

Hepatocyte Growth Factor (HGF) Modulates Leydig Cells Extracellular Matrix Components

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Abstract

Hepatocyte growth factor (HGF) is a pleiotropic factor which plays multiple roles during mammalian development. We have previously demonstrated that in the postnatal testes the HGF receptor, c-met, is expressed by Leydig cells and HGF increases the steroidogenic activity of the cells. In the present paper we report that HGF modify the composition of the extracellular matrix of cultured Leydig cells. We show that HGF increases metabolic activity of isolated Leydig cells; in particular the factor increases uPA and MMP 2 secretion. We have also shown that the levels of active TGF- β are increased by HGF. On the contrary, utilizing the western blotting technique, a strong reduction of fibronectin present in the culture medium of cells cultured in the presence of HGF has been detected. The presented data demonstrate that HGF modulates several functional activity of Leydig cells, further supporting the hypothesis that this factor exerts a relevant role in the regulation of mammalian spermatogenesis.

Running title: HGF and Leydig cells

Keywords: Leydig cells, TGF- β , uPA, MMP, fibronectin

Introduction

Hepatocyte growth factor (HGF) is a pleiotropic factor which plays a role in organogenesis and development (Zarnegar and Michalopoulos, 1995). The multiple actions of HGF are exerted by the c-met receptor, a transmembrane glycoprotein with tyrosine kinase activity, encoded by the MET protooncogene (Weidner et al. 1993; Hartmann et al. 1994). As we previously demonstrated, 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 (Catizone et al. 1999, 2000, 2001). In the Sertoli cells c-met expression is developmentally regulated and is detectable in cells isolated from pubertal animals (Catizone et al. 2005). Mitotic and meiotic rat germ cells express c-met as well as testicular and epididymal spermatozoa (Catizone et al. 2002, 2006) and HGF regulates proliferation and apoptosis of prepubertal rat germ cells (Catizone et al. 2006). HGF is also expressed during embryonic development (Sonnenberg et al., 1993) and we previously demonstrated that in the embryonic mouse testis HGF induces testicular cell proliferation and acts as a morphogenetic factor (Ricci et al. 1999, 2002, 2004).

Matrix metalloproteinases (MMPs) are a large group of enzymes able to cleave several constituents of the extracellular matrix (ECM), regulating in this way the composition of the matrix and, consequently, the interactions between matrix and cells. In this way MMPs control the signals starting from the matrix molecules which regulate many aspects of cell activity including cell proliferation and differentiation (Chakraborti et al., 2003). MMPs are secreted as latent enzymes which are activated by physiological activators including plasmin and plasminogen activators (PAs). MMP 2 is constitutively synthesized by many cells, is activated in different ways and is modulated by growth factors and cytokines (Overall et al., 1991). HGF increases MMP 2 mRNA levels in endothelial cells (Wang and Keiser, 2000), in normal human glomeruli (Esposito et al, 2005) and in glioma cells (Hamasuna et al., 1999; Yano et al., 2001) and decreases the expression of tissue inhibitor of MMP 2 (Gong et al., 2003).

Leydig cells, the testosterone-producing cells of the mammalian testis, during postnatal development undergo to a series of morphological and functional transformations. They are present as proliferating precursors in prepubertal rats and as immature and mature adult Leydig cells in pubertal rats (Ariyaratne and Mendis-Handagama 2000; Haider 2004). In both stages the cells are able to produce testosterone, but differ in the levels of testosterone synthesis (Haider 2004). Leydig cells secrete a variety of proteases including plasminogen activators (Odet et al., 2006; Le Magueresse-Battistoni, 2007) and their functions are regulated by several hormones among them interleukins (Svechnikov et al. 2001; Walch and Morris 2002), transforming growth factors beta (Khan et al. 1992; Dickson et al. 2002), insulin-like factors (Khan et al. 1992; Ge and Hardy 1997) and ghrelin (Barreiro et al. 2004). We have recently demonstrated that in the embryonic testes HGF regulates testosterone production of foetal Leydig cells (Ricci et al. 2006) and reported that, in the postnatal testes, c-met is expressed by Leydig cells isolated from pubertal rats and is functionally active. In fact HGF increases the steroidogenic activity of Leydig cells and prevents apoptosis of Leydig cells (Del Bravo et al., 2007). By culturing testicular fragments we recently reported that in the testis HGF regulates Sertoli-Sertoli tight junctions influencing the synthesis of occludin and other molecules involved in junction dynamics such as TGF β and urokinase type PA (uPA) (Catizone et al. 2008).

