Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 6
Sofia • 2001

Mullerian Inhibitory Substance Down-Regulates Seminiferous Growth Factor-Stimulated Rat Prospermatogonial Cell Proliferation In Vitro

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The effect of seminiferous growth factor (SGF) and transforming growth factor β (TGFб) family, namely TGFб1, activin, inhibin and Mullerian inhibiting substance (MIS) on rat prospermatogonial stem cell proliferation was studied. Testes from 3-day-old rats were cultured in vitro in DMEM, supplemented with SGF or other potential growth promoters. MIS (holo MIS, C- and N-terminal domains) was tested additionally in presence of maximally stimulating dose of SGF. DNA synthesis was assayed by the incorporation and immunocytochemical detection of BrdU. SGF in a dose of 60pM increased the percentage of labelled prospermatogonia up to 55, activin in a dose of 3nM increased the same cells up to 40%, while MIS, TGFβ1 and inhibin had no effect. The combination of SGF and holo MIS and C-terminal domain down-regulated SGF-stimulated germ cell proliferation while N-terminal domain was noneffective.

Key words: Mullerian Inhibitory Substance, Seminiferous Growth Factor, Spermatogenesis

Introduction

Mullerian inhibitory substance (MIS) also known as antimullerian hormone (AMH) is a homodimeric glycoprotein, member of the transforming growth factor type β (TGFβ) family. Mature MIS undergoes glycosylation and dimerization and is secreted as a 140 kDa dimer (holo MIS) of two identical disulfide-linked subunits. After plasmine cleavage holo MIS has been separated to 110 kDa N- and 25 kDa C-terminal dimers [9]. In organ culture assay 25 kDa C-terminal domain has been shown consistent activity while 110 kDa N-terminus is inactive [7]. MIS is secreted by Sertoli cells in high amounts from the beginning of testicular differentiation until puberty when its production declines to undetectable levels. It is generally accepted that the primary function of MIS is to regress the presumptive female reproductive tract during male fetal development [1]. In addition to well-defined role in Mullerian duct regression, MIS has been considered to participate in germ cell and lung maturation, in gonadal morphogenesis and testicular descent [7].

Another members of TGFβ family, namely TGFβ1, inhibin and activin are significant growth factors in the local control of spermatogenesis. The action of these factors includes the modulation of Leydig cell function and spermatogonial proliferation [13], but their effects on the prospermatogonial stem cells are unknown.

Earlier we have been shown that Sertoli cell secreted proteins, SGF and LIF [6, 11, 14] stimulate in a dose-dependent manner quiescent rat prospermatogonial stem cell to proliferate. In the present study using the same model system we have tested the possibility of MIS (holo MIS, C- and N-terminal domains), TGFβ1, inhibin and activin to participate in regulation of quiescent germ cell proliferation in vitro.

Material and Methods

Materials

Male Wistar rats were supplied by Camm Laboratories Animal Research (Wayne, NJ). Dulbecco's modified Eagle's medium (DMEM) and bovine serum albumin (BSA)(Gibco BRL, Grand Island, NY); Bacto-Agar (Difco Laboratories, Detroit, MI); OCT Compound (Miles Scientific, Naperville, Il); Procelloidin (Fluka Chemica-Biochemica, Ronconcoma, NY); Organ culture dishes (Falkon, Becton-Dickinson, Oxnard,CA); Cell proliferation kit (Amersham, Arlington Heights, IL). Purified recombinant activin and inhibin were provided by Dr. Jennifer P. Mather (Genentech, South San Francisco, CA), human recombinant transforming growth factor β (TGFβ) from R & D Systems (Minneapolis, MN) was provided by Dr Richard Assoian (Columbia University, NY). Holo MIS, C- and N-terminal domains were kindly provided by Dr Patricia Donahoe (Massachusetts General Hospital, Boston, MA). SGF was purified from testes of 1- to 2-week-old bovine calves as described previously [3].

Tissue culture

Tissue cultures were prepared as previously described [14]. Briefly, rat Day-3 testes were cut into four segments, placed on permeable celloidine membranes and overlayed with 2% low-melting-point agar in 0.9% NaCl (vol/vol). Each preparation was placed in an organ culture dish containing DMEM supplemented with 2% BSA and 5-bromo-2-deoxyuridine (BrdU) 1:500 (negative control).

