Hepatocyte growth factor modulates *in vitro* survival and proliferation of germ cells during postnatal testis development

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Abstract

The hepatocyte growth factor (HGF) is a pleiotropic cytokine that influences mitogenesis, motility and differentiation of many different cell types by its tyrosine kinase receptor c-Met. We previously demonstrated that the c-Met/HGF system is present and functionally active during postnatal testis development. We found also that spermatozoa express c-Met and that HGF has a positive effect on the maintenance of sperm motility. In the present paper, we extend our study on the germ cells at different stages of differentiation during the postnatal development of the testis. We demonstrate that c-met is present in rat spermatogonia, pachytene spermatocytes and round spermatids and that HGF significantly increases spermatogonial proliferation in 8- to 10-day-old pre-pubertal rats. At this age HGF does not affect Sertoli cells and peritubular myoid cells proliferation. In addition,

we studied the effect of the factor on germ cell apoptosis and we show that HGF prevents the germ cell apoptotic process. We also studied the effect of HGF on 18- to 20-day-old and 28- to 30-day-old rat testes. At these ages also the factor significantly increases germ cell duplication and decreases the number of apoptotic cells. However, the effect on programmed cell death is higher in the 8- to 10-day-old rats and declines in the older animals.

In conclusion, we report that rat germ cells (spermatogonia, pachytene spermatocytes and round spermatids) express c-met and that HGF modulates germ cell proliferating activity and apoptosis *in vitro*. These data indicate that the c-Met/HGF system is involved in male germ cell homeostasis and, consequently, has a role in male fertility.

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Introduction

During the first wave of spermatogenesis, germ cell apoptosis appears to be necessary for normal germ cell maturation. When apoptosis is eliminated, an abnormal number of germ cells accumulates in the seminiferous tubules and infertility occurs. Similar results, i.e. germ cell apoptosis and abnormal spermatogenesis, have been obtained by altering the expression of molecules regulating the apoptotic process (Furuchi *et al.* 1996, Rodriguez *et al.* 1997, Russell *et al.* 2002). Recently, it has been demonstrated that in the rat testis apoptosis peaks at 18 days of postnatal age, mediated by activation of caspase 3 and involves pachytene spermatocytes and type A spermatogonia in a stage-dependent manner (Jahnukainen *et al.* 2004).

In the adult rat testis, germ cell maturation is dependent on the hormonal levels. It has long been known that testosterone and follicle-stimulating hormone (FSH) regulate germ cell apoptosis, respectively inducing and preventing apoptosis of the germ cells (Madhwa Raj & Dym 1976, Russell *et al.* 1987, Henriksen *et al.* 1995, 1996).

Recently, it has been reported that leukemia inhibitory factor (LIF) and stem cell factor (SCF) promote primordial germ cell survival preventing programmed cell death (Matsui *et al.* 1991, Pesce *et al.* 1993, Hakovirta *et al.* 1999, Yan *et al.* 2000). Endocrine and paracrine/autocrine factors also regulate germ cell proliferation: FSH stimulates spermatogonial proliferation both *in vivo* and *in vitro* (van Alphen *et al.* 1988, Arslan *et al.* 1993, Boitani *et al.* 1993) and many other testicular cytokines and growth factors affect germ cell proliferation (Pollanen *et al.* 1989, Parvinen *et al.* 1991, Boitani *et al.* 1995, Huleihel & Lunenfeld 2004, Kubota *et al.* 2004, Puglisi *et al.* 2004).

Hepatocyte growth factor (HGF), originally identified as a mitogenic factor for hepatocytes (Nakamura *et al.* 1984, 1989), is a pleiotropic cytokine able to regulate the functional activities of many different cellular types (Zarnegar & Michalopoulos 1995, Matsumoto & Nakamura 1996). HGF is expressed during embryonic development (Sonnemberg *et al.* 1993) and we have previously demonstrated that HGF is synthesized by the embryonic mouse testis, induces testicular cell

proliferation and acts as a morphogenetic factor (Ricci *et al.* 1999, 2002).

The multiple actions of HGF are mediated by its receptor, c-met, a transmembrane glycoprotein with tyrosine kinase activity, encoded by the MET protooncogene (Weidner *et al.* 1993, Hartmann *et al.* 1994). As we previously demonstrated, the HGF receptor is expressed in the postnatal rat testis and is detectable in the interstitial tissue and in the peritubular myoid cells of the seminiferous tubules. In the Sertoli cells, c-met expression is detectable in cells isolated from pubertal animals and is involved in postnatal testis development and function (Catizone *et al.* 1999, 2001). We also reported that in Sertoli cells HGF expression is developmentally regulated since its expression is only detectable at puberty (Catizone *et al.* 2005).