In the present paper we report our recent investigations on the effects of HGF on isolated Leydig cells showing that HGF increases the amount of uPA and MMP 2 secreted by the cells and increases TGF- β activity. We also demonstrate that fibronectin secretion is strongly reduced by HGF.

Materials and Methods

Animals

Wistar rats were housed at the “Sapienza” University of Rome. 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 killed by CO₂ asphyxia before testes removal. Usually three or four 28-30-day-old rats of 100-120 grams of body weight were used for each experiment.

Leydig cell isolation and culture

Leydig cells were isolated as previously reported (Morris et al., 1997) with slight modifications. Briefly, decapsulated testes were incubated with Minimum Essential Medium (MEM, Gibco, Invitrogen s.r.l., Milan, Italy) containing 0,18 % trypsin at 32 °C in a shaking water bath (90 cycles/min) for 15 min. After dissociation, the enzyme was diluted with culture medium and the seminiferous tubules were removed by sedimentation at gravity (4 min). Tubules were washed again to detach the interstitium and the two supernatants collected and centrifuged (300 x g, 10 min). The pellet was suspended in MEM containing bovine serum albumin (BSA) 0,1% and DNase 0,01% and the cell suspension was loaded on top of a discontinuous Percoll gradient (20-86% Percoll) and centrifuged at 800 x g for 20 min at 18 °C. After centrifugation, fractions at 1.056 and at 1.068 g/ml were collected, washed with buffer and counted. Isolated Leydig cells were suspended in MEM culture medium containing 15 mM Hepes, non essential aminoacids, 5 µg/ml gentamicin, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded on Falcon culture plates (Becton Dickinson and Co., Lincoln Park, NJ) at the concentration of 1.2x10⁶ cells/ml of medium. Usually the cells were cultured at a density of 0.1x10⁶ cells/cm². Viability of Leydig cells was assessed by trypan blue dye exclusion method. Briefly, isolated Leydig cells were mixed with an equal volume of 0.4% trypan blue (Flow Laboratories, Irvine, Scotland), incubated for 5 min at 37 °C and examined under a microscope. After 24 h of culture Leydig cells were almost totally viable. To assess the effects of HGF *in vitro*, cells were cultured for 26 h at 32 °C in a humidified 5% CO₂-95% air atmosphere in the presence of the growth factor (100 U/ml) for the last 24 h of culture. At the end of incubation the conditioned media were collected and utilized for the different assays.

Evaluation of isolated Leydig cell purity

Purity of Leydig cell preparation was routinely assessed on the basis of positive staining of cells for the enzyme 3β- *hydroxy steroid dehydrogenase* (HSD) (Payne et al., 1980). Briefly, an aliquot of Leydig cell fraction was added to a tube containing 0.5 ml of β-nicotinamide adenine dinucleotide (NAD, 9 mg/ml, Sigma-Aldrich Co., St. Louis, MO), 0.2 ml of dehydroepiandrosterone (DHEA, 1mg/ml in methanol) and 0.25 ml of nitroblue tetrazolium (NBT, 2 mg/ml in phosphate buffer pH 7.4, Sigma-Aldrich Co.). The reaction mixture was allowed to stand for 1 h at 37 °C. The percentage of positively stained cells was examined under the microscope. The cell purity was consistently higher than 90% (fig.1). In several experiments the purity of our cell populations was also evaluated by immunocytochemistry for cytochrome P450 side chain cleavage (P450_{scc}) as we previously described (Del Bravo et al., 2007).

