IGF-I, IGF-II and insulin promote differentiation of spermatogonia to primary spermatocytes in organ culture of newt testes

YUKI NAKAYAMA[1], TAKASHI YAMAMOTO[2] and SHIN-ICHI ABÉ[1]*

[1]*Department of Materials and Life Science, Graduate School of Science and Technology* and [2]*Department of Biological Science, Faculty of Science, Kumamoto University, Kumamoto, Japan*

ABSTRACT   Recombinant human insulin-like growth factors (rhIGF-I and rhIGF-II) and human insulin promoted the differentiation of spermatogonia into primary spermatocytes in newt testes fragments cultured in a chemically defined medium. The biological potency for promoting differentiation was dose-dependent for all the ligands with the highest potency displayed by IGF-I, followed by IGF-II, and the least by insulin. The difference in potency was larger between IGF-II and insulin than that between IGF-I and IGF-II. This order of biological potency was in good accordance with the order of affinity in binding specificity of [^{125}I]IGF-I to the testicular membrane fractions: IGF-II and insulin competed the binding of [^{125}I]IGF-I only at concentrations 20-fold and 100-fold higher, respectively, than IGF-I. Specific binding was observed in both somatic cells (mostly Sertoli cells) and germ cells (spermatogonia and primary spermatocytes), though the binding to somatic cells was about 2.7 times higher than that to germ cells. These results indicate that (1) specific binding sites for IGF-I are present in the newt testes, (2) IGF-II and insulin also bind to these receptors but to a lesser degree, and (3) IGF-II and insulin as well as IGF-I promote spermatogonial differentiation into primary spermatocytes by binding to the IGF-I receptor.

KEY WORDS: *IGFs, insulin, meiosis initiation, newt testes, organ culture*

Introduction

Spermatogenesis commences with the proliferation of spermatogonia which enter meiosis and differentiate into primary spermatocytes. The number of mitoses that spermatogonia undergo before entering meiosis is species-specific (Roosen Runge, 1977), but the exact mechanisms controlling this proliferation and entrance into meiosis requires elucidation. It is known that germ cell proliferation and differentiation are controlled mainly by Sertoli cells which remain closely attached to germ cells throughout spermatogenesis (Parvinen *et al.,* 1986; Griswold *et al.,* 1988; Skinner, 1991; Jégou, 1993). It is believed that Sertoli cells are stimulated by follicle-stimulating hormone (FSH) and androgens to produce paracrine or autocrine factors which in turn induce the proliferation and differentiation of germ cells (Bellvé and Zheng, 1989; Skinner, 1991; Jégou, 1993).

The subject of our current investigation focuses on insulin-like growth factor I (IGF-I), a factor thought to play autocrine and paracrine roles during spermatogenesis. On one hand, IGF-I is produced by testicular cells (Ritzén, 1983) and was purified from conditioned medium from rat (Smith *et al.,* 1987) and porcine (Chatelain *et al.,* 1987) Sertoli cells. On the other hand, IGF-I can be accumulated from the medium by cultured peritubular rat myoid (Skinner and Fritz, 1986; Cailleau *et al.,* 1990), Sertoli (Tres *et al.,* 1986; Smith *et al.,* 1987; Cailleau *et al.,* 1990) and Leydig cells (Cailleau *et al.,* 1990). Consistent with the uptake of IGF-I by testicular cells are reports demonstrating the presence of specific IGF-I receptors on Leydig (Handelsman *et al.,* 1985; Lin *et al.,* 1986a,b; Kasson and Hsueh, 1987; Saez *et al.,* 1988) and Sertoli cells (Borland *et al.,* 1984; Oonk and Grootegoed, 1988): rat Sertoli cells and pachytene spermatocytes display binding sites for exogenous IGF-I, but spermatogonia fail to do so (Tres *et al.,* 1986). In agreement with the binding studies, IGF-I was shown to have several biological functions, such as promoting DNA synthesis (Borland *et al.,* 1984; Jaillard *et al.,* 1987) and glucose transport (Mita *et al.,* 1985) in cultured rat Sertoli cells, and stimulating the

