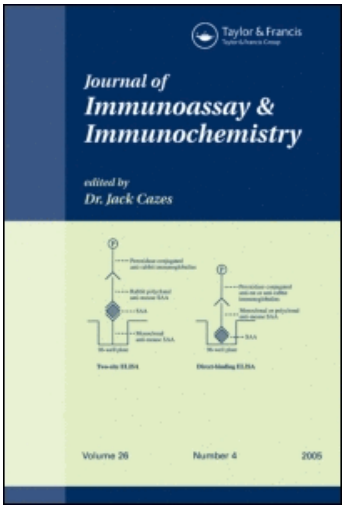

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

IMMUNOASSAY FOR MEASURING THE HEPARIN-BINDING GROWTH FACTORS HARP AND MK IN BIOLOGICAL FLUIDS

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Online publication date: 04 March 2002

To cite this Article Soulié, Patrick , Héroult, Mélanie , Bernard, Isabelle , Kerros, Marie-Emmanuelle , Milhiet, Pierre Emmanuel , Delbé, Jean , Barritault, Denis , Caruelle, Danièle and Courty, José(2002) 'IMMUNOASSAY FOR MEASURING THE HEPARIN-BINDING GROWTH FACTORS HARP AND MK IN BIOLOGICAL FLUIDS', Journal of Immunoassay and Immunochemistry, 23: 1, 33 — 48
To link to this Article: DOI: 10.1081/IAS-120002273
URL: http://dx.doi.org/10.1081/IAS-120002273

J. IMMUNOASSAY & IMMUNOCHEMISTRY, 23(1), 33–48 (2002)

IMMUNOASSAY FOR MEASURING THE HEPARIN-BINDING GROWTH FACTORS HARP AND MK IN BIOLOGICAL FLUIDS

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ABSTRACT

Heparin-affin regulatory peptide (HARP) and Midkine (MK) belong to a family of growth/differentiation factors that have a high affinity for heparin. The involvement of these molecules in various proliferative diseases prompted us to develop an assay for measuring the concentrations of these factors in biological fluids and culture media. This report describes an immunoassay that uses only commercially available materials, based on the high affinity of certain molecules for heparin. It consists of adsorbing heparin-BSA covalent complexes to microtiter

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plate wells and to quantify the heparin bound HARP or MK by using appropriate antibody. The method is specific and measures concentrations ranging from 40–1200 pg/mL HARP and from 25–1200 pg/mL MK and various parameters are investigated. The within-assay coefficient of variation was less than 5% for both assays. The method was checked by measuring the concentrations of these growth factors in the sera of healthy humans and in patients with cancer. As previously reported, we confirmed that the serum concentrations of MK are higher in patients with tumours ($n = 139$) than in controls ($n = 19$). The synthesis of HARP and MK by various cells in culture was also analysed.

INTRODUCTION

Heparin-affin regulatory peptide (HARP; or pleiotrophin) and Midkine (MK) belong to a new family of growth factors that bind strongly to heparin and glycoaminoglycans (GAGs).(1–3) MK and HARP have 10 conserved cysteine residues and a signal peptide.(4) They promote neurite outgrowth and the survival of embryonic neurones and are also mitogens for various cells. MK causes the proliferation of fibroblasts and neuroectoderm-derived cells *in vitro*.(5) MK is abundantly produced during the mid-gestation period of mouse development and may be involved in organogenesis.(6) The biological properties of HARP have been a matter of controversy until recently.(7–9) It was initially described as a molecule that induced neurite outgrowth from embryonic neurones (10), but others, including ourselves, reported that HARP was a mitogen for epithelial, fibroblastic, and endothelial cells.(1,11,12)

HARP was also found to have angiogenic activity,(13,14) Other reports indicated that the C-terminal part of the molecule enhanced the concentration of plasminogen activator and inhibited the synthesis of plasminogen activator inhibitor-1 by bovine aorta endothelial cells in culture.(15) Similar results have been obtained with MK.(15) HARP and MK are involved in tumour progression, probably because of their angiogenic and mitogenic activities(13) and the serum concentrations of these two growth factors are elevated in several proliferative diseases, including carcinomas.(16–19)

The biological properties and the pathophysiological relevance of HARP and MK prompted us to develop an assay that could easily be used by other laboratories. This report describes a simple immunoassay for heparin binding molecules like MK or HARP that uses only commercially available materials.

