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Functional Role of Hepatocyte Growth Factor Receptor During Sperm Maturation

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ABSTRACT: Mammalian spermatozoa acquire motility and fertilizing capacity during their transit through the epididymis. Hepatocyte growth factor (HGF) is a pleiotropic cytokine with potent motogenic capacities that has been identified in different organs, including the mammalian male genital tract. In mice, HGF is present in the testis and, in large amounts, in the distal part of the epididymis. In prepuberal rats, we have demonstrated that HGF is synthesized by the peritubular myoid cells and in men, HGF is present in significant quantities in seminal plasma. It has been suggested that in mice, HGF has a role in initiating sperm motility, whereas in men, no significant correlations between HGF concentration and sperm motility have been found. In the present paper we report that in rats, HGF receptor, c-met, is expressed in testicular and epididymal spermatozoa. Through immunocytochemistry, we have found that c-met is exclusively localized on the head in testicular sperm. A different localization of c-met has been found in sperm isolated from caput and cauda epididymidis. Cells isolated from epididymal caput show a c-met localization exclusively restricted to the head in most cells. In a minority of caput epididymis spermatozoa the receptor is localized both in the cell head and along the flagellum. Spermatozoa isolated from the epididymal cauda were quite homogeneous, showing the receptor localized along the entire cell surface. We also report that HGF is synthesized and secreted by the rat epididymis as indicated by the scatter effect of epididymal cell homogenate and culture medium on MDCK cells. To clarify whether HGF is involved in the acquisition of sperm motility in the epididymis, its maintenance, or both, spermatozoa isolated from caput epididymidis have been cultured in medium alone or supplemented with HGF. The results obtained indicated that HGF has a positive effect on the maintenance of sperm motility which, in the absence of HGF, significantly decreases during the first hour of culture, whereas it is maintained for at least 3 hours when HGF is present in the culture medium. We also report that HGF does not influence spermatozoa viability as indicated by the cytometrical analysis of propidium iodide-labeled sperm; an equal number of dead cells appeared in control and in HGF-treated preparations. In conclusion, our data strongly support the hypothesis that HGF positively influences sperm motility maintenance during sperm transit through the epididymis, indicating that c-met receptor and its ligand, HGF, have a role in male fertility.

Key words: C-met, spermatozoa, rat, testis, epididymis.

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It is well known that mammalian spermatozoa acquire motility and fertilizing capacity in the epididymis (Orgebin-Crist, 1967; Robaire and Hermo, 2002). The percentage of motile sperm increases during their transit through the epididymis, reaching maximum levels in the corpus or in the cauda of the organ, depending on mammalian species (Chevrier and Dacheux, 1992; Yeung et al, 1992). Maturation of spermatozoa depends on the epididymal fluid and involves a remodeling of their plasma membrane due to different factors such as the uptake of secreted epididymal glycoproteins, variation in phospholipid composition, and processing and reposition of both protein and lipid components of the plasma membrane (Jones, 1998). Moreover, during their transit through the epididymis, spermatozoa lose the cytoplasmic droplet formed in the testis as a consequence of the morphogenetic process (Bloom and Nicander, 1961). As recently demonstrated by Syntin and Robaire (2001), sperm motility changes in older sperm, as evidenced by a marked increase in abnormal cells, a decrease in the percentage of motile cells, and in the number of sperm that retain the cytoplasmic droplet.

Hepatocyte growth factor (HGF), also known as scatter factor, is a pleiotropic cytokine with potent motogenic capacities that has been identified in a variety of tissues and organs (Bhargava et al, 1991; Di Renzo et al, 1991; Naldini et al, 1991; Lail-Trecker et al, 1998). HGF acts on different cell types through a receptor, c-met, which is a transmembrane glycoprotein with tyrosine kinase activity, a product of the met proto-oncogene (Park et al, 1987). It is known that HGF and its receptor are expressed in the mammalian male genital tract. In the genital tracts of mice, HGF is expressed in a region-specific manner, with slight or no expression in testes and caput