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Address for reprints: Department of Materials and Life Science, Graduate School of Science and Technology, Kumamoto University, Kurokami 2-39-1, Kumamoto 860-8555, Japan. FAX: 81-96-342-3437. e-mail:abeshin@gpo.kumamoto-u.ac.jp
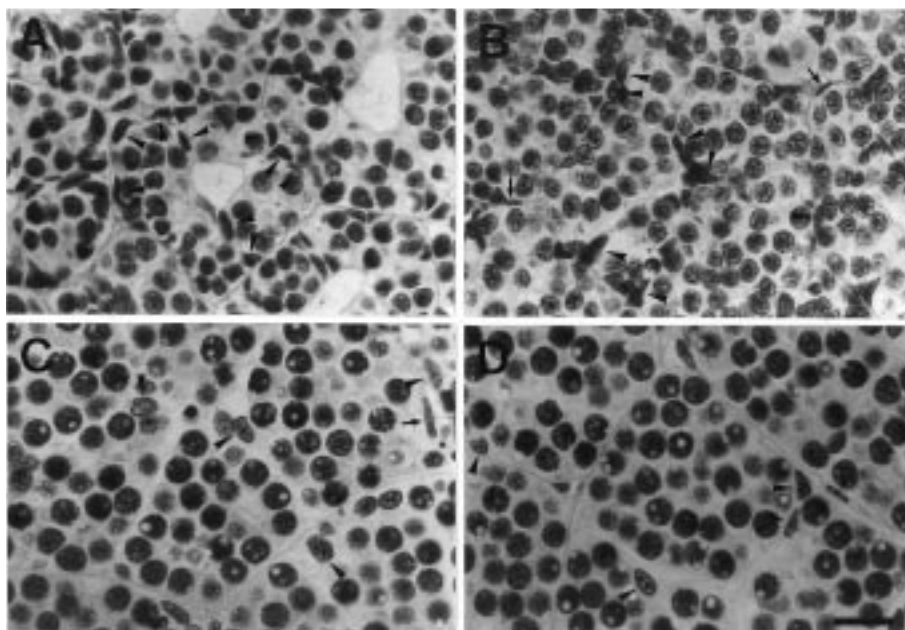
Fig. 1. Photomicrographs of histological sections from testis fragments on 0 day (A) and after 2 weeks of culture (B-D). (A) *Most of the cysts contained secondary spermatogonia.* **(B)** *In control medium, most of the germ cells remained as secondary spermatogonia.* **(C)** *In medium containing 100 ng/ml IGF-I.* **(D)** *In medium containing 100 ng/ml IGF-II. In* **(C)** *and* **(D)**, *most of the germ cells are primary spermatocytes in pachytene stage. Arrows and arrowheads show pericystic cells and Sertoli cells, respectively. Bar, 50 μm.*

investigator to analyze individual zones, each containing germ cells in the same stage. Using this advantageous organization in urodeles, we previously showed in cultures of newt testes fragments that mammalian FSH stimulates spermatogonial proliferation and their differentiation into primary spermatocytes (Ji *et al.,* 1992; Abé and Ji, 1994). Furthermore, we demonstrated that this proliferation and differentiation is mediated by Sertoli cells (Maekawa *et al.,* 1995; Ito and Abé, 1999), suggesting that paracrine factors produced by Sertoli cells stimulate this proliferation and differentiation. Here we report that human recombinant IGF-I, IGF-II and insulin promote differentiation of newt spermatogonia into primary spermatocytes in cultures of testes fragments.

Results

Differentiation of secondary spermatogonia into primary spermatocytes in organ culture of testis fragments by IGF-I, IGF-II an insulin.