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EXPERIMENTAL

Materials

Horseradish peroxidase-conjugated rabbit anti-goat immunoglobulins were obtained from Jackson (West Groove, PA, USA). Porcine mucosal heparin was a kind gift from M. Petitou (Sanofi, France). Bovine serum albumin (BSA), heparan sulfate (HS) from bovine intestinal mucosa, keratan sulfate (KS) from bovine cornea, chondroitin sulfate A (CS-A) from bovine trachea, dermatan sulfate (DS) from porcine skin, and chondroitin sulfate C (CS-C) from shark cartilage was purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Dihydrochloride, BCA protein assay, and 3,3′,5,5′-tetramethyl benzidine were from Pierce, Interchim (Montluçon, France). Anti-HARP antibody and anti-MK antibody were from R&D (Abingdon, UK). Recombinant HARP and MK, produced by Escherichia Coli, were prepared and purified according to the modified procedure previously described.(20)

Patients and Sample

The majority of patients had advanced, recurrent, and metastasic disease ($n = 139$) and were entering into chemotherapy regimens. All information concerning the cancer patients were retrospectively obtained from clinical charts. The mean age of the patients was 55.8 (standard deviation 11.2 years) and the male/female ratio was 0.16. The venous blood was drawn into a serum separator tube (type vacutainer, Becton-Dickinson) and immediately centrifuged at 1200 g for 10 min. The serum was then collected and aliquoted into 1 mL fractions and stored at -20°C. Nineteen serum samples were randomly selected from blood donors and used as control.

Cell Cultures

Bovine brain capillary endothelial cells (BBC) and arch bovine aorta endothelial cells (ABAE) were a generous gift from Dr Bohlen and were cultivated as previously described.(1) The Eahy 926 human endothelial-derived cell line was obtained from C.-J. S. Edgell and was grown in Dubelcco's modified Eagle's medium (DMEM), supplemented with 1 g/L glucose and 10% fetal calf serum (FCS). Human umbilical endothelial cells (HUVEC) were from Clonetics (BioWhittaker Europe, Belgium) and were grown in EGM-2 Bulletkit medium (BioWhittaker Europe, Belgium).

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The human epithelial cell line derived from normal prostate (PNT-1A) and the tumoral prostate cell lines (DU 145, PC3 and LN CaP) were a generous gift from O. Cussenot and were cultured in RPMI-1640 medium containing 10% FCS. C2C7 and SW 13 were kindly donated by M. Lazar and A. Wellstein, respectively, and were cultured in DMEM medium supplemented by 10% FCS. CHO-K1 cells were a generous gift from T. Melot and were cultivated in HAM-F12 supplemented by 10% FCS. Bovine epithelial lens cells were cultivated as previously described.(1) MDA MB 231, CCL64 and HISM were from the American Type Cell Collection and cultured as recommended. Cells were grown during 72 h and the supernatants were collected, centrifuged at $100 \times g$ for 5 min. Samples were stored at 4°C until used. Cell densities were determined using a hematocytometer.

Western Blot Analysis

Human recombinant MK (20 ng) or human recombinant HARP (20 ng) were run on a SDS-polyacrylamide gel and electro-transferred to an Immobilon-P membrane (Millipore, Saint-Quentin en Yvelines, France) in 10 mM CAPS buffer, pH 11, 10% methanol. Non-specific binding was prevented by incubating the membrane in Superblocker (Pierce, PerBio Science, France) for 20 min at room temperature. The membrane was then incubated overnight at 4°C with goat anti-HARP immunoglobulins at 1/1000 dilution in PBS-T (phosphate-buffer saline, 0.2% Tween-20) plus 3% Superblocker and washed in PBS-T. Bound antibodies were visualised with peroxidase-conjugated rabbit anti-goat immunoglobulins (Jackson, West Groove, PA, USA) diluted 1/100 000 in PBS-T plus 3% Superblocker using the chemioluminescence procedure (Boehringer Mannheim, Mannheim, Germany). The membrane was then processed for autoradiography using Kodak X-Omat film.

