use at the Central Institute for Experimental Animals, Kanagawa, Japan were used in September 1996 (n=2), February (n=2) and June (n=2), 1997. Testicular tissues obtained were immediately fixed for 13 hr in Bouin's solution for histological and immunohistochemical observation. All procedures were carried out in accordance with the guidelines established by Tokyo University of Agriculture and Technology.

Histology

Testicular samples were dehydrated in an ethanol series and embedded in paraffin wax. Sections (4–6 μ m) of testes were routinely stained with hematoxylin-eosin (HE) for histological observation. Serial sections of testes were mounted on slides coated with poly-L-lysine (Sigma, St. Louis, MO, U.S.A.) for immunohistochemistry.

Immunohistochemistry

The serial sections of the testes were incubated with 10% normal goat serum to reduce background staining caused by the second antibody. The sections were then incubated with primary antibody (1:500 or 1:1000) raised against bovine

adrenal cholesterol side-chain cleavage cytochrome P450 (P450_{scc}) [14], human placental 3 β -hydroxysteroid dehydrogenase (3 β HSD) [15], porcine testicular 17 α -hydroxylase cytochrome P450 (P450_{c17}) [16], or human placental aromatase cytochrome P450 (P450_{arom}) [17] for 16 hr at room temperature. The antibodies of 3 β HSD and P450_{arom} were kindly supplied by Dr. J. I. Mason (Edinburgh University, Edinburgh EH3 9YW, U.K.) and Dr. N. Harada (Fujita Gakuen Health University, Aichi 470–1100, Japan), respectively. Antibodies of P450_{scc} and P450_{c17} were kindly supplied by Dr. S. Kominami (Hiroshima University, Hiroshima 236–8982, Japan). The sections were then incubated with a second antibody, goat anti-rabbit IgG conjugated with biotin and peroxidase with avidin, using a rabbit ExtrAvidinTM staining kit (Sigma), followed by visualization with 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 ml of 0.05 mol Tris-HCl l⁻¹ buffer, pH 7.6, plus 30 μ l H₂O₂. Finally, the reacted sections were counterstained with haematoxylin solution (Merck, Tokyo, Japan). The control sections were treated with normal rabbit serum (Sigma) instead of the primary antisera.

Results

Histology

A number of seminiferous tubules and the interstitial connective tissue were observed in the testes examined. The most prominent cells in the interstitium were Leydig cells. Each Leydig cell had a large round nucleus and a large cytoplasm. Normal spermatogenesis was observed in all testes collected in September, February and June. Spermatogonia, spermatocytes, round spermatids and elongating spermatids were present in seminiferous tubules of all testes. Seasonal changes of spermatogenesis were not observed in these testes of GM pigs (Fig. 1a).

Immunohistochemistry

P450_{scc}, 3 β HSD, P450_{c17} and P450_{arom} were immunolocalized in the cytoplasm of Leydig cells of all testes (Figs. 1b–e, respectively). Immunoreactivity for P450_{scc}, 3 β HSD, P450_{c17} or P450_{arom} was not observed in any Sertoli cells or germ cells. The intensities of the immunohistochemical signals for P450_{scc}, 3 β HSD,

Fig. 1. Immunolocalization of steroidogenic enzymes in the testes of the Götting miniature pigs collected in June. Immunostaining for cholesterol side-chain cleavage cytochrome P450 (P450scc), human placental 3β -hydroxysteroid dehydrogenase (3β HSD), porcine testicular 17α -hydroxylase cytochrome P450 (P450c17), and human placental aromatase cytochrome P450 (P450arom) were immunolocalized in the cytoplasm of Leydig cells (b, c, d, e, respectively). Control incubated with normal rabbit serum in place of primary antiserum (a). All micrographs were taken at the same magnification and the scale bar represents 40 μ m. LC: Leydig cell.

P450c17 or P450arom did not appear markedly different among the testes collected in September 1996, February and June, 1997. No immunostaining was detected in control sections when normal rabbit serum was substituted for the primary antibody (Fig. 1a).