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epididymis, and a strong expression in corpus and cauda epididymidis. The presence of large amounts of HGF in the distal part of the epididymis, together with the acquisition of motility by immotile spermatozoa cultured in the presence of HGF, suggests that this growth factor is involved with the induction of sperm motility (Naz et al, 1994). C-met expression was not detected in the testis of adult mice (Iyer et al, 1990), but more recently, its expression has been reported in the testes of prepuberal and adult mice and in cell lines derived from Sertoli, peritubular, and Leydig cells (van der Wee and Hofmann, 1999). In rats, as we demonstrated, HGF is present in the prepuberal testis, and it is exclusively expressed by the peritubular myoid cells of the seminiferous tubules, whereas its receptor is expressed in peritubular myoid cells and in the interstitial compartment (Catizone et al, 1999). In a study using rats of different postnatal ages, we recently demonstrated that c-met is expressed in myoid cells at all the ages, whereas in Sertoli cells, c-met expression appears to be developmentally regulated, and is detectable only in cells isolated from testes of animals between 25 and 35 days of age (Catizone et al, 2001). In men, c-met is expressed in germinal cells at different stages of differentiation and in spermatozoa (Depuydt et al, 1996), in which it is predominantly localized to the acrosomal region (Herness and Naz, 1999). In human seminal plasma, HGF is present in significant quantities (Depuydt et al, 1997; Wiltshire et al, 2000), and its levels are correlated with various andrological diseases (Depuydt et al, 1998). The role of HGF in relation to sperm motility is at the moment unclear because the motility of ejaculated human spermatozoa seems not to be influenced by HGF levels (Kitamura et al, 2000; Wiltshire et al, 2000).

In this paper we report our studies on c-met expression in testicular and epididymal spermatozoa from rats. We report that c-met is expressed in both testicular and epididymal spermatozoa. We found it interesting that c-met is differentially localized on cells isolated from the caput or the cauda of the epididymis. We also report that HGF is synthesized and secreted by epididymal rat cells and has a positive effect on epididymal sperm motility.

Materials and Methods

Materials

Dulbecco modified Eagle medium (DMEM), fetal calf serum (FCS), and sodium pyruvate were purchased from Gibco BRL Life Technologies (Gaithersburg, Md). Culture plates were purchased from Corning (Corning, NY). Nitrocellulose transfer membranes were purchased from Schleicher and Schuell (Dassel, Germany), PharMingen Cytofix/Cytoperm were from PharMingen, Beckton and Dickinson (San Diego, Calif), antibody against c-met was from Santa Cruz Biotechnology (Santa Cruz, Calif), and alkaline phosphatase (AP)–conjugated secondary antibody was from Zymed (San Francisco, Calif). Western blot chemiluminescence reagent was obtained from NEN Life Science Products (Boston, Mass). Bovine serum albumin (BSA), hepatocyte growth factor (HGF), fluorescein isothiocyanate (FITC)–conjugated antibody, rabbit immunoglobulin (Ig) G, propidium iodide (PI), and all other reagents were purchased from Sigma Chemical Company (St Louis, Mo).

Sperm Collection

Testes and epididymides from 60-day-old rats were removed, trimmed free of fat, and placed in a 35-mm Petri dish containing DMEM. The caput and cauda of each epididymis and decapsulated testes were then transferred to separate Petri dishes containing 3 mL of fresh medium supplemented with 1 mM sodium pyruvate. A scalpel blade was used to pierce several tubules of the tissues. Epididymal sperm were allowed to disperse into the medium for 5 minutes, whereas testicular fragments were gently shaken for the same amount of time. The tissues were then removed and epididymal sperm were diluted to approximately 10×10^6 sperm/mL and cultured for different times periods (1–5 hours). Testicular cells were washed twice with phosphate-buffered saline (PBS), treated for 2–3 minutes with a hypotonic solution (Galdieri et al, 1981), washed again with PBS, and fixed in methanol at −20°C for 10 minutes.

Organ Culture and Homogenate Preparation

Epididymides were sectioned according to the method described by Hinton et al (1979). Small portions of caput and cauda were placed on steel grids that were previously coated with 2% agar. Grids were then placed in organ culture dishes with 0.8 mL of medium (DMEM supplemented with 2 mM glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin). Tissues on grids were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air for 48 or 96 hours. At the end of the culture time, conditioned culture media were collected, centrifuged at $5000 \times g$, and their scatter activity was analyzed. Portions of the same epididymides (caput and cauda) were immediately homogenized in culture medium using a tissue homogenizer in ice, centrifuged at $20\,000 \times g$, the supernatant was collected, and the protein content was measured by the method described by Bradford (1976). The supernatants were used for scatter activity at the same protein concentrations (usually 0.2–0.4 μg/mL).

