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The roles of pericystic cells and Sertoli cells in spermatogonial proliferation stimulated by some growth factors in organ culture of newt (*Cynops pyrrhogaster*) testis

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ABSTRACT

We have previously shown FSH promotes spermatogonial proliferation and their differentiation into primary spermatocytes in organ culture of newt testicular fragments. Several growth factors identified in newt testis, such as stem cell factor (SCF), insulin-like growth factor (IGF)-I, and neuregulin (NRG)1, also stimulate spermatogonial proliferation in the organ culture. However, any growth factor added in vitro might not work on spermatogonia directly, but act on somatic cells such as Sertoli cells or pericystic cells, because size-selective barrier exists around a cyst which is enclosed by Sertoli cells. In order to determine the target somatic cells of the growth factors as well as the role of pericystic cells in spermatogonial proliferation and differentiation, we searched for agents that kill pericystic cells selectively. We found that treatment of the testicular fragments with trypan blue (TB) caused cell death of only pericystic cells and significant abolishment of the activity of SCF to stimulate spermatogonial proliferation, while the activities of neither IGF-I nor NRG1 were affected. In addition, the potency of neither IGF-I nor FSH to stimulate the differentiation of spermatogonia into primary spermatocytes was abolished by TB treatment. Consistent with these results, only the mRNA expression of c-kit was reduced by TB treatment, whereas those of FSH receptor, SCF, IGF-I, IGF-I receptor, Immunoglobulin-like domain-containing NRG1, ErbB2, and ErbB4 were unaffected. These results indicate that SCF stimulates pericystic cells, while IGF-I and NRG1, as well as FSH, activate Sertoli cells, resulting in stimulation of spermatogonial proliferation in organ culture of testicular fragments.

1. Introduction

Spermatogenesis involves interactions between germ cells and somatic cells, such as Sertoli cells and Leydig cells, in the testis, and actions of hormones secreted by the pituitary and testis (Parvinen et al., 1986; Skinner, 1991; Jegou, 1993). It is well known that gonadotropin is a pituitary glycoprotein hormone that regulates gonadal development and function in vertebrates. In mammals, two chemically distinct gonadotropins, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are synthesized in and released from the pituitary gland to regulate the spermatogenesis. FSH targets Sertoli cells which have FSH receptor (FSHR), while LH targets Leydig cells with LH receptor to stimulate androgen secretion.

In newt, presence of both FSH and LH was also demonstrated by isolation of their cDNAs and immunocytochemical detection of the antigens in the pituitary (Saito et al., 2002). Injections of mammalian FSH into hypophysectomized *Pleurodeles* and *Ambystoma tigrinum* stimulate spermatogenesis, while addition of LH caused 3β-HSD reactions in the glandular tissue (Andrieux et al., 1973; Moore, 1975). Also injection of frog LH stimulates testosterone production and induces spermiation, while frog FSH supports spermatogenesis (Tanaka et al., 2004). Our previous studies showed porcine follicle-stimulating hormone (pFSH) promotes spermatogonial proliferation and differentiation into primary spermatocytes in organ and reaggregate culture systems (Ji et al., 1992; Ito and Abé, 1999). The endocrine regulation of testis function has effects on cell–cell interactions and is affected by local cell–cell interactions. FSH actions on Sertoli cells also promote cell–cell interactions that influence germinal cell development, peritubular myoid cell differentiation and Leydig cell function (Parvinen et al., 1986; Sanborn et al., 1986; Skinner, 1991). Various paracrine factors are known to be involved in these interactions, such as activin, transforming growth factor-β (TGF-β), stem cell factor (SCF), insulin-like growth factor (IGF)-I, etc. (Jegou, 1993).