In the first group of experiments, the medium was additionally supplemented with two doses SGF: maximal effect dose (60 pM) and half maximal effect dose (30 pM), TGFβ1 (100 pM), inhibin (3 nM), activin (3 nM) or holo MIS (100 pM).

In the second group of experiments the same maximal effect dose (60 pM) of SGF was used simultaneously with different doses of holo MIS, C- or N-terminal domains (20, 50, 100, 150, 200, 300, 500, 1000 and 2000 pM).

The explants were cultured 24 h at 37 °C with 5% CO_2. At the end of culture period the segments were immersed in OCT compound, snap-frosen in liquid nitrogen and cryosectioned at 5μm.

Immunocytochemistry

Sections mounted on slides were fixed in Carnoy's fixative and incubated in 0.03% H_2O_2 for 30 min to block endogeneous peroxidase activity. Detection of incorporated BrdU was achieved with a monoclonal antibody incubation to BrdU followed by anti-mouse IgG2a antibodies conjugated with horse radish peroxidase and stained with DAB/substrate/intensifier. The sections were counterstained with Harris's haematoxylin, dehydrated and mounted in Permount.

Statistical analysis

Labelled and unlabelled prospermatogonial nuclei were counted and compared. The counts were made of 100 cells in three different testicular segments, each done in triplicate. Statistical analysis was performed by Student's t-test. Photographs were taken on an Opton microscope at a magnification ×400.

Results

Prospermatogonial cells in 3-day-old rat testis are easily distinguished by their localization centrally in the seminiferous cords and by their big size (20-25 μm in diameter). In comparison Sertoli cells are smaller in size (7-8 μm in diameter) and are situ-

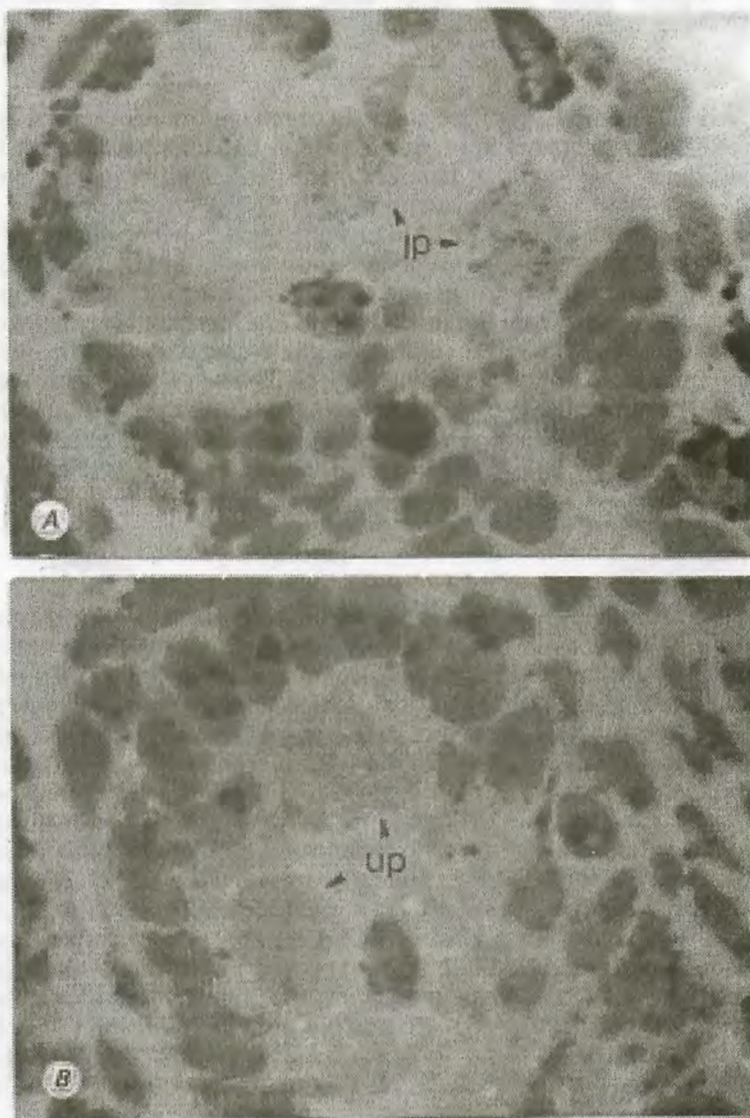

Fig.1. Light micrographs of stained frozen sections prepared from Day-3 rat testis after incubation in BrdU with different growth factors *A* — positive control with 60 pM SGF, nearly 56% of the germ cells are labelled; *B* — with 60 pM SGF and 200 pM C-terminal domain of MIS, about 12% of the prospermatogonia incorporated BrdU × 400; up unlabelled prospermatogonia; lp — labelled prospermatogonia