Testes fragments containing germ cells in the spermatogonial stage (Fig. 1A) were cultured in the absence or presence of IGF-I, IGF-II or insulin. After 2 weeks most of the germ cells remained as spermatogonia in control medium (Fig. 1B). On the other hand, in the presence of IGF-I (100 ng/ml) spermatogonia differentiated into primary spermatocytes in the pachytene stage (Fig. 1C) in more than 50% of the cysts (Fig. 2). IGF-II (100 ng/ml; Fig. 1D) as well as insulin (500 ng/ml) also promoted the differentiation of spermatogonia into primary spermatocytes. The effects of IGF-I, IGF-II and insulin were dose-dependent (Fig. 2). The extent of differentiation with 100 ng/ml IGF-I was almost comparable to that with FSH (5 μg/ml); the most advanced stage attained in both cases was the pachytene stage. The percentage of cysts that differentiated into primary spermatocytes was the highest in 100 ng/ml IGF-I, intermediate in 100 ng/ml IGF-II and the lowest in 500 ng/ml insulin. Even 100 ng/ml insulin promoted less differentiation than 10 ng/ml IGF-I, and the value attained with 500 ng/ml insulin was almost comparable to that with 10 ng/ml IGF-I. These results indicate that IGF-I is more potent than IGF-II in promoting the differentiation of spermatogonia into primary spermatocytes, whereas insulin is the least potent, i.e. approximately 9 (6.5–11.6) and 6 (4.6–7.5) fold less than IGF-I and IGF-II, respectively.

Binding of [^{125}I]IGF-I to testicular cells

The binding of [^{125}I]IGF-I to membrane fractions prepared from the testes in the spermatogonial stage was examined. Unlabeled competitors with increasing concentrations inhibited [^{125}I]IGF-I binding. RhIGF-I was most efficient in the range of 0.1-10 ng/ml while rhIGF-II was about 20 times less potent than IGF-I (Fig. 3A). Insulin also competed with radiolabeled IGF-I but at concentrations about 100-fold larger than that of IGF-I (Fig. 3A). These results indicate that the binding sites recognized all three ligands but had a higher affinity for IGF-I than for IGF-II or insulin, and that IGF-II

basal and hCG-supported cAMP and testosterone production in cultured Leydig cells (Bernier *et al.,* 1986; Lin *et al.,* 1986a,b; Chatelain *et al.,* 1987; Kasson and Hsueh, 1987; Smith *et al.,* 1987).

With respect to the action of IGF-I on germ cells, Söder *et al.* (1992) reported first in mammals that IGFs stimulate premitotic DNA synthesis and may maintain premeiotic DNA synthesis in cultured segments of rat seminiferous tubules. In fishes, rhIGF-I increases DNA synthesis in cultures of premeiotic dogfish spermatocysts (Dubois and Callard, 1993). Furthermore, IGF-I stimulated DNA synthesis in trout spermatogonia and primary spermatocytes in a dose-dependent manner, indicating that IGF-I acts directly as a stimulator of proliferation (Loir, 1994). In fact, rhIGF-I was shown to bind specifically to rainbow trout spermatogonia and primary spermatocytes (Loir and Le Gac, 1994), and IGF-I mRNA and receptors are present to a greater extent in Sertoli cell-enriched populations and in spermatogonia with primary spermatocytes (Le Gac *et al.,* 1996). Thus, the above studies implicate IGF-I as an important factor in governing the proliferation of male germ cells and support the view that Sertoli cells have both an autocrine and paracrine role as both targets and sources of growth factors.

The structure of mammalian testes is quite complex and consists of seminiferous tubules, each containing many generations of spermatogenic cells residing with Sertoli cells; this organization involves complex interactions among testicular cells in various stages (Callard, 1991). In contrast to mammalian testes, the structure of testes in fish and urodeles is quite simple, and consists of spermatocysts each comprising a clone of germ cells and a stage-synchronized clone of Sertoli cells (Callard, 1991). Furthermore, spermatogenesis progresses unidirectionally from the cephalic to the caudal region of fish and urodele testes, permitting the

and insulin exert their effects on spermatogonia by binding to the IGF-I receptor.