Synthesis of Heparin-BSA Complex

Heparin was covalently bound to BSA essentially as described by Gray (21) and by Najjam.(22) Heparin (912 mg) and BSA (34 mg) were dissolved in 5 mL potassium phosphate buffer (0.2 M, pH 8.0). Sodium cyanoborohydride (25 mg) was added and the mixture was incubated for 2 days at 37°C, dialysed against water, and freeze dried. The residue was dissolved in 50 mM *tris*-HCl, pH 7.4. The bicinchoninic acid assay was used to quantify the heparin-BSA complex with BSA as standard. The solution was aliquoted and stored at −80°C.

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Assay Procedure

Samples of heparin-BSA complex (100 μL containing 0.25 μg BSA equivalent or the concentrations defined in the text) in 50 mM *tris*-HCl, pH 7.4, supplemented with 12.7 mM EDTA, were incubated in 96 well plates at 4°C overnight. The heparin-BSA solution was then removed and each well was washed with washing buffer (PBS containing 0.05% Tween-20) and blocked (1 hour at room temperature) with 3% BSA.

For assays, samples (100 μL) were added to the wells, and incubated at 4°C overnight. The plates were washed three times with washing buffer, and the anti-HARP antibody was added (100 μL/well, 250 ng/mL in PBS containing 1% BSA). The plates were incubated for 2 h at room temperature, washed three times with washing buffer, and peroxidase-labelled rabbit anti-goat (100 μL/well diluted 1/20 000 in PBS containing 1% BSA) was added. The plates were again washed 3 times with washing buffer and peroxidase activity was detected using 3,3′,5,5′-tetramethyl benzidine dihydrochloride according to the supplier. Absorbances were read at 450 nm after 12 min and the concentration of HARP was determined with a titration curve established with various concentration of HARP ranging from 12.5 to 1500 pg/mL in presence of serum diluted twice with PBS for titration of HARP in serum or in culture medium for titration in conditioned cell media. The same procedure was followed for MK, except that anti-MK antibody was used.

Statistical Analysis

Serum concentration analysis was performed using GraphPad Prism software, USA 1996. Mann-Whitney *U* test and Person test were used as indicated in the legend of figure.

RESULTS

Heparin-ELISA Assay: Sensitivity, Specificity, and Validation

The assay is based on the heparin affinity for certain polypeptides. Heparin-BSA was adsorbed onto the wells of microtiter plates, samples containing the heparin-binding molecules were added, and the bound molecules were quantified using affinity-purified antibodies. As the sequence of HARP is more than 50% identical to that of MK, the specificities of the antibodies used were first analysed by Western blotting. As shown

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in Figure 1, anti-MK antibodies did not recognise HARP (panel A, lane 2) and anti-HARP antibodies did not recognise MK (panel B, lane 1). Similar results were obtained using a direct ELISA assay (not shown). We established the optimum assay conditions by determining the concentration of heparin-BSA that gave optimal ligand binding. Heparin-BSA complex (0.06–1 μg/mL) was added to each well of 96-well microtiter plates and allowed to stand overnight. Various concentrations of HARP were then added and bound HARP was then detected as described in Experimental (Figure 2).

Maximal binding occurred with 0.25 μg/mL heparin-BSA as coating complex. The maximal signal was obtained for 12 and 3.5 ng/mL HARP using 0.06 and 0.25 μg/mL coated heparin-BSA. Since optimal sensitivity was obtained using 0.25 μg/mL heparin-BSA, this concentration was used to produce standard curves for HARP (Figure 3A) and MK (Figure 3B). The coefficient of variation was less than 5% for both assays and the minimal detectable concentrations were 40 pg/mL HARP and 25 pg/mL MK. The method estimated concentrations 40 to 1200 pg/mL HARP and 25 to 1200 pg/mL MK and the correlation coefficients (r^2) were 0.99 for HARP and 0.94 for MK.