Western blot analysis

Leydig cell conditioned medium prepared as indicated in the ‘Leydig cell culture’ paragraph, was 10 fold concentrated utilizing Centricon-30 concentrators (Amicon, Beverly, MA., U.S.A.). Equal amounts of concentrated medium (40µl) were resolved by 7% SDS-PAGE (Laemmli 1970) and transferred to nitrocellulose membranes. After blocking, the membranes were incubated (16 h at 4 °C) with 2,5 µg/ml rabbit polyclonal anti-fibronectin antibody (Chemicon, Billerica, MA., U.S.A. AB1954,

1:600) and successively for 1 h at RT with conjugated AP goat anti-rabbit secondary antibody (Zymed, Invitrogen, Paisley, U.K. 626122, 1:2000). AP was visualized with the CDP-Star chemiluminescence Reagent (PerkinElmer LAS Inc., Boston, MA) according to the manufacturer's instructions. Fibronectin content was determined densitometrically utilizing AIDA software (Advanced Image Data Analyzer). The optical densities over the entire bands are integrated, arbitrarily considering as 100 the control value.

Bioassay for TGF- β

Active TGF- β was assayed according to Abe et al. (1994) utilizing a Mink Lung Epithelial Cell (MLEC) line steadily transfected with the Luciferase gene. MLECs were suspended at 8×10^4 cells/ml and 100 μ l of the cell suspension was plated in 96-well plates. Cells were cultured for 24 h and washed twice with MEM before the addition of 100 μ l of our conditioned media. Conditioned media were prepared culturing 3×10^6 Leydig cells for 24 h as described above. After 16 h of culture, MLECs were washed twice with phosphate-buffered saline (PBS) and lysed in 60 μ l of lysis buffer (Promega, Madison, WI). Cell extracts were assayed for luciferase activity with an assay kit obtained from Promega (Madison, WI) and using a Netzschalter 090003 luminometer (GSG Nuclear, Milan, Italy). Parallel MLECs were incubated with known increasing concentrations of TGF- β (Sigma-Aldrich Co, T1654, ranging from 0.0625 to 4.0 ng/ml) to obtain a standard curve.

Zymography analysis of plasminogen activator (PA)

For zymography of plasminogen activator, 20 μ l of the same conditioned medium used for TGF- β bioassay were separated by 10% sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions according to the procedure of Laemmli (1970). Molecular weights were calculated from the position of pre-stained molecular weight markers (Sigma-Aldrich Co) subjected to electrophoresis in parallel lanes. PA was then visualized by placing the Triton X-100 washed gel on a casein-agar-plasminogen underlay, as previously described (Granelli-Piperno and Reich, 1978; Vassalli et al., 1984). The densitometric analysis of the band was performed.

Gelatin zymography for MMPs detection

Gelatinolytic activity was assayed using the Heussen and Dowdle (Heussen and Dowdle, 1980) method, adapted for a minigel format. Twenty-microliter aliquots of conditioned media were applied directly, without prior heating or reduction, to 10% (w/v) acrylamide gels containing 2 mg/ml of gelatin (Sigma-Aldrich Co). After removal of SDS from the gel by incubation in 2.5% (v/v) Triton X-100 for 30 min, the gels were incubated at 37°C for 18 h under continuous stirring in 50 mM Tris-HCl, pH 7.6, containing 0.2 M NaCl, 5 mM CaCl₂ and 0.02% (w/v) Brij-35. Gels were stained for 30 min in Coomassie brilliant blue diluted in 30% methanol in 10% glacial acetic acid 0.5% (v/v) and then destained in the same solution without the dye. The gelatinolytic activity of each gelatinase, identified by the molecular weight, was evident as a clear band against the blue background of stained gel. The intensity of the bands was quantified with specific densitometry (Molecular Analyst Software; Bio-Rad, Hercules, CA, USA).

Statistical analysis

All experimental data were expressed as the mean \pm SE of at least three separate experiments. Statistical analysis was performed by Student's t-test. Differences were considered significant at P-value < 0.05.

Results

Purity of Leydig cell preparations

The purity of the Leydig cell preparations was always checked looking at the positivity of the cells for the enzyme 3 β -HSD as described in the materials and methods section. In fig.1A the positivity (black asterisks) and negativity (white asterisks) of a representative cell preparation is shown. In fig.1B the phase contrast microscopy of the same field is presented.