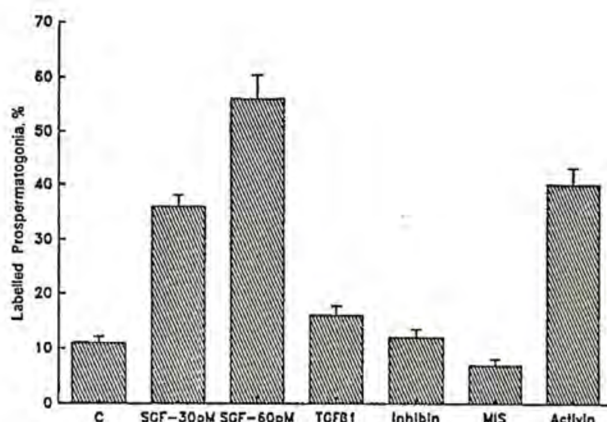

Fig. 2. Percentage (mean ± s.d.) of labelled prospermatogonia of 3-day-old rat testes after culture for 24h in the presence of 30 and 60pM SGF, 100pM TGFβ1, 3nM inhibin, 3nM activin or 100pM holo MIS. Data derived from three experiments each done in triplicate

ated in the periphery of the cords. In organ culture prospermatogonia from Day 3 rat testis incorporated BrdU at normal levels with about 10% of germ cells labelled . The addition of SGF at 60 pM increased the percentage of labelled prospermatogonia up to 55% (Fig.1*A* — positive control; Fig.2) The effect appear to be peptide-specific, since no other factors tested in physiological concentrations as TGFβ1, inhibin and MIS caused an increase in the incorporation of BrdU. The exception was an activin which increased the prospermatogonial labelling up to 40% (Fig.2).

The results from the second group of experiments are shown on Fig.3. It is obvious that holo MIS and C-terminal domain down-regulate the incorporation of BrdU into prospermatogonia, stimulated by SGF. An inhibition with holo MIS and C-terminal domain is occurred in a dose-dependent manner over range of 100 to 1000 pM and 20 to 200 pM respectively (Fig.1b). The application of N-terminal domain of MIS in the range of 50 to 1000 pM does not down-regulate SGF-stimulated germ cell proliferation.

Discussion

Rat primordial germ cells migrate from the yolk sac endoderm to the gonadal ridges during Days 9 to 11 of the fetal life. On the reaching the gonad, the germ cells continue to proliferate for another five to six days and then pass into a phase of quiescence that lasts until day 5 after birth [2]. On day 5 post partum (pp) germ cells in the testis coordinately reinitiate DNA synthesis, undergo a series of cell divisions and migrate to the periphery of the seminiferous cords. The latter cells, assigned as prospermatogonia act as progenitors to those destined to enter spermatogenesis in the pubertal and adult animals. The process of stem cell renewal and proliferation occurs in a "cyclic wave" within the seminiferous epithelium, in a pattern that is consistent with the existence of positive and negative regulators of spermatogonial proliferation. Activin was shown to stimulate spermatogonial proliferation in co-cultures of germ and Sertoli cells from 20-day-old rat testes [12]. Our results show that activin may stimulate proliferation of quiescent prospermatogonia — an earlier stage in the germ cell development. These data support the finding about the expression of activin II B receptors on type A spermatogonia in prepubertal testes [10]. Thus activin together with FGF2, LIF, SLF, and SGF may positively regulate spermatogonial proliferation in mammalian testis. Holo MIS, TGFβ1 and inhibin, tested separately do not stimulate quiescent germ cell proliferation.