Various parameters of the heparin-ELISA assay were checked. As the HARP-heparin interaction is partly charge dependent, the ionic strengh of the binding buffer conditions that may affect the binding and, consequently,

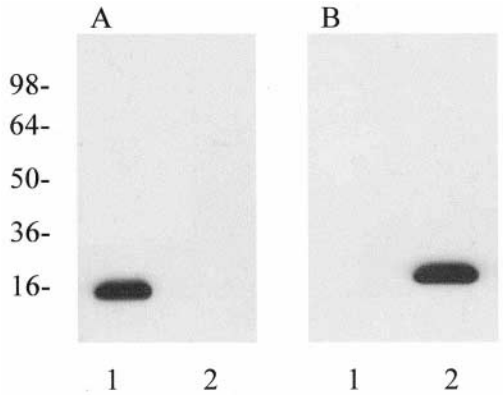

Figure 1. Validation of anti-MK and anti-HARP antibodies by Western blotting analysis. Human recombinant MK (100 ng; lane 1) and human recombinant HARP (100 ng; lane 2) were analysed by Western blotting experiment. Immuno-detection was performed using anti MK (A) or anti-HARP (B) antibodies as described in Experimental. Molecular weight markers are given in kilodaltons.

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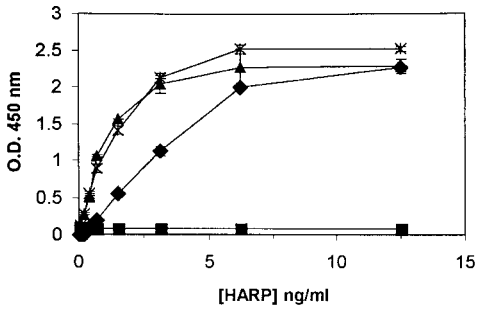

Figure 2. Effect of increased concentration of immobilised heparin-BSA on the binding of HARP. Various concentrations of HARP were incubated in coated well with 0 (■), 0.06 (◆), 0.25 (×), or 1 (▲) μg/mL of heparin-BSA complex. Each well was saturated with BSA and HARP was incubated at various concentration ranging from 12.5 to 0.01 ng/mL. After washes in PBS-T, bound HARP was then revealed using specific HARP immunoglobulins as described in Experimental.

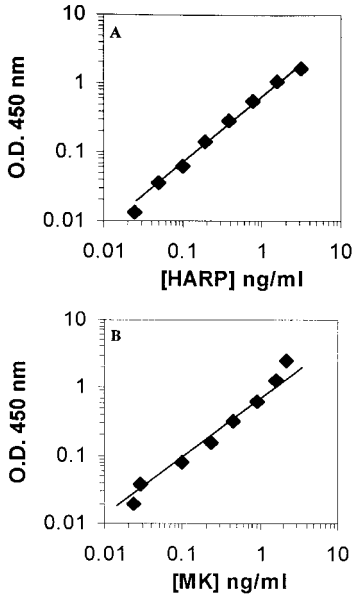

Figure 3. Standard curve for HARP (A) and for MK (B). Various concentration of HARP ranging from 12.5 to 1250 pg/mL were incubated in well precoated with 0.25 μg/mL of heparin-BSA complex. After three washes, bound HARP (A) or MK (B) were revealed using specific antibodies as described in Experimental. The coefficient of correlation (r^2) was 0.99 to 0.94 for HARP and MK, respectively.

the sensitivity of the assay, was investigated. The binding of HARP to heparin-BSA complex depended on the ionic strength. It was constant from 0.15 to 0.3 M, slightly but significantly increased between 0.3 and 0.38 M, and reduced above 0.38 M (Figure 4). Only 15% of the binding obtained under physiological conditions was detected in 0.52 M NaCl. We also studied the effects of molecules that bind avidly to heparin on the binding of HARP or MK to immobilised heparin. Incubation of various concentrations of MK, ranging from 0 to 1000 ng/mL with 1 ng/mL HARP, did not significantly modify the signal obtained using HARP alone (Figure 5A). As shown in Figure 5B, similar results were obtained using HARP as competitor for MK binding. A 1000-fold excess of competitor did not alter the signal obtained with the protein (HARP or MK) alone. The same result has been obtained using FGF-2 or $VEGF_{165}$ as competitor of the HARP binding (not shown).

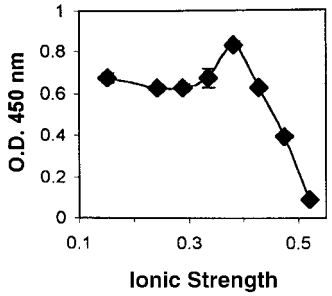

Figure 4. Effect of the ionic strength on the binding of HARP to heparin-BSA complex. The binding of 500 pg/mL HARP was investigated in various ionic strengths ranging from 0.15 to 0.5 M. After washes bound HARP was revealed as described in Experimental.