In humans, c-met is expressed in germ cells at different stages of differentiation and in spermatozoa (Depuydt *et al.* 1996, Herness & Naz 1999). In rats, we have demonstrated that c-met is differently localized in spermatozoa isolated from the caput or the cauda of the epididymis and we have shown that HGF positively influences sperm motility (Catizone *et al.* 2002).

In the present paper, we report that mitotic and meiotic rat germ cells express c-met and we investigate the role of HGF on proliferation and apoptosis of germ cells during the first spermatogenic wave.

Materials and Methods

Animals

Wistar rats were housed at the University of Rome 'La Sapienza'. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for Care and Use of Laboratory Animals and were killed by CO₂ asphyxia before testes removal. Ten male 8- to 10-day-old rats of 18–22 g body weight (BW), five to ten male 18- to 20-day-old rats of 30–40 g BW and five male 28- to 30-day-old rats of 100–120 g BW were used for each experiment.

Cell preparation and culture

Germ cells suspension was prepared from 8- to 10-day-old and 28- to 30-day-old male Wistar rats, according to the technique previously described (Galdieri & Monaco 1983).

To purify the spermatogonia, the cell suspension obtained from 8- to 10-day-old rats was cultured for 2 h on lectin-coated dishes to allow somatic cell adhesion according to a previously published technique (Scarpino *et al.* 1998). Floating germ cells were then collected and utilized for immunocytochemistry experiments. Utilizing 28- to 30-day-old rats, purified populations of pachytene spermatocytes and round spermatids were obtained by

sedimentation of the total germ cells suspension in an albumin gradient, according to the method of Lam *et al.* (1970). The fractions containing pachytene spermatocytes and round spermatids were collected, washed in modified Eagle's medium (MEM) (Gibco) and utilized for c-met detection. The purity of the fractions was evaluated by light microscopy and was 80–85% for pachytene spermatocytes and 90–95% for round spermatids.

Immunocytochemistry

Germ cells prepared as indicated above were suspended in saline solution and approximately 15 000 cells were air dried on a slide, fixed in ethanol-acetone (1:1) according to the suggestions of the antibody manufacturer for 10 min at -20°C , treated with 5% BSA (Sigma-Aldrich Chemical Co) for 30 min at room temperature to minimize the non-specific binding and were then exposed to a polyclonal antibody against the carboxy terminus of c-met (sc-162, 1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 16 h at 4°C . At the end of the incubation period, the cells were washed extensively with PBS and incubated for 45 min at room temperature with a fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum (Sigma-Aldrich Chemical Co). The cells were rinsed again with PBS and mounted in buffered glycerol (pH 9). As a negative control, the primary antibody was omitted and substituted with rabbit immunoglobulin G or 10-fold excess by weight of the blocking peptide (sc-162P; Santa Cruz Biotechnology). Samples were analyzed using a Zeiss Axioplan fluorescence microscope.

Western blotting

Germ cells isolated from at least five rats were purified as described in the 'Cell preparation and culture' section and centrifuged for 20 min at $300 \times g$. The pellet was washed twice and suspended in a solution containing PBS, 1% Igepal, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 2 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 5 $\mu\text{g}/\text{ml}$ aprotinin, 500 $\mu\text{g}/\text{ml}$ leupeptin (Sigma-Aldrich Chemical Co) and sonicated on ice. The sonicated cells were centrifuged for 2 min at $15\,000 g$ at 4°C and the supernatant was stored at -20°C until use. Protein concentration was determined by the method of Bradford (1976). Usually, 100 μg protein were solubilized in boiling Laemmli buffer (Laemmli 1970) containing 5% β -mercaptoethanol and then separated on 7% SDS PAGE. The proteins were electrotransferred to a nitrocellulose membrane. Non-specific binding was blocked by incubation with 5% BSA in TBS buffer (20 mM Tris pH 7.6, 150 mM NaCl). After blocking, the membrane was incubated with rabbit anti-met polyclonal antibody against the carboxy terminus of c-met p140 of mouse origin (Santa Cruz sp-260, 1:1000) or with a mouse

monoclonal antibody anti-tubulin (Sigma-Aldrich T5168, 1:3000) in TBS, 5% BSA overnight at 4 °C. The membrane was washed three times with TBS for 20 min each at room temperature and then incubated with the AP-conjugated secondary antibody (Sigma-Aldrich Chemical Co) (1:2000) for 1 h at room temperature. After washing with TBS, immunocomplexes were detected by Western blot chemiluminescence reagent (CDP-star; NEN, Boston, MA, USA) following the manufacturer's instructions.