Western Blotting

Epididymal spermatozoa from at least 4 rats were collected from whole epididymides as described in the ''Sperm Collection'' section and centrifuged for 20 minutes at $300 \times g$. The pellet was washed twice, suspended in a solution containing PBS, 1% Igepal, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL aprotinin, and 500 μg/mL leupeptin, and incubated for 30 minutes at 4°C. The spermatozoa were then sonicated (250×10^6 sperm/mL) for 30 seconds and incubated again for 30 minutes on ice with PMSF (1 mg/mL). After incubation, the sonicated cells were centrifuged for 20 minutes at $15\,000 \times g$ at 4°C and the supernatants were stored at −20°C until use. Protein concentration was determined by the method described by Brad-

ford (1976). Usually, 150 μg of protein were solubilized in boiling Laemmli buffer (Laemmli, 1970) containing 5% β-mercaptoethanol and then separated on 7% SDS with polyacrylamide gel electrophoresis (PAGE). The proteins were electrotransferred to nitrocellulose membrane. Nonspecific 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 (1:1000; SP260; Santa Cruz Biotechnology) in TBS and 5% BSA for 1 hour at room temperature. The membrane was washed 3 times with TBS for 20 minutes each and then incubated with the AP-conjugated secondary antibody (1:2000) for 1 hour at room temperature. After washing with TBS, immunocomplexes were detected with Western blot chemiluminescence reagent following the manufacturer's instructions.

Indirect Immunofluorescence

Testicular and epididymal (caput and cauda) spermatozoa, isolated as previously described, were washed twice in PBS, placed on slides, and fixed in methanol according to the suggestions of the antibody manufacturer for 10 minutes at −20°C. Cells were then washed twice in PBS and treated with 5% BSA for 30 minutes at room temperature to minimize nonspecific binding. After washing in PBS, the cells were exposed to a polyclonal antibody against the carboxy terminus of c-met (1:50 dilution) for 16 hours at 4°C. At the end of the incubation, the cells were washed extensively with PBS and incubated for 45 minutes at room temperature with an FITC-conjugated secondary antibody. The cells were rinsed again with PBS and mounted in buffered glycerol (pH 9). As a negative control, the primary antibody was omitted. Samples were analyzed using a Zeiss axioskop 2 fluorescence microscope (Zeiss, Hallbergmoos, Germany).

Scatter Activity

The scatter activity of homogenates and conditioned media were measured on colonies of canine kidney epithelial cells (MDCK cells) as previously described (Bhargava et al, 1992). A suspension of MDCK cells in DMEM supplemented with 10% FCS was prepared, and 9000 cells were plated in 24-well plates containing 0.5 mL of culture medium. Plates were incubated at 37°C. Cell homogenates, both containing 0.2 μg/mL of proteins, were added to MDCK cells as well as culture media previously centrifuged at 2000 × *g* for 5 minutes As a standard for scatter activity, HGF (100 U/mL, equivalent to 30 ng/mL) was added to some of the cells.

Flow Cytometric Analysis

Flow cytometry of rat spermatozoa was performed using a Coulter EPICS XL flow cytometer (Beckman Coulter Company) equipped with the standard rectangular flow cell with a 250 μm square channel. To determine the correct side scatter and forward scatter of the cells, epididymal rat spermatozoa resuspended in medium were back-gated from the area in which sperm cells appeared. Spermatozoa stained with PI or FITC were back-gated from the area in which the fluorescently stained population appeared in the orange or green channels. A 488 nm filter was used to excite FITC and PI. Filters set to 525 nm and 620 nm were used for determining FITC and PI excitation, respectively. Data were analyzed with the WinMDI 2.8 Software (Verity Software House, Inc, Topsham, Maine). Briefly, the spermatozoa obtained from caput and cauda epididymis were washed twice in PBS containing 0.1% BSA at 4°C. After washing, the samples were fixed and permeabilized with PharMingen Cytofix/Cytoperm solution for 20 minutes at 4°C, and washed with Perm/Wash solution. After washing, the samples were incubated with rabbit anti-met polyclonal antibody (1:50 dilution) for 30 minutes at 4°C, washed twice in Permwash, and then incubated with FITC-conjugated goat anti-rabbit antibody (1:200) for 30 minutes at 4°C. After 2 washes in Permwash, 2×10^4 spermatozoa per sample were immediately subjected to flow cytometry. Spermatozoa incubated with rabbit IgG and secondary antibody were used to determine background fluorescence.

Motility Analysis

Aliquots of freshly prepared samples (5×10^6 sperm in 0.5 mL of DMEM) were plated in 24 multiwell plates supplemented with various quantities of HGF (75, 150, or 300 U/mL) and incubated at 37°C in an atmosphere of 5% CO_2 in a humidified incubator. The number of motile cells was evaluated with optical microscopy at various culture times (0, 1, 3, or 5 hours) using a Bürker chamber. In the 5 experiments performed in triplicate, usually 600–700 spermatozoa were observed.