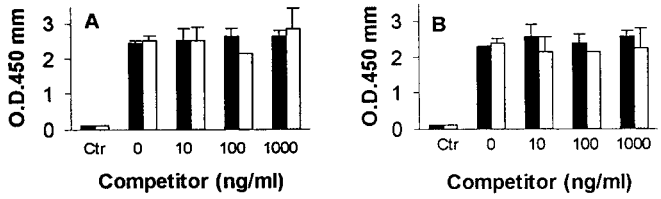

Figure 5. Specificity of the assay. The binding of 1 ng/mL HARP (A) or MK (B) to heparin-BSA complex were studied in presence (close bar) or in absence (open bar) of various concentrations of MK (A) or HARP (B) ranging from 0 to 1000 ng/mL. The binding was revealed using specific antibodies as described in Experimental.

We also checked the binding of HARP to heparin-BSA complex in the presence of GAGs or heparin (Figure 6). HARP was displaced from the immobilised heparin by heparin, dermatan sulphate, chondroitin sulphate A, and heparan sulphate. The IC_{50} for the HARP competition by soluble GAGs was 300 ng/mL and close to 100 μg/mL for heparin and dermatan sulphate, respectively. The IC_{50} of chondroitin sulphate A and heparan sulphate were up to 1 mg/mL. In contrast, chondroitin sulphate C was not a competitor.

Determination of HARP and MK in the Sera of Healthy Humans and Patients with Cancer

Although the background level decreased when serum was added, standard serum curves for HARP and MK measurement were routinely obtained (not shown) using sera that contained undetectable HARP or MK concentration and diluted 2-fold with PBS. In this condition, the minimum detectable concentrations were typically 45 pg/mL for HARP and 25 pg/mL for MK. The serum HARP and MK concentrations of

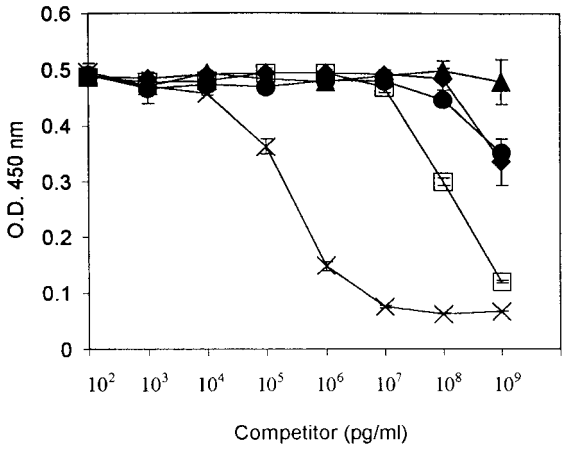

Figure 6. Effect of GAGs on the binding of HARP to heparin-BSA complex. Various concentrations of heparin (×), dermatan sulfate (□), chondroïtine sulfate A (◆), heparan sulfate (●), and chondroïtine sulfate C (▲), ranging from 10^2 to 10^9 ng/mL, were incubated in presence of 500 pg/mL of HARP in well precoated with 0.25 μg/mL of heparin-BSA complex. After washes, bound HARP was revealed as described in Experimental.

non selected healthy blood donors ($n = 19$) and patients with various neoplasic diseases ($n = 139$) were measured (Figure 7A and 7B). The mean HARP and MK concentrations in the control were 99.7 ± 57.3 pg/mL (95% confidence interval = 72–127 pg/mL) for HARP and 26.4 ± 4.86 pg/mL (95% confidence interval = 24–29.4 pg/mL) for MK.

