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Hemodialysis stimulates hepatocyte growth factor release

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Hemodialysis stimulates hepatocyte growth factor release. Studies were performed in 26 patients on regular dialysis treatment with cuprophane (CU), polymethylmetacrilate (PMMA) or cuprammonium (CAM) dialyzers. Controls were six patients with chronic renal failure but not on regular dialysis treatment (CRF) and six healthy subjects (N). Blood was collected at the start (T_0), and at 15 (T_{15}) and 240 (T_{240}) minutes of dialysis to measure the serum hepatocyte growth factor (HGF) concentration and to study HGF production by peripheral blood mononuclear cells (PBMC) *in vitro*. The form of HGF (that is, inactive/monomeric, active/dimeric) present in the serum was analyzed by immunoblotting. In addition, the ability of serum to stimulate proliferation of tubular cells (HK-2) and HGF release by PBMC and fibroblasts (MRC-5) was investigated. At T_0, serum HGF levels were identical to that of the controls. In patients treated with CU, serum HGF rose from 0.24 ng/ml at T_0 to 7.44 ng/ml at T_{15}, and remained high at T_{240}. PBMC collected at T_{15} and T_{240} released significantly more HGF *in vitro* than those collected at T_0. Serum at T_{15} stimulated proliferation of HK-2 cells and the release of HGF by PBMC and MRC-5 cells. The PMMA and CAM dialyzers had similar effects as the CU. These results indicate that dialysis induces a striking rise in serum HGF and a prompt circulation of factor(s) stimulating HGF release. Dialysis-activated PBMC release HGF.

Hepatocyte growth factor (HGF) was originally identified in the serum of partially hepatectomized rats, and was characterized as a potent stimulator of DNA synthesis in cultured hepatocytes [1]. Subsequent studies have shown that HGF has a mitogenic action on several other epithelial cells, including renal tubular cells [2], and that HGF is a pleiotropic factor that induces the scattering of cell colonies, regulates the synthesis of acute phase proteins, plays a role in embryonic development and tumor propagation, and has morphogenic, that is, angiogenic and tubulogenic, properties [3–7]. Hepatocyte growth factor is produced in various organs by cells of mesenchymal origin, such as Kupffer's cells, lung fibroblasts, and mesangial cells [1, 8]. Experimental studies have shown that tissue injury (for example, in the liver or the kidney) is associated with a marked increase in HGF expression not only in the injured tissue, but also in distant organs, such as lung and spleen [9]. The humoral factors signaling tissue injury to HGF producing cells are ill-defined substances named "injurins" [10].

Key words: biocompatibility, dialyzers, fibroblasts, peripheral blood mononuclear cells, injurins, acute renal failure, hepatitis.

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Hepatocyte growth factor normally circulates as an inactive, single-chain protein, and is converted to a mature, heterodimeric, active form by a serine-protease homologous to Factor XII, which in turn derives from an inactive precursor activated by thrombin. Hepatocyte growth factor has a high degree of structural homology with plasminogen, and urokinase type plasminogen activator can also convert HGF into its active form [11–13].

Extracorporeal circulation in hemodialysis is associated with leukocyte activation and the release in blood of cytokines [14]. Activated peripheral blood leukocytes are a possible source of HGF, because HGF is produced by leukocytes in diseased liver tissue [1]; cytokines may act as injurins [15]. Thus, dialysis may induce the release of HGF either from activated peripheral blood leukocytes or from tissue cells stimulated by cytokines. In addition, dialysis may cause the release of coagulation products that convert HGF into its active form. Increased HGF activity induced by dialysis may be pathophysiologically relevant in patients with acute renal failure [16], or in patients on regular dialysis treatment who have viral hepatitis. Thus, in the current study we investigated whether hemodialysis stimulates HGF release, and whether leukocytes are a source of dialysis-induced HGF.

METHODS

Patients

The study was performed in 26 patients on regular dialysis treatment (RDT). Six patients with chronic renal failure on conservative treatment (CRF) and six healthy subjects (N) were used as controls. All subjects were recruited after clinical, serological and echographic exclusion of liver disease. Patients on RDT underwent four-hour hemodialysis sessions thrice weekly with cuprophane (CU; $N = 11$), polymethylmetacrilate (PMMA; $N = 9$), or cuprammonium (CAM; $N = 6$) dialyzers. Since heparin may cause the shedding of HGF into the circulation [17], six patients were dialyzed with CU without using heparin, that is, extracorporeal blood coagulation was prevented by washing the dialyzer with saline at regular intervals.