Sperm Viability

Viability was evaluated by labeling cells cultured in the absence or in the presence of HGF (300 U/mL) with 1 μg/mL of PI for 10 minutes at room temperature and assessing their fluorescence labeling using flow cytometry. Each stained sample was also microscopically evaluated to determine the staining pattern of PI-stained spermatozoa using a Zeiss axioplan fluorescence microscope.

Statistical Analysis

Data were analyzed using the Sigma Plot 5.0 software package, and the Student's *t* test was employed.

Results

C-Met Expression in Epididymal Spermatozoa

Western blot analysis of proteins extracted from epididymal spermatozoa isolated from 60-day-old rats showed the presence of c-met protein in these cells. One molecular species with a molecular weight of 145 kd, coincident in weight with the c-met β chain, was demonstrated by electrophoresis under reducing conditions of the proteins extracted from the spermatozoa (Figure 1A). Liver proteins were used as a positive control and gave the expected positive signals (Figure 1B), whereas no signals were detected using the control rabbit IgG.

Spermatozoa were also isolated from caput and cauda epididymis, and Figure 2, A and C, respectively, show phase contrast microscopy of these cells. By immunolocalization, the presence of c-met protein was detected on

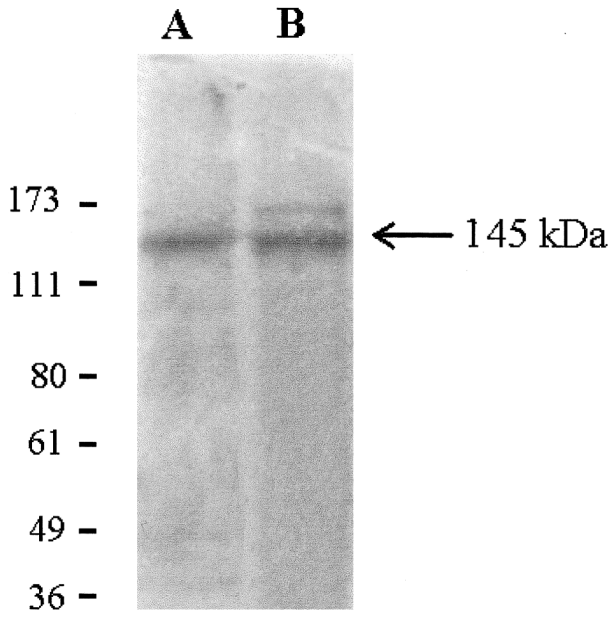


Figure 1. Western blot analysis of proteins extracted from epididymal rat spermatozoa **(A)**. Proteins were separated by SDS-PAGE under reducing conditions and probed with anti-met polyclonal antibody. The arrow indicates the mature c-met β chain (p145). Rat liver proteins **(B)** were used as a positive control.

both cell populations but with a different cellular localization. In fact, the spermatozoa isolated from caput epididymis consisted of 2 populations; one of approximately 90% of the total cells in which the protein was almost exclusively localized to the cell heads (Figure 2B, h), and a second in which the protein was localized both to the head and to the principal part of the flagellum (Figure 2B, h and f). In contrast, in spermatozoa isolated from cauda epididymis, only one population was detectable, and the positivity was localized along the entire surface of the cell (Figure 2D). C-met expression was also evaluated in spermatozoa isolated from testes (Figure 2E), and in these cells, the protein was exclusively localized to sperm heads (Figure 2F).

Flow Cytometric Analysis

Spermatozoa obtained from caput and cauda epididymidis were labeled with anti-met polyclonal antibody or with isotype rabbit IgG and subjected to flow cytometry. Figure 3.1 shows the FITC-fluorescence profile histogram of c-met expression of cells isolated from epididymal caput, which reveals 2 distinct peaks with different levels of mean fluorescence intensity (peak A and peak B). On the contrary, only one peak of fluorescence was identified in the histogram of spermatozoa isolated from epididymal cauda (Figure 3.2). The histogram of cells incubated with the isotype (I) is also shown.

Effect of HGF on Epididymal Spermatozoa

Motility of spermatozoa freshly isolated from the caput epididymis was evaluated. The cells obtained from the upper part (caput) of the organ were mostly immotile, with only approximately 40% of cells exhibiting motility. Cells were cultured in the absence or in the presence of HGF (300 U/mL), and their motility was evaluated at different culture times (1–5 hours). The results obtained indicate that HGF has a significant effect in maintaining sperm motility for at least 3 hours (Figure 4). Lower doses of HGF (75–150 U/mL) had no effect or a slight effect in maintaining sperm motility (Table).