Spermatogenesis in urodeles proceeds synchronously within a spermatocyst, the smallest unit of the testis, in close contact with some Sertoli cells which enclose a clone of germ cells (Callard et al., 1978). A larger unit of the testis is a lobule that consists of some cysts and is ensheathed by a basement membrane. Outside

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the lobules are Leydig-like cells that are also called pericystic cells or lobule boundary cells (Lofts, 1987). The testis of *Cynops pyrrhogaster* in spring consists of two main parts; the anterior immature part consisting of many cysts containing spermatogonia and sometimes spermatocytes, and the posterior mature part containing mature sperm. As in most salamanders, pericystic cells in the immature part are small and poorly developed when spermatogenesis progresses (Pudney and Callard, 1984). When spermiation occurs, however, pericystic cells undergo hypertrophy and lobules degenerate, resulting in the formation of a layer of differentiated Leydig cells called the glandular tissue. These cells of the glandular tissue show morphological and histochemical characteristics with steroid hormone-secreting cells; Δ5-3β-hydroxysteroid dehydrogenase (Δ5-3β-HSD) activity is positive (Imai and Tanaka, 1978). But the pericystic cells in immature part during spermatogenesis are negative for the Δ5-3β-HSD activity. Thus the roles of pericystic cells in immature part during spermatogenesis are not known.

Newt spermatogonia enter meiosis in the 8th generation after 7 mitotic divisions (Abé, 2004), when FSH alone is added, in organ and reaggregate culture systems with chemically defined medium (Ji et al., 1992; Ito and Abé, 1999). Since FSH acts on Sertoli cells which in turn produce some paracrine factors to activate spermatogonia, we have looked for those factors and found that SCF, IGF-I and neuregulin (NRG)1 are expressed in newt testes.

SCF is a ligand synthesized by Sertoli cells in the testis and c-kit is a receptor for SCF that is expressed by spermatogonia and Leydig cells (Loveland and Schlatt, 1997). SCF/c-kit system plays a pivotal role in survival (Packer et al., 1995; Hakovirta et al., 1999), proliferation and differentiation of germ cells (Yoshinaga et al., 1991; Manova et al., 1993; Vincent et al., 1998; Sette et al., 2000) and Leydig cells (Yan et al., 2000) in mammalian testis. IGFs are thought to play autocrine and paracrine roles in spermatogenesis (Spiteri-Grech and Nieschlag, 1992). In mammalian testes, IGFs stimulate mitotic DNA synthesis in cultured segments of rat seminiferous tubules (Söder et al., 1992). The IGF-I receptor (IGF-IR), a transmembrane tyrosine kinase, is widely expressed across many cell types in fetal and postnatal tissues (Adams et al., 2000). IGF-I has been found to play a key role in the interaction between Sertoli cells and Leydig cells (Oonk and Grootegoed, 1988). NRG1 is one of the members of the NRG genes (also called NDF, heregulin, GGF, and ARIA) which belong to epidermal growth factor (EGF) family and induce growth and differentiation of epithelial, glial and muscle cells in culture (Falls, 2003). The family of EGF receptors includes four closely related transmembrane tyrosine kinases: ErbB1, ErbB2, ErbB3, and ErbB4.

Treatment of newt testis in organ culture with recombinant human (rh) SCF (rhSCF) stimulates the proliferation of spermatogonia, but not the initiation of meiosis (Abe et al., 2002). Our unpublished study indicated that the mRNA and protein of both SCF and c-kit are expressed in spermatogonial stage. In newt testis, we also identified two different isoforms of NRG1, Immunoglobulin (Ig)-like domain-containing type and cysteine-rich domain (CRD)-containing type, and showed Ig-type NRG1 mRNA expression was observed only in somatic cells, while CRD-type NRG mRNA was observed both in spermatogonia and somatic cells (Oral et al., in press). In testicular organ culture FSH elevates Ig-type NRG1 mRNA expression, and EGF domain of NRG1 stimulates proliferation of spermatogonia, but not the initiation of meiosis (Oral et al., in press). On the other hand, rhIGF-I promotes the proliferation of spermatogonia and their differentiation into primary spermatocytes in organ culture of newt testes (Nakayama et al., 1999). IGF-I mRNA expression is elevated in somatic cells by treatment with FSH as well as with IGF-I in testicular organ culture (Yamamoto et al., 2001).