Urokinase type plasminogen (uPA) activator production

Leydig cells were cultured for 24h in medium alone or in medium supplemented with HGF (100 U/ml). At the end of incubation, the culture media were collected and utilized for uPA evaluation. Fig.2A shows the results of the three experiments performed: the cells cultured in the presence of HGF secrete a significantly higher amount of uPA respect to the control samples. In fig.2B the dose-response curve of uPA secreted in the presence of different doses of HGF ranging from 25 to 200 U/ml is presented. The amount of uPA produced was significantly higher at 100-200 U/ml of HGF.

Metalloproteases (MMPs) production

The culture media of Leydig cells cultured for 24h in control medium or in medium supplemented with HGF (100 U/ml) were utilized for the detection of MMPs 2 and 9. In the six experiments performed we have detected MMP 2 and always found a significant increase of both proenzyme and enzyme although the levels of the increase were different in the different experiments (fig.3). On the contrary, MMP 9 was clearly detectable only in one of the experiments performed (not shown).

Transforming Growth Factor- β (TGF- β) production

Leydig cells were cultured for 24h in control medium or in medium supplemented with HGF (100 U/ml) and the secretion of active TGF- β was evaluated using a bioassay based on the use of MLEC cells as described in the methods section. The results obtained in the four experiments performed indicate that HGF significantly increases the amount of the active TGF- β secreted by the cells in the culture medium (fig.4).

Fibronectin production

Aliquots of Leydig cell culture media were utilized to evaluate the amount of fibronectin produced by Leydig cells cultured for 24h in control medium or in medium supplemented with HGF (100 U/ml). A representative western blot experiment is shown in fig.5A. In the four experiments performed the amount of fibronectin detected in the media of cells cultured in the presence of HGF was approximately 25% of the amount present in the media of control cells (fig.5B).

Discussion

We have previously demonstrated that HGF has a crucial role in the regulation of male fertility modulating the functional activities of somatic and germ cells of the rat testis (Catizone et al., 2001, 2002, 2005, 2006) and modulating Sertoli-Sertoli tight junction dynamics (Catizone et al., 2008). Recently we have studied the effects of HGF on isolated Leydig cells reporting that the factor acts preventing Leydig cell apoptosis and increasing the steroidogenic activity of the cells cultured either isolated or in explants of testicular tissue that is in a more physiologic condition (Del Bravo et al., 2007). In the present paper we better define the effects of HGF on isolated Leydig cells showing that HGF increases the amount of urokinase type plasminogen activator secreted by the cells. We have previously shown that HGF increases the amount of uPA secreted by the testicular tissue therefore we have considered of interest to know if the factor had the same effect on the uPA secretion of Leydig

cells. A significant increase of uPA was found when the cells were cultured in a HGF-supplemented medium therefore we conclude that, in the interstitial tissue, HGF positively regulates the production of this protease. Plasminogen activators are serine proteases involved in multiple physiological processes (Blasi et al., 1987; Vassalli et al., 1991; Le Magueresse-Battistoni, 2007) including the activation of inactive TGF- β (Odekon et al., 1994; Nunes et al., 1995). It is well known that mammalian Leydig cells produce TGF- β s (Hedger and Meinhardt, 2003) therefore we investigated the role of HGF in the regulation of TGF- β levels and we found that HGF significantly increases the amount of the active TGF- β present in the culture medium of HGF-treated cells. Considering that we have found an increase of uPA, we hypothesize that the increased amount of the active TGF- β could not be a direct effect of HGF but a consequence of the increased uPA activity.