When HARP or MK was not detectable, the detection limit for each molecule was entered for further analysis, i.e., 45 pg/mL for HARP and 25 pg/mL for MK. Patients with neoplasms had serum HARP concentrations of 45 to 1250 pg/mL (mean 169 pg/mL and median 45 pg/mL) and MK concentration of 25 to 1400 pg/mL (mean 181 pg/mL and median 98 pg/mL). The mean serum MK concentrations of the neoplastic and normal groups

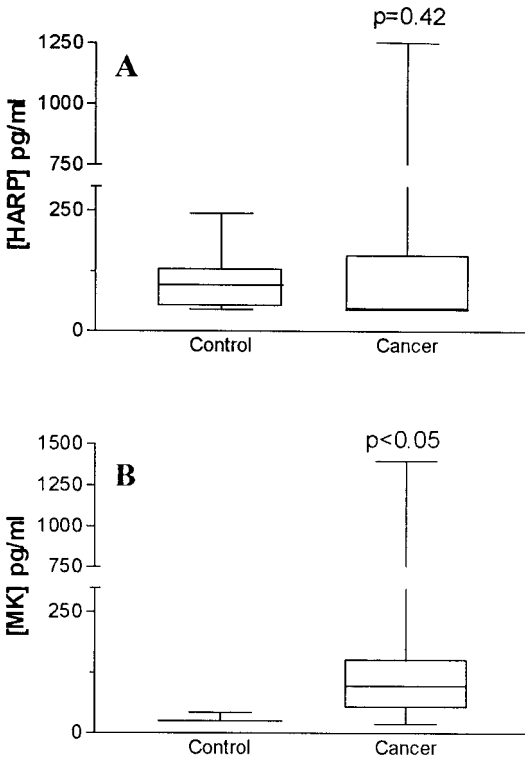

Figure 7. Level of HARP and MK in normal group (control) and carcinoma group (cancer). For the assay, sera (50 μL) were diluted with an equal volume of PBS. The box represents 50% of the population, the bar inside represents the median, and the error bars represent the extreme values. Statistical analysis were performed using Mann-Whitney *U* test.

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were significantly different ($p < 10^{-4}$), but the HARP concentrations were not ($p = 0.42$).

HARP and MK Produced by Cells in Culture

The synthesis of HARP and MK by various cells, including endothelial, epithelial, muscular, and fibroblasts was measured by heparin-ELISA assay (Table 1). HARP was undetectable in the medium of endothelial cells from bovine brain capillaries (BBC), bovine aorta (ABAE), and human umbilical (HUVEC and Eahy926). In contrast, HARP was detected in the conditioned medium of epithelial cells, with 141 ± 1.8 pg/mL per 10^6 cells in the medium of PNT-1A, 51.5 ± 1.8 pg/mL per 10^6 cells in that of MDA MB 231, 40.9 ± 0.8 pg/mL per 10^6 cells in that of PC3, 40.5 ± 0.4 pg/mL per 10^6 cells in the medium of CCL64 cells.

Table 1. Presence of HARP and MK in Supernatants of Various Cells

Cell Type	Current Name	HARP Concentration pg/10^6 Cells	MK Concentration pg/10^6 Cells
Endothelial	BBC	ND	–
	ABAE	ND	–
	Eahy960	ND	590 ± 0.4
	HUVEC	ND	219 ± 1.9
Epithelials	PNT-1A	141 ± 1.8	28.7 ± 0.4
	MDA MB 231	51.5 ± 1.8	3.5 ± 0.4
	PC 3	40.9 ± 0.8	152 ± 7.3
	CCL64	40.5 ± 0.4	–
	LN CaP	12.9 ± 0.2	9.8 ± 0.3
	BEL	6.75 ± 0.3	9.4 ± 0.9
	DU 145	0.86 ± 0.4	11.8 ± 1
	SW13	ND	3.1 ± 0.4
	CHO-K1	ND	–
Muscular	C2C7	472 ± 13.8	–
	HISM	9580 ± 490	313 ± 4.5
	VSML	21.1 ± 0.22	–
Fibroblast	COS	35.5 ± 2.2	–
	NIH 3T3	130 ± 6	–
	Balb C 3T3	64 ± 0.01	–

Cells were grown for 96 h as described in Experimental and counted at the end of the experiments. The data were expressed in pg/mL per 106 cells. ND: not detected, (–): not determined.