To evaluate whether HGF affects cell viability, we used PI, which stains dead cells, and used flow cytometry to determine the percentages of PI-stained and PI-unstained sperm in control and treated cells (Figure 5). The percentages of live (peak A) and dead cells (peak B) were similar both in the control (dotted line) and in the cells treated with HGF for 3 hours. The results obtained with flow cytometric analysis were usually well-correlated with the results obtained with direct microscopic examination.

HGF Production

Small portions of caput and cauda epididymidis were cultured for 48–96 hours on steel grids placed in organ culture dishes. At the end of incubation time, the conditioned medium of both samples was administered to MDCK cells to evaluate its scatter activity. After 16–20 hours of incubation, MDCK cells cultured in conditioned medium showed a scattered appearance (Figure 6, D and E, respectively) in both cases, whereas control cells showed that morphology was unchanged (Figure 6A). Higher scatter effects were obtained when the supernatant obtained from the homogenate of caput and cauda epididymis, both containing 0.2 μg/mL of protein, was added to MDCK cells (Figure 6, B and C, respectively). The presence of HGF in the homogenates of caput and cauda epididymis was also detected by Western blotting (data not shown).

Discussion

In this paper we report that epididymal spermatozoa in rats express the HGF receptor, c-met. C-met expression was detected with Western blotting and confirmed through immunocytochemistry experiments. With immunocytochemistry, we found that spermatozoa isolated from the epididymal caput can be divided into 2 different populations according to their c-met expression: the receptor appears exclusively localized to the heads of a large portion of cells, and to the entire cell surface in a smaller part of the total cell population. On the contrary,

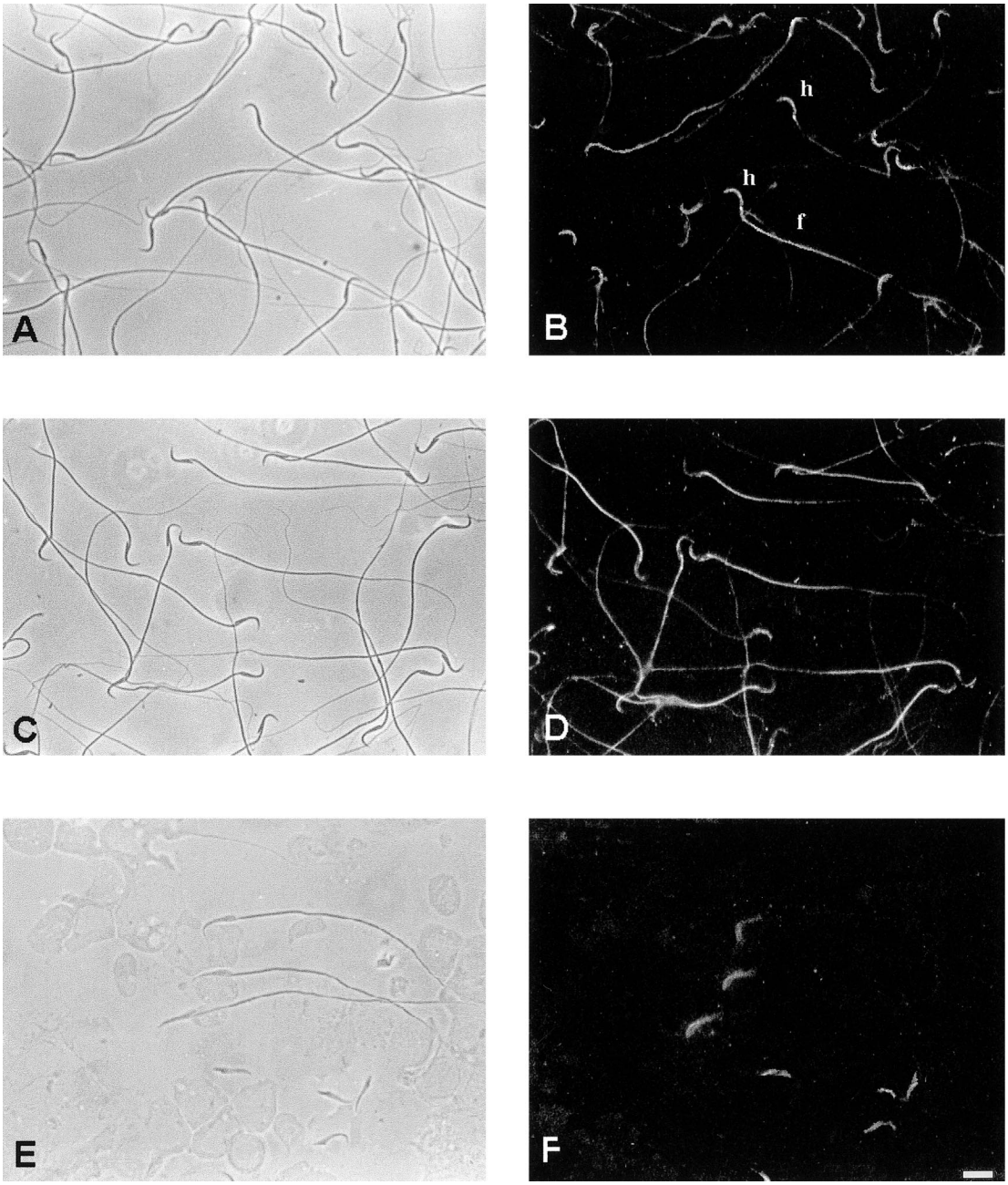
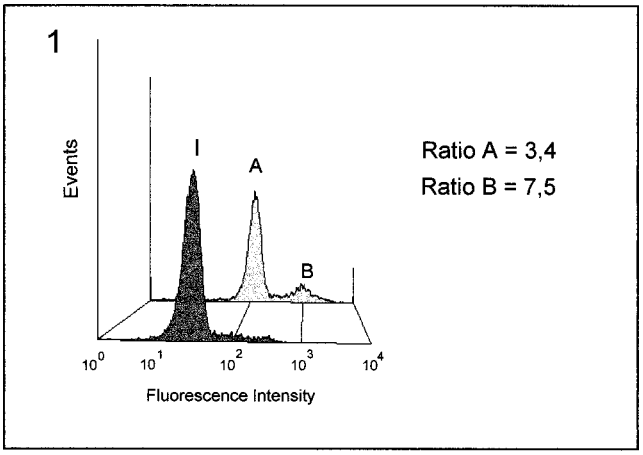