Since we discovered a size-selective barrier which allows small molecules (~500 Da) to get into cysts through Sertoli cells' barrier, but not larger ones (>1.9 kDa) in newt testis (Jin et al., 2008), those growth factors found in newt testis (SCF, IGF-I, and NRG1), with high molecular weight, may not act directly on spermatogonia which are enclosed by Sertoli cells. Thus the growth factors may work on spermatogonia indirectly via Sertoli cells or pericystic cells to stimulate spermatogonial proliferation and/or differentiation. But it has not yet been determined through which somatic cell type those growth factors work on spermatogonia. Though we have previously implicated the pivotal role of Sertoli cells in the FSH-dependent hormonal regulation of newt spermatogenesis (Nakayama et al., 2000; Yazawa et al., 2002), our knowledge about the function of pericystic cells is still limited due to the lack of the appropriate method of the fractionation of Sertoli cells and pericystic cells.

In order to determine which cell type is the target of those growth factors, Sertoli cells or pericystic cells, and the role of pericystic cells in spermatogonial proliferation, we looked for agents to kill pericystic cells only; we found that pericystic cells can be killed by trypan blue (TB) treatment. Hence, in the current study, we examined the effect of TB on causing death of pericystic cells in detail and studied the effects of rhSCF, rhIGF-I, and newt NRG1 (nNRG1) on spermatogonial proliferation and their differentiation into primary spermatocytes in organ culture of testicular fragments deprived of pericystic cells by TB.

2. Materials and methods

2.1. Animals

Adult male newts, *C. pyrrhogaster*, were purchased from a dealer (Hamamatsu Seibutsu Kyozai Ltd., Hamamatsu, Japan) and kept at 8 °C. Prior to be used for all the experiments, newts were transferred to 22 °C and fed frozen Tubifex for about 1 week. Experiments were carried out under the control of the Guideline to Animal Experiment in Kumamoto University.

2.2. Treatment with trypan blue (TB) and organ culture of testicular fragments

About 20 newts were used for one experiment; 10 newts for treatment with TB and the other 10 for control. The immature part of testis containing late spermatogonial stage was cut into 2.0–2.5 mm diameter fragments. TB (Nacalai Tesque, Kyoto, Japan) was added at 0.1% to the fragments in Leibovitz's L-15 culture medium (Gibco) supplemented with 10 mM Hepes, pH 7.4, and incubated for 0, 5 or 9 h.

Fragments treated with or without TB were washed twice in L-15 and placed on floaters of nucleopore filters (Whatman, Nucleopore Track-Etch Membrane) (4 fragments/filter) in a 35-mm plastic dish (#1008; Falcon, Lincoln Park, NJ) containing 2 ml medium including 0.1% BSA, and cultured at 18 °C for 1 and 2 weeks in the absence or presence of either porcine FSH (National Hormone & Peptide Program, Harbor-UCLA Medical Center, CA) at 200 ng/ml, rhSCF (Strathmann Biotech, Hamburg, Germany) at 100 ng/ml, rhIGF-I (Genzyme, Cambridge, MA) at 100 ng/ml, or recombinant protein of β-type EGF-like domain of newt NRG1 (nNRG1-EGF) at 2.5 μg/ml.

Expression of the recombinant nNRG1-EGF as a poly(histidine) fusion protein in bacterial cells was performed as described in Oral et al. (in press). The cDNA encoding amino acid residues 103–161 (EGF domain of nIg-NRG1) was amplified by PCR at 45 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s using the isolated cDNA inserted in pT7Blue vector as a template with a sense and an antisense primer 5′-CATATGACAGCTGGGCCAGGTCAC-3′ and 5′-ATGGCCAGCTTCTACAAATAAGGATCC-3′ designed to provide the PCR product with a NdeI restriction site at the 5′-end and a BamHI