Experimental design

Blood was collected from controls after overnight fasting, and from patients on RDT just before starting a dialysis session (T_0), at 15 minutes of dialysis (T_{15}), and at 240 minutes of dialysis (T_{240}). Part of blood was immediately centrifuged and the serum was stored at −80°C until processed for HGF measurement or used for conditioning cell cultures; the other part was used to isolate peripheral blood mononuclear cells (PBMC). The release

of HGF from dialysis-activated PBMC was measured after a 48-hour incubation with 1% fetal calf serum. The ability of serum of dialysis patients to stimulate HGF release was investigated by conditioning PBMC of normal volunteers and human lung fibroblasts (MRC-5; ATCC, Rockville, MD, USA) with the serum of patients (5% in PBMC studies, 10% in MRC-5 studies) for 48 hours, and measuring HGF in the supernatant.

Hepatocyte growth factor measurement

Hepatocyte growth factor was measured in serum and in cell culture supernatant by a commercial EIA (R&D Systems, Minneapolis, MN, USA). The immunoassay uses recombinant human HGF expressed in Sf 21 cells that produce a mixture of monomeric and dimeric (that is, inactive and active) HGF, and a monoclonal antibody specific for HGF that binds with no apparent preference both the monomeric and the dimeric forms. Therefore, the immunoassay measures the sum of active and inactive HGF.

Hepatocyte growth factor characterization

Hepatocyte growth factor is released as an inactive monomer of 92 kDa that is transformed in an active heterodimer composed of a 69 kDa α-subunit and a 32 to 34 kDa β-subunit. The form of HGF (that is, monomeric/inactive or dimeric/active) present in blood was characterized by affinity purification and Western blotting. Since coagulation products may activate HGF, experiments were performed both on serum and plasma obtained by addition of EDTA. The samples were repeatedly centrifuged in order to eliminate all traces of clotted material. Serum and plasma samples (about 5 ml each) were incubated for 16 hours at 4°C with 50 μl of immobilized heparin (Pierce), in the presence of 350 mM NaCl added in order to inhibit aspecific binding. Beads were then precipitated by centrifugation and, after three washes with PBS (with 350 mM NaCl added), the protein samples were subjected to SDS denaturation (5 min at 95°C in Laemmli buffer) and eventually separated by SDS-PAGE electrophoresis under reducing conditions. Proteins were then transferred to nitrocellulose by Western blotting using a semidry apparatus (Millipore). According to standard protocols, filters were subsequently blocked with 10% BSA-TBS and incubated with the HGF beta-chain specific polyclonal antiserum H04, generously provided by Dr. A. Galvani (Pharmacia-Upjohn, Milan, Italy). After incubation with HRP-conjugated Protein-A (Amersham) and an appropriate number of washes, chemioluminescent detection with ECL-Plus reagents (Amersham) was eventually performed.

Serum biological activity

The ability of serum to induce the proliferation of HGF-sensitive cells was tested using a proximal tubular cell line, derived from normal human kidney, transfected with HPV-16 (HK-2 cells; ATCC). Cells were grown in keratinocyte-serum free medium (Gibco BRL, Basel, Switzerland) supplemented with recombinant EGF (5 ng/ml; Gibco BRL) and bovine pituitary extract (40 μg/ml; Gibco BRL). Cells were plated on 96-well plates (Costar, Bodenheim, Germany) at a density of 10^4 cells/well and after 24-hour incubation basal cell number/well was counted in a NewBauer chamber; cells were then conditioned with medium containing recombinant human HGF (3 and 10 ng/ml; Sigma), or 10% serum (control serum from normal volunteers and serum sampled during hemodialysis at T_0 and T_{15}). Cell number/well was