Discussion

This is the first immunohistochemical description of the localization of steroidogenic enzymes in testicular tissues of the GM pig. The principal findings are that Leydig cells contain P450scc, 3 β HSD, P450c17 and P450arom, suggesting that synthesis of progesterin, androgen and estrogen occurs in Leydig cells which are an important source of steroid hormones in GM pigs.

The biosynthesis of sex steroid hormones such as progesterin, androgen and estrogen requires the activity of each specific steroidogenic enzyme. Immunolocalization of steroidogenic enzymes in GM pigs was first observed in this study, and it showed that P450scc, 3 β HSD, P450c17 and P450arom are localized in Leydig cells but not Sertoli cells. These findings are similar to those which have been observed in other species such as brown bears [4], black bears [5–7], raccoon dogs [8], humans [18, 19] and rats [20]. The Leydig cells of the testis are the only cells in the male with the capacity to synthesize testosterone from cholesterol [21]. P450scc converts cholesterol to pregnenolone, and is located in the inner mitochondrial membrane of the Leydig cell, while the other biosynthetic enzymes such as 3 β HSD, P450c17 and P450arom, are situated in the endoplasmic reticulum. Androgens and estrogen are synthesized in the endoplasmic reticulum of Leydig cells [22]. Our immunohistochemical results, that P450scc, 3 β HSD, P450c17 and P450arom are localized in Leydig cells, indicate that the synthesis of androgen and estrogen occurs in the Leydig cells of GM pigs. In addition, the intensity of the immunohistochemical signal for P450scc, 3 β HSD, P450c17 or P450arom did not appear markedly different among testes collected in September, February and June, suggesting that steroidogenesis does not show seasonal changes in GM pigs.

The survival of spermatogenic cells is dependent

on gonadotropins as well as intratesticular androgens induced by luteinizing hormone (LH) [23, 24]. Testosterone is necessary to maintain normal spermatogenesis in mature animals. An important function of testosterone is the conversion of round spermatids to elongated ones in the rat [25]. Testosterone also plays an important role in preventing apoptotic cell death in androgen-dependent tissues [26–28]. In this study, all types of spermatogenic cells were found, including mature-phase spermatozoa in seminiferous tubules of all testes, indicating that the adult GM pig has the ability to produce germ cells and is a continuous breeder throughout the year. Immunoreactions for P450scc, 3 β HSD and P450c17 were present in Leydig cells of all testes, indicating that testosterone, which is essential for maintenance of spermatogenesis, is synthesized in Leydig cells of the GM pig. These results also suggest that testosterone regulates germ cell apoptosis in a stage-specific manner [22].

Normal spermatogenesis requires estrogen, and timing, amount, and duration of estrogen action must be tightly coordinated for the development and maintenance of normal sperm production [22]. P450arom is a key enzyme responsible for the formation of estrogens from androgens, and has been immunolocalized in Leydig cells of many species as well as in germ cells of mice [29], rats [30], brown bears [4], black bears [5–7] and raccoon dogs [8]. Aromatase activity has been measured *in vitro* in Leydig cells and Sertoli cells of immature and mature rats, whereas in pigs, rams and humans the enzyme activity is only present in Leydig cells [1]. In the present study, P450arom was only extensively immunostained in Leydig cells, not in Sertoli cells and germ cells. These results are in accordance with the results of other studies on P450arom in this species [31]. Thus, the present results indicate that Leydig cells are a major source of estrogen, and estrogen may play an important physiological role in normal spermatogenesis in GM pigs. Future studies should elucidate whether P450arom mRNA is expressed in Sertoli cells and germ cells of GM pigs.

In conclusion, immunoreactions for P450scc, 3 β HSD, P450c17 and P450arom were present in Leydig cells of GM pigs. These results suggest that the synthesis of progesterin, androgen and estrogen occurs in the Leydig cells of GM pigs.

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