Organ culture

Fragments of approximately 1 mm³ were isolated from ten testes of 8- to 10-day-old rats, or from eight testes of 18- to 20- or 28- to 30-day-old rats and placed on steel grids previously coated with 2% agar. Grids were then placed in organ culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ, USA) with 0.8 ml medium necessary to wet the grid. The chemically defined medium was MEM (Gibco) supplemented with glutamine (2 mM), Hepes (15 mM), non-essential amino acids, penicillin (100 IU/ml) and streptomycin (100 µg/ml). HGF (150 U/ml; Sigma-Aldrich Co) was added to the culture medium when indicated. Samples were cultured for 24 h at 32 °C in a humidified atmosphere of 5% CO₂ in air. Bromodeoxyuridine (BrdU) was then added to the culture medium for 4 h before harvesting. The protocol specified by the manufacturer (Cell Proliferation Kit; Amersham Corp) was used. After culture, the samples were washed twice in PBS, fixed overnight in Bouin's fixative, dehydrated, embedded in paraffin, sectioned at a thickness of 8 µm and processed according to the manufacturer to detect BrdU-labeled cells. The sections were counterstained with hemalum, dehydrated, mounted with Histovitrex (Carlo Erba, Milano, Italy) and analyzed using a Zeiss Axioscope microscope. The number of BrdU-positive cells in at least 300 transverse tubules of three different experiments was counted.

The sections were also utilized for TUNEL assay. The TUNEL method utilized for apoptotic cell analysis was the Q-BIOgene Apoptosis Kit (ApopTag Peroxidase; MP Biochemicals, Italy). As positive controls, samples were treated with DNase I. Negative controls were carried out omitting terminal deoxynucleotidyl transferase enzyme in the reaction mixture. The sections were counterstained with hemalum, dehydrated, mounted with Histovitrex (Carlo Erba) and analyzed using a Zeiss Axioscope microscope. At the different ages, at least three experiments in triplicate were performed and the number of TUNEL-positive cells/250 transverse tubules was evaluated.

At all the ages studied the morphology of the samples appeared well preserved. Somatic and germ cells were identified by cell size, nuclear morphology and localization within the seminiferous tubules, according to the description by Chiarini-García *et al.* (2003).

Statistical analysis

Data were analyzed using the Sigma Plot 5.0 software package (Systat Software GmbH, Erkrath, Germany); Student's *t*-test was employed.

Results

c-met expression in germ cells

The presence of *c-met* protein was detected by immunolocalization on spermatogonia obtained in three separate isolations from 8- to 10-day-old rats (Fig. 1D) and on pachytene spermatocytes and round spermatids isolated from animals of 28- to 30 days of age (Fig. 1E, F). In all the cell types the protein was present and showed a spotted distribution. Phase contrast microscopy of the same cells is shown in Fig. 1 (A-C) and negative controls are presented in Fig. 1 (G-J). The controls were invariably negative using either isotype serum or the blocking peptide. The presence of *c-met* on three different preparations of spermatocytes and spermatids was also evaluated by Western blotting. Figure 2 shows that the anti-*met* antibody detects a positive single band of 145 kDa, coincident in weight with the mature *c-met* β chain, in the proteins extracted from both germ cell populations (Fig. 2A,B). The tubulin content of the cells was also evaluated. Therefore, the presence of *c-met* was confirmed by two different techniques. Liver proteins were used as a positive control and gave the same positive signal. An additional signal at a molecular weight close to the *c-met* single-chain precursor is also present in the liver (Fig. 2C).

HGF effects

At least three separate experiments were performed to investigate the role of HGF on germ cell duplication and apoptosis. Small explants of testes isolated from animals of different ages have been cultured for 24 h in medium alone or supplemented with HGF (150 U/ml). In Fig. 3 the results obtained utilizing prepubertal rats (8- to 10-days-old) are presented. The left panel of the figure (A) shows the morphological appearance of the control (C) and the HGF-treated samples (HGF) in which it is evident that the presence of HGF in the culture medium increases the number of proliferating cells. The number of BrdU-positive cells present in the tubules has been counted and in the HGF-treated samples spermatogonia proliferate to almost twice as many in respect to the germ cells present in the control samples (Table 1). To ascertain whether HGF also affected the somatic cells of the tubules, the number of BrdU-labeled Sertoli cells and peritubular myoid cells, identified by morphological criteria, has been counted (250 transverse tubules of both samples).