Figure 2. C-met distribution on spermatozoa isolated from epididymis and testis. Phase contrast microscopy of cells isolated from caput **(A)** and cauda **(C)** epididymidis, and testis **(E)**, and c-met indirect immunofluorescence on spermatozoa of the same origins **(B, D, F)** are shown. Bar = 15 μm.

spermatozoa isolated from the epididymal cauda are quite homogeneous, showing c-met localized both on the head and the flagellum of the entire cell population. Cytometric analysis of spermatozoa isolated from epididymal caput confirmed the existence of 2 different cell populations according to their fluorescence intensity, whereas only 1 homogeneous cell population was detected when spermatozoa isolated from the epididymal cauda were analyzed. It is well known that spermatozoa undergo several changes during their passage through the epididymis both at molecular, morphological, and physiological levels. By losing their cytoplasmic droplets, they acquire their final morphology (Bloom and Nicander, 1961), and by remodeling the plasma membrane, they acquire the molecular pattern of the membrane of a mature cell (Dacheux and Volgmayr, 1983; Syntin et al, 1996; Jones, 1998). Acquisition of specific glycoproteins on the plasma membrane is necessary for the HGF effect, as suggested by the data shown by Rosen et al (1990), who reported that HGF action on MDCK cells is inhibited by blocking the



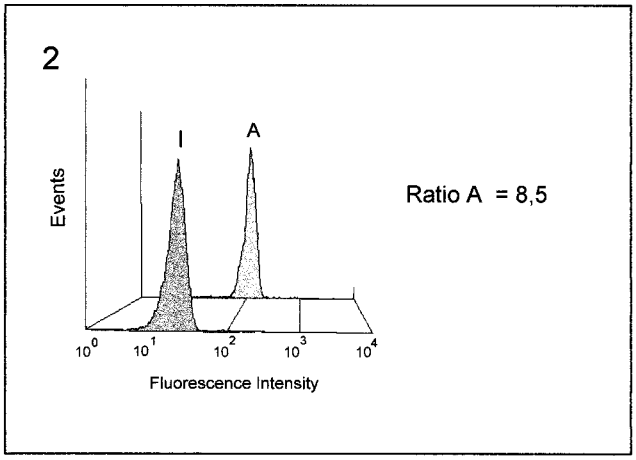


Figure 3. Flow cytometric analysis of c-met expression. 1: Representative histograms of spermatozoa isolated from caput epididymidis after incubation with anti c-met antibody (peaks A and B). 2: Representative histograms of spermatozoa isolated from cauda epididymis after incubation with anti-c-met antibody. The histogram obtained after incubation of spermatozoa with isotype is also shown (peak I). Measurements (±SE) of mean cell fluorescence were performed in triplicate.