Besides uPA we looked at the effect of HGF on other proteases secreted by Leydig cells, in particular on matrix metalloproteinase 2 and 9, also named gelatinases A and B for their action on gelatine (Chakraborty et al., 2003). We found that MMP 2 activity is increased by HGF and the data was not surprising since it has been reported that MMPs are modulated by different molecules: in human fibroblasts MMP 2 is increased by TGF- β at the transcriptional and post-transcriptional levels (Overall et al., 1989; 1991) and HGF increases MMP 2 and MMP 9 transcription in human glomeruli thus modulating matrix turnover (Esposito et al., 2005). It is also known that HGF modulates matrix MMPs and plasminogen activator/plasmin system in pathological conditions such as renal interstitial fibrosis (Gong et al., 2003). The pathology is ameliorated by HGF which enhances the catabolism of the extracellular matrix (ECM) via MMPs and PA/plasmin system (Gong et al., 2003). ECM is mainly degraded through the two different pathways based on MMPs and PA/plasmin system (Visse and Nagase 2003; Nagase et al., 2006). Our reports indicate that the extracellular matrix of Leydig cell is modulated by HGF considering that the factor modulates either PA/plasmin system and the levels of MMPs, in particular MMP 2 levels. MMP 2, having a part the gelatinolytic activity, shows collagenolytic activity for different types of collagen including type IV collagen, important feature of basement membrane remodelling (Chakraborty et al., 2003; Nagase et al., 2006; Page-McCaw et al., 2007). Therefore MMPs activity, inducing the degradation of the ECM components, can either modify the tissue architecture and create molecular fragments with biological activity (as a review see Page-McCaw et al., 2007). Different types of molecules can be substrates of MMPs including growth factors, tyrosine kinase receptors and cell-adhesion molecules: fibronectin is degraded by MMP 2 (Page-McCaw et al., 2007) and active TGF- β can be proteolytically produced from the inactive complex by MMP 2 and MMP 9 activity (Yu and Stamenkovic, 2000). Therefore also in our system the increase of MMP 2 could be responsible for the increase we found of TGF- β levels (levels that could also be controlled by uPA). Interestingly, we have also demonstrated that in the presence of HGF the fibronectin present in the medium of HGF-treated Leydig cells is strongly reduced therefore the low amount of fibronectin detected in the media of cells treated with HGF could be due to the high levels of proteases present in the medium.

In conclusion in this paper we report that HGF influences the metabolic activity of isolated Leydig cells, that is uPA and MMP 2 secretion, and increase TGF- β activity. Also fibronectin is modulated by HGF as indicated by the strong reduction of fibronectin detected in the culture medium. All together the data presented indicate a relevant role of HGF in the regulation of Leydig cell metabolic activity and of the composition of the interstitial tissue. Therefore our data further indicate an important role of HGF on the regulation of mammalian spermatogenesis.

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Figure legends

Fig.1. Purity of Leydig cell preparations. Phase contrast microscopy (B) of the cells and their positivity (black asterisks) and negativity (white asterisks) for the enzyme 3 β -HSD (A) are shown. Bar=10 μ m. A representative micrograph is shown.

Fig.2. Urokinase type plasminogen activator secreted by isolated Leydig cells. A: In the left part of the panel the zymography of uPA of control (C) and HGF-treated (HGF) sample is shown whereas in the right part the densitometric analysis is reported. The levels are expressed as 'arbitrary unit' considering 100 the control level. The data represent the mean \pm S.E. of three different experiments. B: Dose-response curve of uPA secreted in the presence of different doses of HGF (25 - 200 U/ml). In A, HGF vs C, $p < 0.05$; in B *vs C, $p < 0.05$.

Fig.3. MMP 2 secreted by isolated Leydig cells cultured in control medium (C) and in medium supplemented with HGF (HGF). A: Gelatin zymography of MMP 2 proenzyme (72 KDa) and enzyme (62 KDa) of control (C) and HGF-treated (HGF) sample is shown. B: The activity levels obtained in the four different experiments performed are shown.

Fig.4. TGF β secreted by isolated Leydig cells. The amount of active TGF β of control (C) and HGF-treated (HGF) samples is shown. The data represent the mean \pm S.E. of four different experiments. * vs C, $p < 0.05$.

Fig.5. A: western blot of fibronectin present in the culture medium. B: densitometric analysis of the bands. The data represent the mean \pm S.E. of the different experiments. HGF vs C, $p < 0.001$. The levels are expressed as 'arbitrary unit' considering 100 the control level.