IGF-I binding was also determined for preparations of isolated germ (spermatogonia and primary spermatocytes) and somatic cells (mostly Sertoli cells). As shown in Figure 3B, specific binding was observed not only in somatic cells but also in germ cells, though the specific binding for somatic cells was about 2.7 times higher than that for germ cells. The purity of germ cells (Gm) was 98% (spermatogonia, 62%; primary spermatocytes, 36%; somatic cells, 2%), as was the purity of somatic cells (Sm) (Sertoli cells, 71%; pericystic cells, 27%; primary spermatocytes, 2%). These results indicate that both germ and somatic cells (mainly Sertoli cells) contained binding sites for IGF-I.

Discussion

Our current study demonstrates for the first time in vertebrates that human IGF-I, IGF-II and insulin promote the differentiation of spermatogonia into primary spermatocytes in cultures of newt testes fragments. The biological potency for promoting differentiation was the highest for IGF-I, followed by IGF-II, and the least for insulin. The difference of potency between IGF-II and insulin is larger than that between IGF-I and IGF-II. This order of biological potency accords with the order of affinity in binding specificity of [^{125}I]IGF-I to the testicular membrane fractions. The observed affinity order in competition binding assays (IGF-I > IGF-II >> insulin) is similar to that described for the IGF-I receptor in mammals (Sara and Hall, 1990; Francis *et al.,* 1993), *Xenopus* (Janicot *et al.,* 1991) and rainbow trout (Loir and Le Gac, 1994; Le Gac *et al.,* 1996).

With respect to receptors, IGF-IR, IGF-IIR and insulin receptor have been characterized in mammals; IGF-IR is structurally homologous to the insulin receptor but binds insulin with low affinity (Chernausek *et al.,* 1981; Massague and Czech, 1982; Ullrich *et al.,* 1986). The biological activity of IGFs is mediated largely through their interaction with the IGF-IR (Van Wyk and Casella, 1991; Soos *et al.,* 1992). In the case of *Xenopus laevis* oocytes, IGF-IR mediates the effects of insulin, IGF-I and IGF-II, even

though IGF-IIR is also present (Janicot *et al.,* 1991). Our present data on newt testes indicate that specific binding sites for IGF-I are present, and that IGF-II and insulin promote spermatogonial differentiation to primary spermatocytes through the IGF-I receptor. However, since the effect of IGF-II on spermatogonial differentiation is comparable to that of IGF-I, IGF-II may act through both the IGF-I and IGF-II receptors. This possibility can be evaluated by developing antibodies against the newt IGF-I and IGF-II receptors, and examining the effect of IGF-I, IGF-II and insulin on spermatogonial differentiation in the presence of their antibodies.

Previous studies of testes demonstrated the presence of IGF-IR on both somatic and germ cells, such as mammalian Leydig (Handelsman *et al.,* 1985; Lin *et al.,* 1986a,b; Kasson and Hsueh, 1987; Saez *et al.,* 1988) and Sertoli cells (Borland *et al.,* 1984; Oonk and Grootegoed, 1988), and trout Sertoli cells, spermatogonia and primary spermatocytes (Le Gac *et al.,* 1996). In addition, IGF-I binding was observed on rat pachytene spermatocytes (Tres *et al.,* 1986), and IGF-IR immunoreactivity was localized on human secondary spermatocytes and early spermatids (Vannelli *et al.,* 1988). Similarly, our current studies detected [^{125}I]IGF-I binding to both spermatogonia-rich and Sertoli-rich fractions. These results pose several interpretations regarding the mechanism of IGF-I action on spermatogonial differentiation. First, IGF-I directly stimulates spermatogonial proliferation and/or differentiation into primary spermatocytes. Indeed, Loir (1994) showed initially in vertebrates that IGF-I directly stimulates the proliferation of male germ cells, but the differentiation of spermatogonia into primary spermatocytes was not detected as the spermatogonia were cultured only for 3 days. Second, IGF-I stimulates the proliferation of Sertoli cells. Third, IGF-I activates Sertoli cells which in turn produce factors that stimulate spermatogonial proliferation and/or differentiation into primary spermatocytes. Finally, a fourth possibility is that IGF-I directly stimulates spermatogonial proliferation, whereas Sertoli cells, activated by IGF-I, secrete factor(s) required for the differentiation of spermatogonia into primary spermatocytes.