protein glycosylation pathway. In this paper, we report that the glycoprotein receptor HGF is differentially expressed in sperm isolated from different portions of the epididymis, with a larger expression along the entire cell surface in cells isolated from the cauda of the organ. This finding allows us to hypothesize that the effect of HGF on spermatozoa may increase during their transit in the epididymis, and is directly correlated with higher receptor expression. HGF is present in the male genital tract of mice (Naz et al, 1994) and in large quantities in human seminal plasma (Depuydt et al, 1998; Kitamura et al, 2000; Wiltshire et al, 2000), although it is still not clear which organ or organs produce the factor. In humans, the epididymis, prostate, and seminal vesicles appear to be

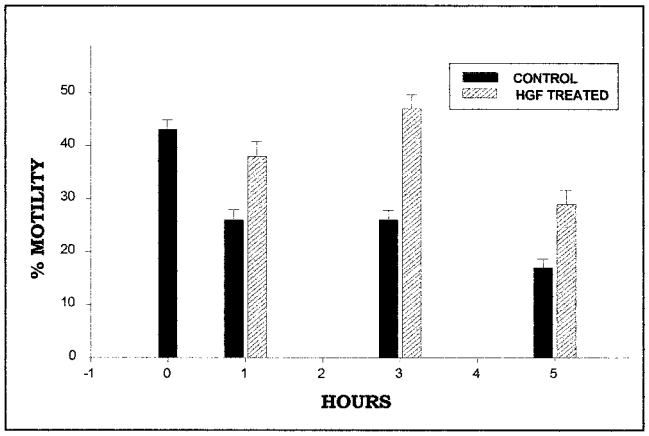


Figure 4. Effect of HGF on sperm motility. Spermatozoa isolated from caput epididymidis were incubated for 1–5 hours with or without HGF (300 U/mL). The motility of freshly isolated cells was also evaluated (time 0). Values represent the mean (±SE) of 5 experiments performed in triplicate.

the main sources of the factor (Wolf et al, 1990; Di Renzo et al, 1991), and in rats, we have previously reported that HGF is produced by the testis (Catizone at al, 1999). We now report that the factor is also synthesized and secreted by the rat epididymis, as indicated by the detected scatter effect of epididymal cell homogenate and culture medium on MDCK cells.

Our data on c-met localization and HGF production by the epididymis, together with the previously reported positive effect of HGF on the acquisition of sperm motility in mice (Naz et al, 1994), suggest that in rats, HGF/c-met are in some way related to epididymal acquisition, maintenance of sperm motility, or both. To clarify this point, spermatozoa isolated from caput epididymidis, most of which are not motile, have been isolated and cultured in medium alone or supplemented with HGF to evaluate the effect of the growth factor on their motility. The results obtained indicated that HGF has a positive effect on maintenance of sperm motility. We have found that sperm motility significantly decreases during the first hour of control culture, whereas it is maintained when HGF is present in the culture medium. On the contrary, the factor does not increase the percentage of motile cells, and this finding suggests that the HGF effect is related only to the maintenance of cell motility. Our data are apparently in contrast with recent data (Kitamura et al, 2000; Wiltshire

Table 1. *Statistical analysis of HGF effect on sperm motility*

Doses of HGF (units)	Time of Culture		
	1 Hour	2 Hours	5 Hours
75	n.s.*	n.s.	n.s.
150	n.s.	$P < .05$	n.s.
300	$P < .01$	$P < .01$	$P < .01$

* n.s. indicates not significant.

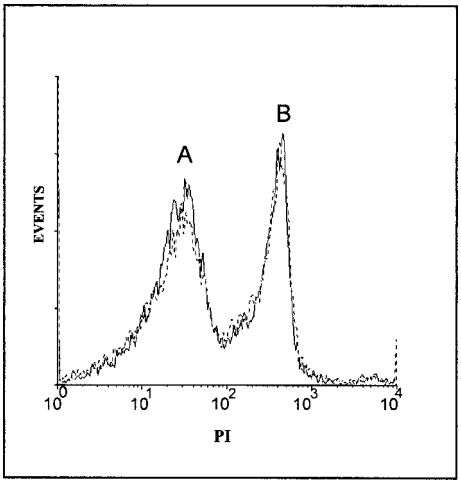


Figure 5. Flow cytometric analysis of rat spermatozoa stained with propidium iodide (PI). Spermatozoa isolated from caput epididymis were cultured for 3 hours with or without 300 U/mL HGF, labeled with PI, and analyzed using flow cytometry. The percentage of live (peak A) and dead (peak B) spermatozoa in controls (dotted line) and in treated preparations are shown. One representative experiment performed in triplicate is reported.