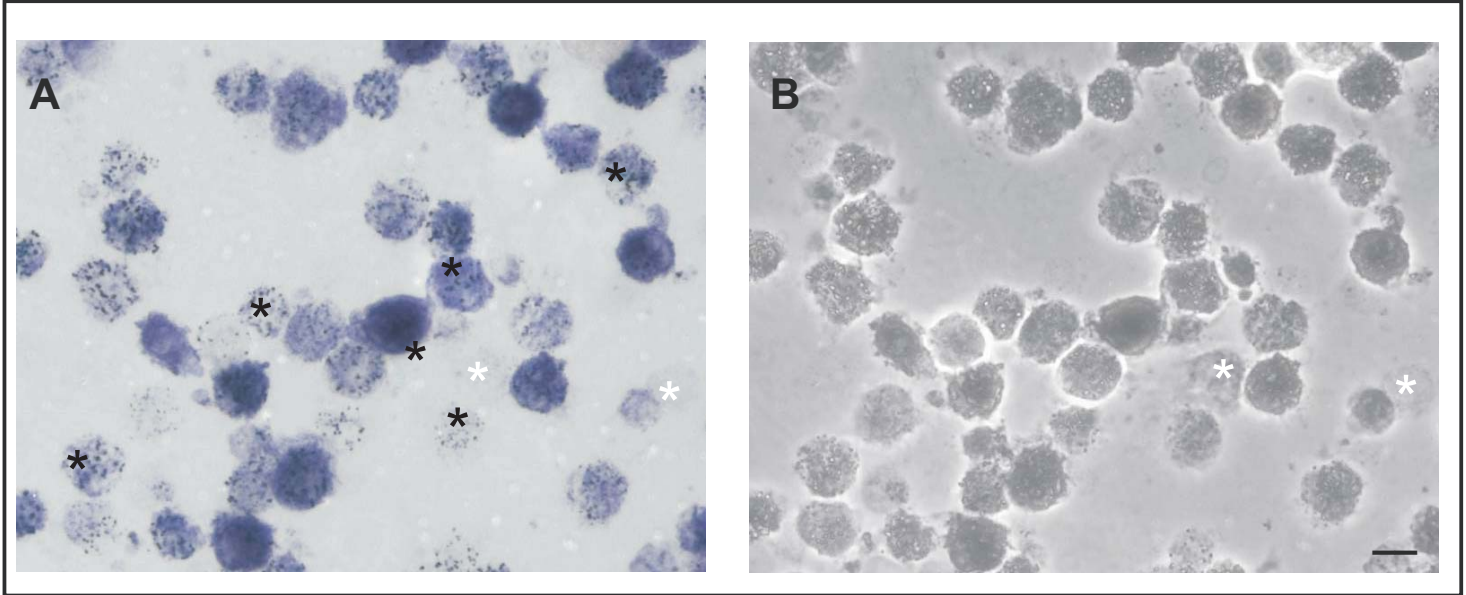


Fig. 1

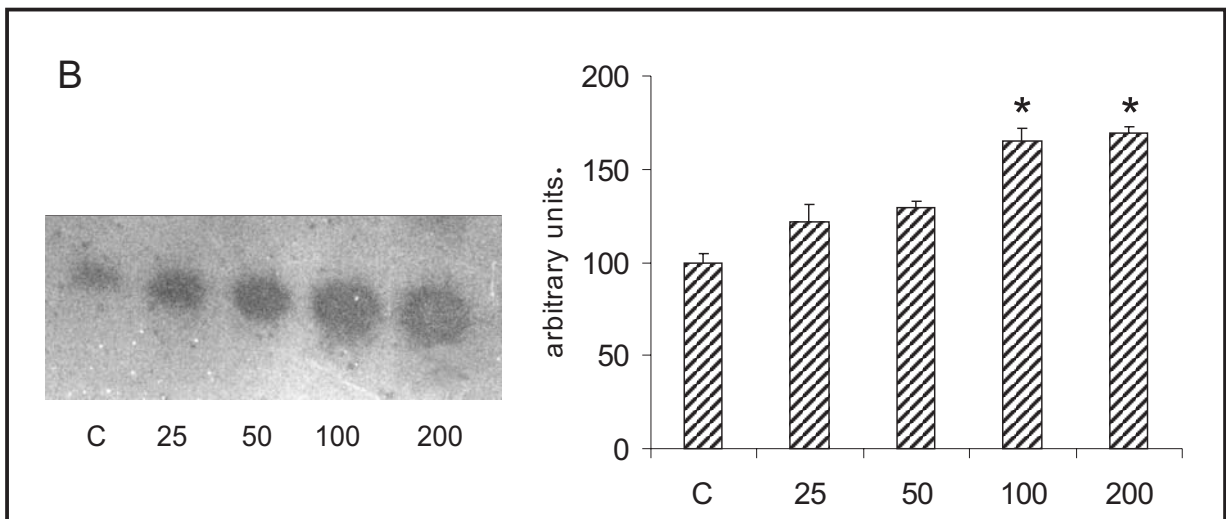
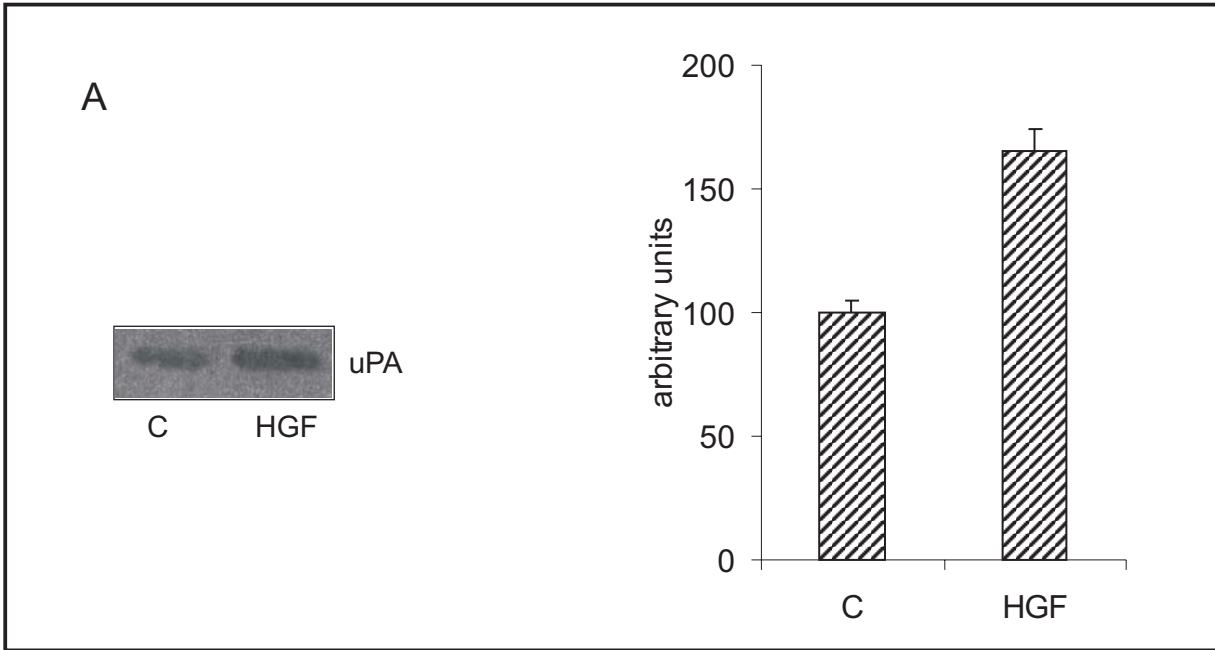
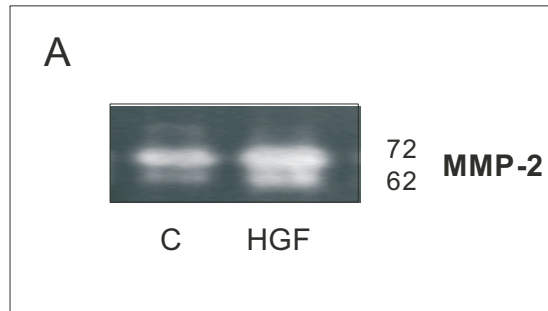


Fig.2



B

PROENZYME

ENZYME

Experiments	Control	HGF	Control	HGF
1	+	++	+	+++
2	+	+++	+	+++
3	+	+++	+	++
4	+	+++	+	++