Recently we established reconstituted cultures composed of newt spermatogonia and somatic cells (mainly Sertoli cells) in which FSH stimulates germ cell proliferation and differentiation into primary spermatocytes (Ito and Abé, 1999). Comparative studies involving separate cultures of germ and somatic cells with reconstituted cultures might reveal the role of IGF-I in the proliferation and/or differentiation of spermatogonia, the cell type expressing IGF-I, and the hormone(s) regulating the expression of IGF-I.

Materials and Methods

Animals and reagents

Adult male newts (*Cynops pyrrhogaster*) were purchased from a dealer (Hamamatsu Seibutsu Kyozai Ltd., Hamamatsu, Japan), kept at 22°C under 12L: 12D illumination, and fed frozen *Tubifex*. All chemicals were obtained from Nacalai Tesque, Inc., Kyoto, Japan, unless otherwise stated.

Organ culture of testicular fragments

All operations were carried out in sterile conditions. Testis fragments were cultured as previously described (Ji *et al.,* 1992). In brief, the immature part of each testis that was in the spermatogonial stage was cut into 1-2 mm diameter fragments. Four fragments were placed on a nucleopore filter (pore size 0.2 μm; diameter, 25 mm; Corning, Acton, MA, USA) which was floated on culture medium in a 35 mm dish. Porcine follicle-stimulating hormone (FSH; Sigma, St. Louis, MO, USA), recombinant human insulin-like growth factor-I (rhIGF-I, Genzyme, Cambridge, MA, USA), insulin-like
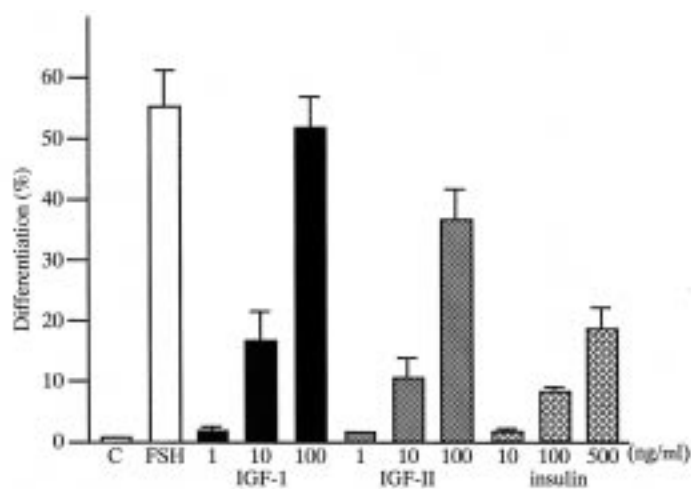

Fig. 2. Effects of FSH, IGF-I, IGF-II and insulin on the percentage of primary spermatocytes after culture for 2 weeks. *Each bar represents the mean ± SEM in triplicate experiments. The concentration of FSH was 5 μg/ml.*
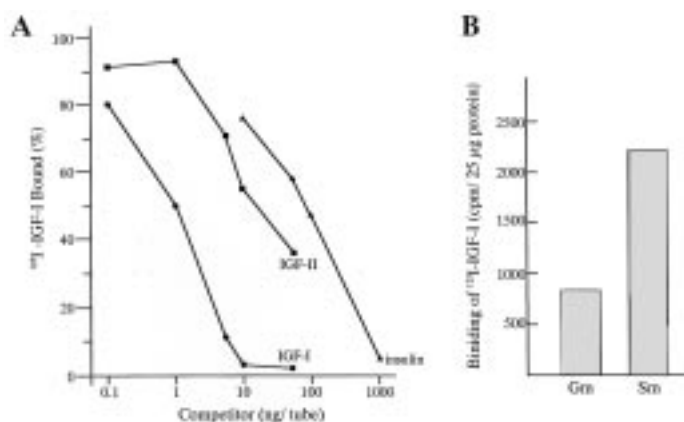
Fig. 3. IGF-I binding to testicular membrane (A) and fractionated populations of testicular cells (B). (A) *Competitive binding assay between [125I]IGF-I and unlabeled IGF-I (●-●), IGF-II (■-■) and insulin (▲-▲) for binding to testicular membrane fraction. (B) Binding of [125I]IGF-I to receptors of membrane preparations from germ cells (Gm; 62% spermatogonia, 36% primary spermatocytes and 2% somatic cells) and those from somatic cells {(Sm; 98% somatic cells (71% Sertoli cells and 27% pericystic cells) and 2% primary spermatocytes)}.*