et al, 2000) reporting that HGF does not significantly maintain the motility of human spermatozoa. The possible explanation for our positive results can be that HGF works differently in humans than it does in rodents, which has also been suggested by the positive effect of HGF on sperm motility that was previously reported in mice (Naz et al, 1994). Moreover, those authors used ejaculated human spermatozoa, which have already undergone the maturation changes that occur during transit through the epididymis.

In this paper we also report that HGF does not influence spermatozoa viability, as indicated by cytometrical analysis of PI-labeled sperm, which indicated an equal number of dead cells in both control and in HGF-treated preparations.

In conclusion, the data we present demonstrate the presence of c-met in rat spermatozoa, and a different distribution of this protein in cells depending on their location in the various portions of the epididymis. The wider distribution of c-met in cauda-derived cells and the longer persistence of the motility of caput-derived cells cultured in the presence of the factor, strongly support the hypothesis that HGF positively influences the maintenance of sperm motility during their transit through the epididymis and indicates that c-met receptor and its ligand, HGF, may play a role in male fertility.

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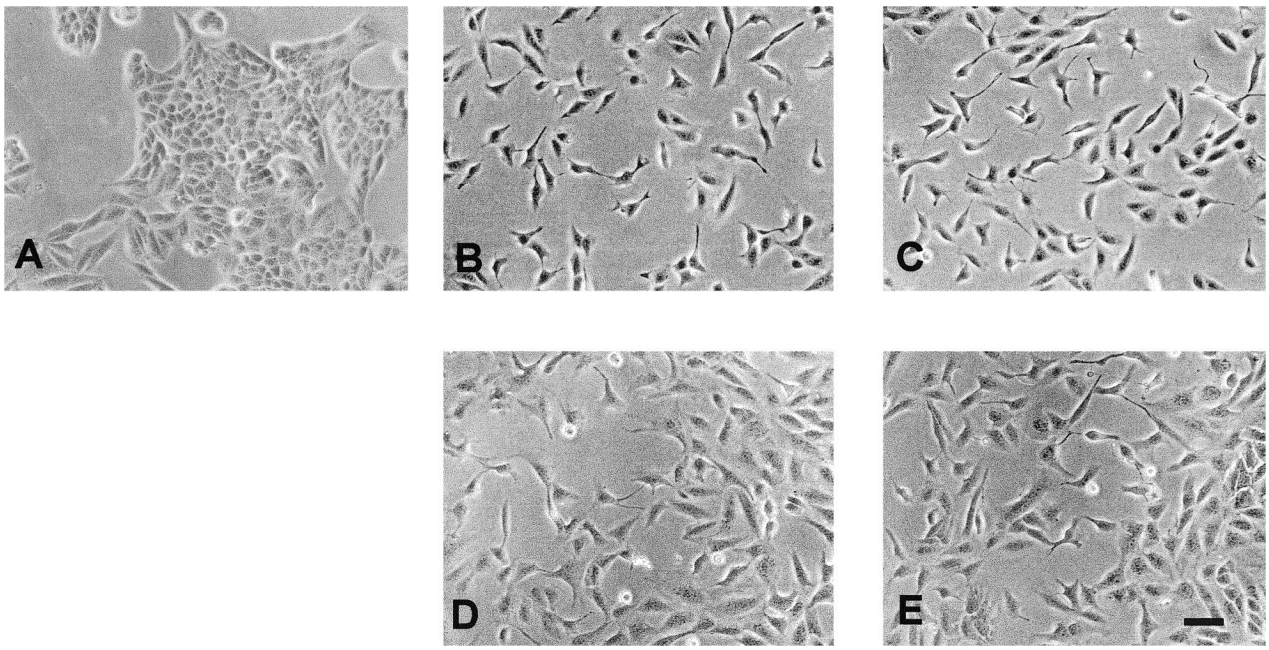

Figure 6. Scattering of MDCK epithelial cells. Morphological appearance of cells cultured for 16 hours in DMEM with 10% FCS added **(A)** or in the presence of the supernatant obtained from the homogenate of caput **(B)** and cauda **(C)** epididymidis. **(D)** and **(E)** show the effect of conditioned culture medium from caput and cauda epididymidis organ cultures. Bar = 38 μm.

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