Figure 1 c-met distribution in germ cells isolated from rat testes. Phase contrast microscopy of spermatogonia (A), pachytene spermatocytes (B) and round spermatids (C) is presented. c-met indirect immunofluorescence on the same cells (D, E, F) is also shown. Negative controls (G, H, J, lower panels) and relative phase contrasts (G, H, J, upper panels) were obtained using the blocking peptide against the anti-met antibody.

The number of proliferating Sertoli and peritubular cells appears not to be modified by HGF treatment (Table 1).

Different sections of the same samples were used to detect the apoptotic cells present in the control and the HGF-treated samples. The morphology, shown in the right hand panels of Fig. 3, appears well preserved and only apoptotic germ cells were detected. The quantification of the effects obtained is shown in Fig. 4A. In the HGF-treated samples the number of BrdU-labeled cells (at least 300 transverse tubules/sample counted) was increased more than 100% whereas the number of TUNEL-positive cells (250 transverse tubules/sample counted) was strongly reduced (approximately 75%).

The morphology of fragments isolated from testes of 18- to 20-day-old rats (late prepubertal rats) is shown in Fig. 5. Cell proliferation (Fig. 5A) and cell apoptosis (Fig. 5B) are presented in the figure. At this age the number of

Figure 2 Western blot analysis of proteins extracted from pachytene spermatocytes (A) and round spermatids (B). Proteins were separated by SDS-PAGE under reducing conditions. The molecular weight of c-met β chain (145 kDa) is indicated. Rat liver proteins (C) were used as a positive control. The tubulin content of the same samples was also evaluated and the results of one representative experiment out of three performed are shown.

Figure 3 Photomicrographs of histological sections of testis fragments from 8- to 10-day-old rats (original magnification $\times 200$) cultured for 24 h in control medium (C) and HGF-supplemented medium (HGF). (A) Sections were immunostained with anti-BrdU. (B) Sections of the same samples in which apoptotic cells were stained by the TUNEL method. Sertoli cells (SC), peritubular cells (PC) and spermatogonia (SPG) are indicated in both panels. MC, mitotic cells.

proliferating and apoptotic cells is increased compared with the 8- to 10-day-old rats and is confined to the germ cells. In the HGF-treated samples (Fig. 4B), the number of BrdU-labeled germ cells was more than 100% increased whereas the number of apoptotic germ cells was decreased (approximately 35%, Fig. 4B).

Similar experiments were performed utilizing testes isolated from 28- to 30-day-old rats and the morphology of the samples (pubertal rats) is presented in Fig. 6A and B. Also at this age we found that the number of proliferating cells is higher with respect to the younger animals, and HGF is able, significantly, to stimulate germ cell

proliferation (Fig. 4C). Germ cell apoptosis is slightly increased at this age compared with 18- to 20-day-old testes and appears to affect both spermatogonia and meiotic germ cells. Also in these pubertal rats, germ cell death is significantly prevented by HGF (approximately 60%) (Fig. 4C).

Discussion

In the present paper, we report that rat germ cells at different stages of differentiation express the HGF receptor. We previously reported that the HGF receptor is

Table 1 Number of BrdU-labelled Sertoli cells, spermatogonia and peritubular myoid cells present in 250 transverse tubules of control and HGF-treated samples

	Number of tubules	Sertoli Cells	Spermatogonia	Peritubular cells
Treatment				
Control	250	357	362	45
HGF	250	363	730	49

Figure 4 Number of BrdU- and TUNEL-positive cells present in testes from 8- to 10-day-old (A), 18- to 20-day-old (B) and 28- to 30-day-old (C) rats. On the left hand side of the three panels the mean of BrdU-labeled cells/tubule is shown. In all the panels, the mean $\pm$ S.E. of positive cells in 300 tubules of each of the three experiments performed is reported. In A and B, $*P < 0.001$ compared with HGF values; in C, $*P < 0.05$ compared with HGF values. On the right hand side of the panels the mean of apoptotic cells/tubule is reported. In all the panels the mean $\pm$ S.E. of positive cells in 250 tubules of each of the three experiments performed is reported. $*P < 0.001$ compared with HGF values.