growth factor-II (rhIGF-II, Genzyme) and insulin (Becton Dickinson, Franklin Lakes, NJ, USA) were added to the culture medium (Leibovitz's L-15) at various concentrations. The dishes were stored in a dark incubator (22°C) and the culture medium was changed once a week.

Histology and quantitative analysis of differentiation

Four fragments from each culture dish were fixed together with the filter in Bouin's solution after 2 weeks of culture. The samples were dehydrated in an ethanol series and embedded in a block of paraffin (paraplast plus tissue embedding medium, Oxford Labware, St. Louis, MO, USA). The blocks were sectioned serially (5 μm) and stained according to the Delafield hematoxylin-eosin method.

To estimate the extent of differentiation occurring after 2 weeks of culture, one section in the central area of each fragment was photographed. The cysts within the fragments were classified into two groups; cysts consisting of spermatogonia and those consisting of primary spermatocytes. The percentage of each group was averaged for all fragments (4 fragments/dish x 2 dishes) examined, and expressed as the extent of differentiation. Triplicate experiments were performed.

Separation of testicular cells

The spermatogenic cells were separated from the somatic cells (mostly Sertoli cells) according to Maekawa *et al.* (1995) with some modifications. Testis fragments rich in secondary spermatogonia and primary spermatocytes were collected and minced in L-15 medium followed by treatment with 0.1% collagenase (type N-2, Nitta Zeratin Co., Tokyo) at 22°C for 2 h. The cell suspension (5 ml) was layered onto 5% Nycodenz (Nycomed Pharma As, Oslo, Norway) in OR-2 (10 ml); this had been underlayered with 10% (10 ml) which in turn was underlayered with 15% Nycodenz (5 ml). The suspension was centrifuged at 1,500*g* for 10 min at room temperature. Spermatogenic cells were recovered from the boundary between 10% and 15%, and somatic cells from the boundary between L-15 and 5%.

IGF-I binding study

Preparation of cellular membranes was performed according to Oonk and Grootegoed (1988). All steps in the preparation were carried out at 0-4°C. Newt testes containing both spermatogonia and primary spermatocytes were homogenized in 5 volumes of ice-cold 0.3M sucrose containing 25 mM Tris-HCl (pH 7.6) with 5 up-and-down strokes of a mechanically driven Teflon/glass homogenizer. The homogenate was centrifuged at 900*g* for 10

min to eliminate nuclei and tissue fragments. The resulting supernatant was centrifuged at 100,000*g* for 60 min, and the pellet was resuspended in 25 mM Tris-HCl (pH 7.6) buffer containing 0.12M NaCl, 2.5 mM KCl and 6 mM MgSO₄, to a protein concentration of 4-10 mg/ml.

Two hundred micrograms of the membrane preparation (200 μl) were incubated for 16 h at 4°C in the presence of 35,000 cpm of [125I] human IGF-I (NEN, Boston, MA, USA) and various concentrations of unlabeled rhIGF-I (Genzyme), rhIGF-II (Genzyme) or human insulin (Becton Dickinson). The reaction was stopped by the addition of 1 ml of 25 mM Tris-HCl (pH 7.6) buffer containing 0.12M NaCl, 2.5 mM KCl, 6 mM MgSO₄ and 0.5% BSA; the tubes were centrifuged at 15,000*g* for 20 min. The supernatant was aspirated and the pellet was washed once with ice-cold buffer. The radioactivity of the pellet was counted using an automatic gamma counter (Wallac, Turk, Finland). Nonspecific binding was determined by the addition of a 2000-fold excess of unlabeled human IGF-I to the reaction mixture.

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