Fig.3

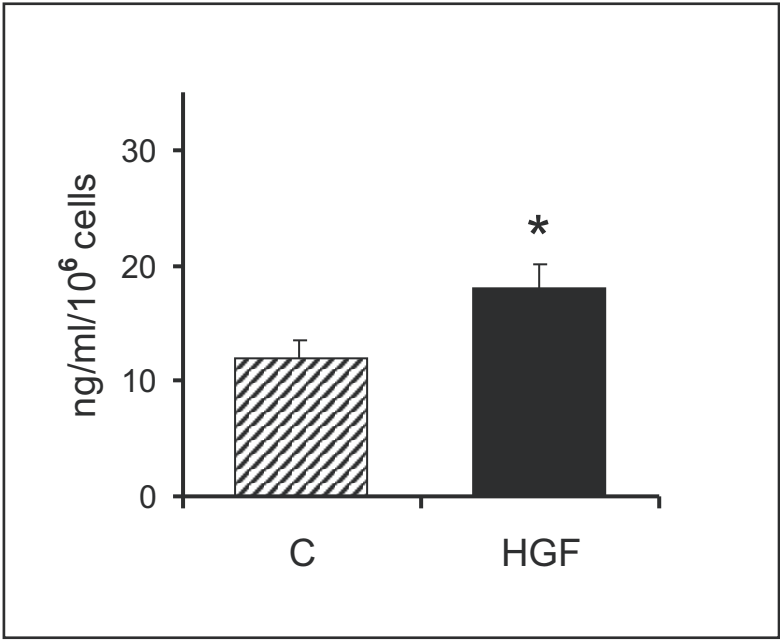


Fig.4

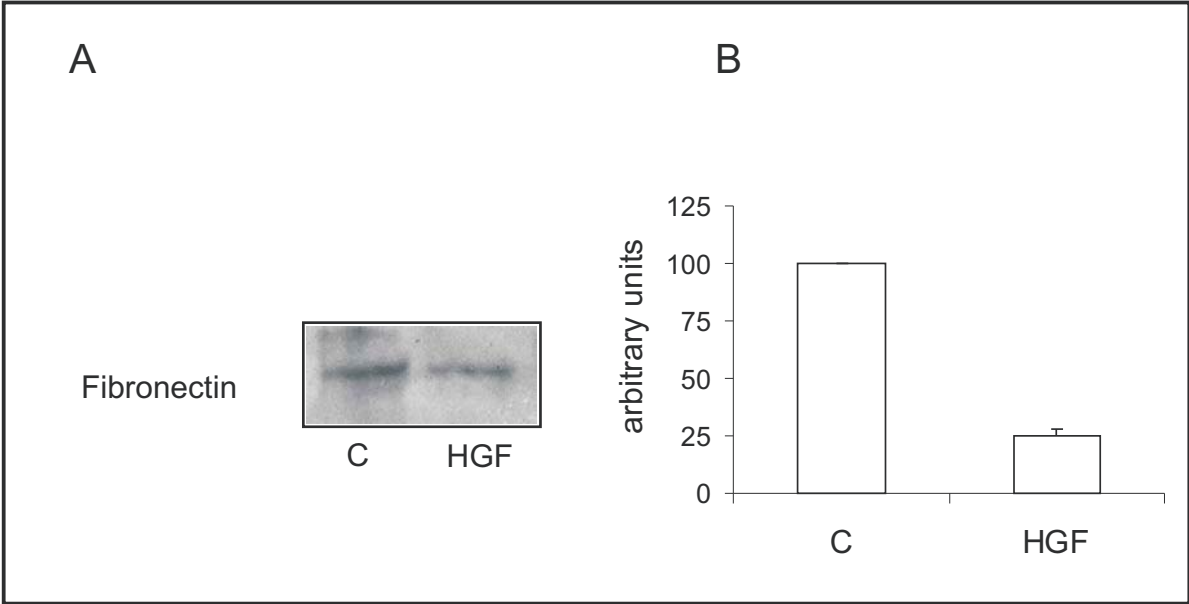


Fig.5