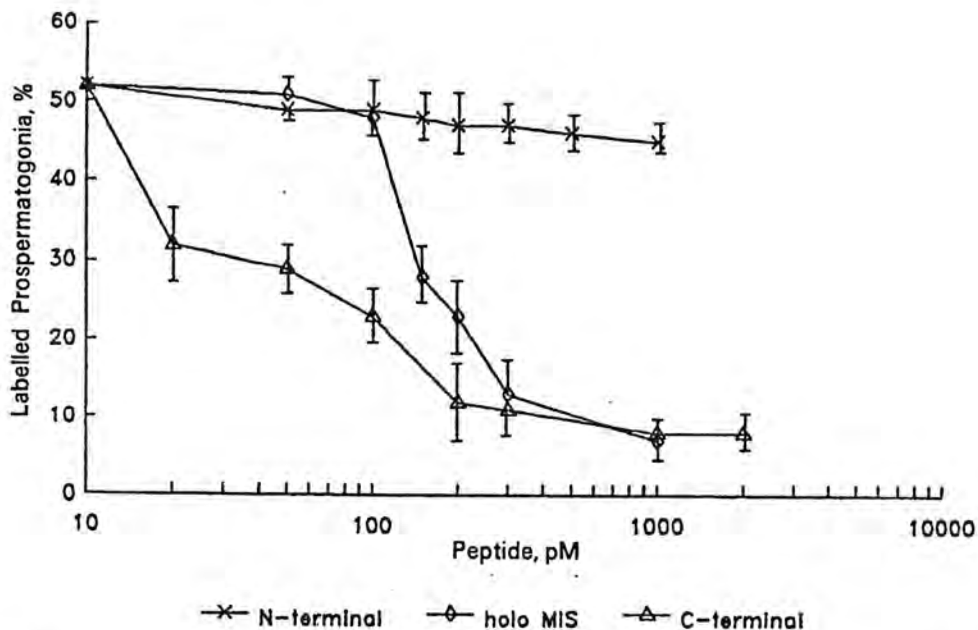

Fig. 3. Percentage (mean ± s. d.) of labelled prospermatogonia of 3-day-old rat testes after culture for 24h in the presence of 60pM SGF and different concentrations of holo MIS, N- and C-terminal domains. Data derived from four experiments each done in triplicate

To elucidate the interactions between SGF and MIS (holo MIS, N- and C-terminal domains), the combination between maximal stimulation dose of SGF and different doses of MIS was studied. The present results show that holo MIS and C-terminal domain down-regulate the incorporation of BrdU into prospermatogonia, stimulated by SGF. This effect was not seen at the same doses of N-terminal domain. These data suggest that interacting with SGF, holo MIS and C-terminal domain suppress indirectly germ cell proliferation. On the other hand, the above results confirm the hypothesis that C-terminal domain of MIS is the active one and may function in organ culture assay [4].

The suppressing effect of MIS on the germ cell proliferation can be explained with the peptide's synthesis and secretion by Sertoli cells in vivo in developing rat testis, which decreases markedly from day 3 to day 5 pp [15]. During puberty MIS expression is negatively regulated by androgens and declines dramatically in seminiferous tubules with meiotic development. Obviously it is an opposite relationship between rapid MIS decrease on day 5 pp and the beginning of prospermatogonial proliferation. On the other hand, the decrease in MIS expression during puberty, which is negatively regulated by androgens, coincides with postnatal differentiation of Leydig cells and active production of testosterone. In addition FGF-2 is known to stimulate Leydig cell steroidogenesis mediated by proteoglicans[8].

Our results are in agreement with the suggestion that MIS could be responsible for germ cell arrest at the prospermatogonial stage [5].

In conclusion the present study show that after birth in males MIS participates in the regulation of germ cell proliferation. Interacting with SGF, another Sertoli cell secreted growth factor, MIS and especially its C-terminal domain down-regulates the

SGF-stimulated prospermatogonial cell proliferation. Thus MIS together with SGF, activin, LIF, FGF-2, and SLF takes an important part in the regulation of germ cell proliferation in mammals.

Acknowledgement. This work reported in this paper was performed in Prof. Anthony R. Bellve's lab — Dept. of Anatomy and Cell Biology, Columbia University, New York and in the Institute of Experimental Morphology and Anthropology, Bulgarian Academy of Sciences, Sofia, Bulgaria.

This study was supported under grant K-612/96 by National Science Fund, Bulgaria.

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