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The concentrations of HARP were low or undetectable in the medium of the other epithelial cells investigated. The highest medium concentration of HARP are 9580 ± 490 pg/mL per 10^6 cells for human intestinal smooth muscle cells (HISM) and 472 ± 13.8 pg/mL per 10^6 cells for mouse skeletal muscle-derived cells (C2C7). The HARP concentrations in the media of fibroblast-derived cells were 130 ± 6–35.5 ± 2.2 pg/mL per 10^6 cells. MK appeared to be a ubiquitous molecule, present at various concentrations in the media of all the cells investigated. The higher concentrations of MK were in the media of human intestinal smooth muscle cells and Eahy926 cells.

DISCUSSION

The sequences of HARP and MK are approximately 50% identical. Both proteins have a strong affinity for heparin, promote neurite outgrowth, and have mitogenic activity.(4,9) According to these structural and biological data, HARP and MK constitute a new family of heparin-binding proteins that cause both cell transformation and neovascularization.(23) We developed a sensitive, reproducible, rapid, and simple assay that uses commercially available materials as a tool for studying HARP and MK and their physiological functions and involvement in various disorders.

This assay is based on the high affinity of these molecules for heparin, and uses a heparin-BSA complex to immobilise heparin binding molecules. This heparin-ELISA assay is at least as sensitive as two other assays, one for MK (24) and the other for HARP. The minimal detectable concentrations are 25 pg/mL for MK and 40 pg/mL for HARP and these two growth factors can be detected in the culture medium of various cells and biological fluids such as serum as presented in this report.

As the specificity of this assay is obtained by the antibody used, the procedure described in this report can be extended to assay other heparin binding molecules. We have successfully used this procedure to assay FGF-2 and $VEGF_{165}$ and have detected these molecules in biological fluids with a detection limit of 25 pg/mL for FGF-2 and 50 pg/mL for $VEGF_{165}$ (not shown). We intend to extend this assay to other heparin-binding molecules.

Validation of this assay was first investigated. In a preliminary experiment, we studied the effect of human serum on the HARP or MK determination. As previously observed for MK (24), addition of serum in the assay caused a high decrease of the signal. Thus, we used normal serum fractionnated by heparin-Sepharose chromatography and we have not detected, in the 2M NaCl eluted fraction, any component that caused significant

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reduction in the binding of HARP to the immobilized heparin (not shown). It was, therefore, concluded that the reduction of the signal observed in presence of serum did not result in the matrix effect induced by a component present in the serum, but was due to a decrease in the non-specific binding of reagents by saturating the plate with serum proteins.

As reported in this study, molecules that interact with heparin-binding protein, such as heparin and GAGs, interfere in this assay. GAGs are extracellular components mainly associated with the cell surface or with the extracellular matrix. Little is known about the difference between the physiological and pathophysiological concentrations of GAGs in serum. In patients with solid tumors, reports were mainly focused on serum hyaluronan determinations which have shown elevated concentrations in mesothelioma and Wilms' tumors.(25) As previously reported, human plasma from blood donors contained 1 to 1.5 μg/mL GAGs, whereas 6 to 14 μg/mL were found in plasmas of patients with chronic myeloid leukemia, as well as chronic lymphocytic leukaemia.(26) Even if we assumed that human serum contained four times the amount of GAGs compared to plasma, as reported by Bjornsson (27), we have not observed, at these concentration levels, significant interference of GAGs in our assay; this allowed us to use this method to assay HARP or MK in biological fluids. Using this assay, we confirm that serum MK concentration in patients with cancer were significantly higher than those of normal serums.(24)

In summary, the assay described in this report is easily and rapidly performed, uses only commercially available materials, and could be extended to assay other heparin binding molecule.

ABBREVIATIONS

MK, Midkine; HARP, Heparin Affin Regulatory Peptide; GAGs, Glycoaminoglycans; CAPS, (3–[cyclohexylamino]-1-propanesulfonic acid); FGF-2, Fibroblast Growth Factor 2; $VEGF_{165}$, Vascular Endothelial Growth Factor 165; PDGF, Platele Endothelial Growth Factor

ACKNOWLEDGMENTS

This work was supported by grants from Association pour la Recherche sur le Cancer (J. Courty, N°5257) and Ministère de l'Education Nationale et de la Recherche (DRED/CNRS). M.H. is a recipient of a grant from Association pour la Polyarthrite Rhumatoide.

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Received March 1, 2001
Accepted April 20, 2001
Manuscript 3037

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