Figure 5 Photomicrographs of histological sections of testis fragments from 18- to 20-day-old rats (original magnification $200\times$) cultured for 24 h in control medium (C) and HGF-supplemented medium (HGF). (A) Sections were immunostained with anti-BrdU. (B) Sections of the same samples in which apoptotic cells are stained by the TUNEL method. SPG, spermatogonia.

expressed in rat spermatozoa (Catizone *et al.* 2002) and we now extend our investigation demonstrating, by different techniques, that c-met is present in rat spermatogonia, pachytene spermatocytes and round spermatids. It was shown in humans that differentiating germ cells, as well as spermatozoa, express c-met (Depuydt *et al.* 1996, Herness & Naz 1999); however, the role of this factor was not investigated. We now report the presence of the receptor on rat germ cells and investigate the functional role of HGF in these cells. By means of culture of small testicular explants we have found that HGF *in vitro* significantly increases spermatogonial proliferation in both prepubertal and pubertal rats. In the 8- to 10-day-old animals Sertoli cells and peritubular myoid cells also proliferate. Therefore, we counted all the proliferating cells and our data demonstrate that HGF exclusively stimulates spermatogonial duplication, as the number of proliferating Sertoli cells and peritubular myoid cells are not significantly different in the control and in the HGF-treated cells. On the other hand, the number of BrdU-labeled spermatogonia is approximately double in the tubules cultured in the presence of HGF. It is well known that several hormones influence spermatogonial apoptosis and

duplication. Testosterone and FSH regulate germ cell apoptosis (Henriksen *et al.* 1995, 1996) probably acting via somatic cells considering the absence on the germ cells of their respective receptors (Heckert & Griswold 1991, de Kretser *et al.* 1998). FSH stimulates spermatogonial proliferation both *in vivo* and *in vitro* (van Alphen *et al.* 1988, Arslan *et al.* 1993, Boitani *et al.* 1993) and many other growth factors such as LIF, interleukins and SCF regulate germ cell duplication (for a review see Huleihel & Lunenfeld 2004). We have previously reported that HGF induces proliferation of the embryonic testicular cells (Ricci *et al.* 2002) and other authors have reported that HGF exerts mitogenic and anti-apoptotic action in endothelial cells (Nakagami *et al.* 2001). The results here presented confirm that HGF has to be included in the list of bioactive molecules acting on spermatogonial duplication in postnatal life, as previously reported by Goda *et al.* (2004). Moreover, we show that the effect of HGF on germ cell proliferation is exerted during the entire first wave of spermatogenesis.

We have also studied the effect of the factor on germ cell apoptosis and have shown that HGF prevents the germ cell apoptotic process. This effect is at its maximum

Figure 6 Photomicrographs of histological sections of testis fragments from 28- to 30-day-old rats cultured for 24 h in control medium (C) and HGF-supplemented medium (HGF). (A) Sections were immunostained with anti-BrdU (original magnification $\times 400$). (B) Sections of the same samples in which apoptotic cells were stained by the TUNEL method (original magnification $\times 200$). SPG, spermatogonia; PSC, pachytene spermatocytes.

(75% reduction of apoptosis) in the 8- to 10-day-old rats and declines in the older animals. The lowest protective effect (35% reduction of apoptosis) was found in late prepubertal rats and this finding is probably due to the fact that the highest level of apoptosis has been described at this age (Jahnukainen *et al.* 2004) or to the presence of a more complex network of pro-apoptotic and anti-apoptotic factors present in the older animals. For example, it is known that paracrine factors secreted by round spermatids promote the survival of spermatocytes in stages VII–VIII of the spermatogenetic process (Zhao *et al.* 1998). On the other hand, a physiological apoptotic wave during the first spermatogenetic maturation is necessary for normal spermatogenesis in the adult individuals and it has been hypothesized that spermatogonial apoptosis during the establishment of spermatogenesis is necessary for the maintenance of a proper ratio between germ cells and Sertoli cells (Rodríguez *et al.* 1997).

It has recently been reported that in the adult rat testis, after experimental induction of cryptorchidism, adenovirally transferred HGF gene expression decreases the number of apoptotic germ cells (Goda *et al.* 2004).

However, these data have been obtained in an experimentally complex way. The authors obtained transient over-expression of HGF in the testis and, a long time after orchidopexy, they reported a decrease in apoptosis in the adenoviral-injected rats. Our data, obtained by culturing testicular fragments isolated from rats of different ages, demonstrate that HGF modulates survival and proliferation of germ cells during the first spermatogenetic wave of postnatal rat testis development.

In conclusion, in the present paper we report that rat germ cells (spermatogonia, pachytene spermatocytes and round spermatids) express the HGF receptor, c-met. Moreover, we report that HGF modulates germ cell metabolic activities *in vitro*, positively regulating germ cell proliferation and decreasing the number of apoptotic germ cells.

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