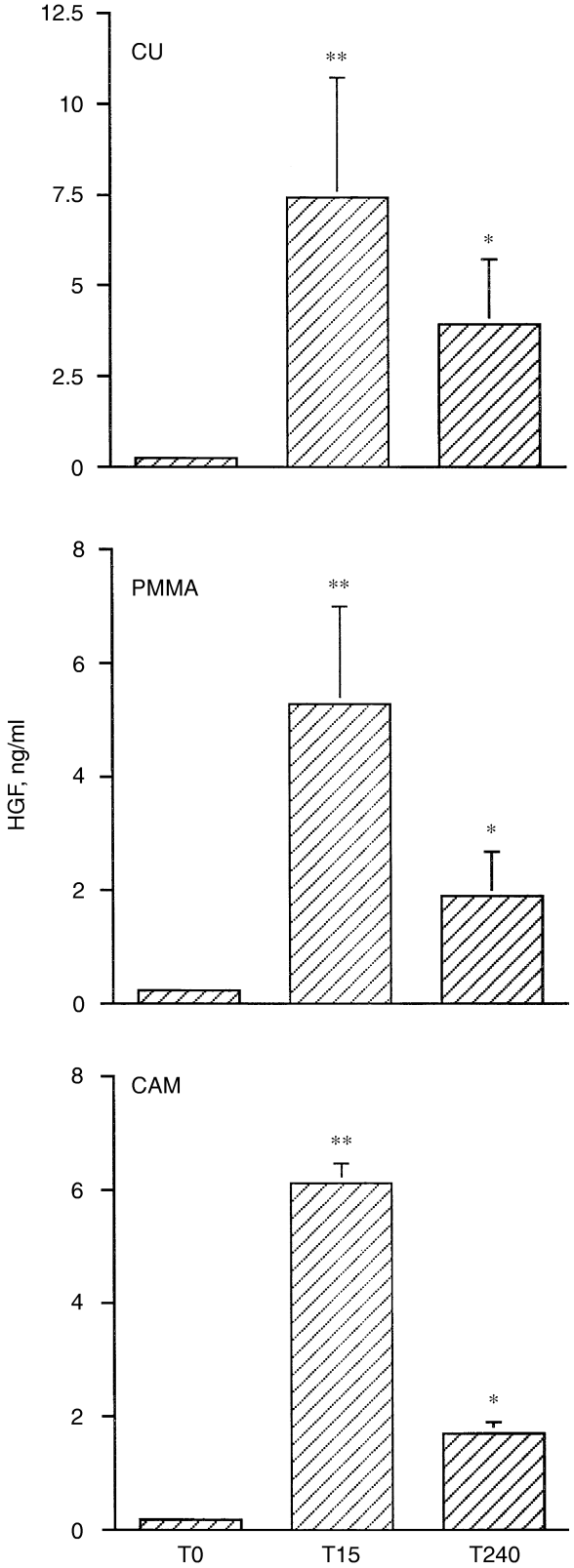

Fig. 1. Effects of hemodialysis on serum hepatocyte growth factor (HGF) concentration. Columns are means, bars are SD. Abbreviations are: CU, cuprophane dialyzer; PMMA, polymethylmetacrilate dialyzer; CAM, cuprammonium dialyzer. T_0, T_{15} and T_{240} indicate timing of blood samples, just before starting dialysis, at 15 minutes of dialysis, and at 240 minutes of dialysis, respectively. $^{**}P < 0.001$ and $^{*}P < 0.01$ versus T_0.

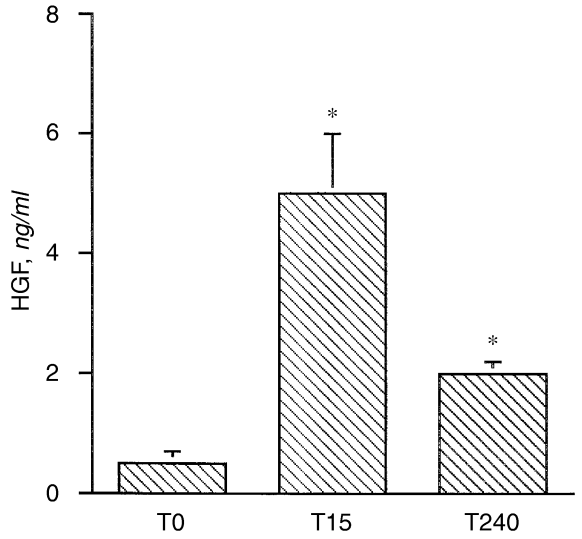

Fig. 2. Effect of no-heparin dialysis on serum concentration of hepatocyte growth factor (HGF). Columns are means, and bars are SD. Abbreviations are in the Figure 1 legend. $^*P < 0.01$ versus T_0.

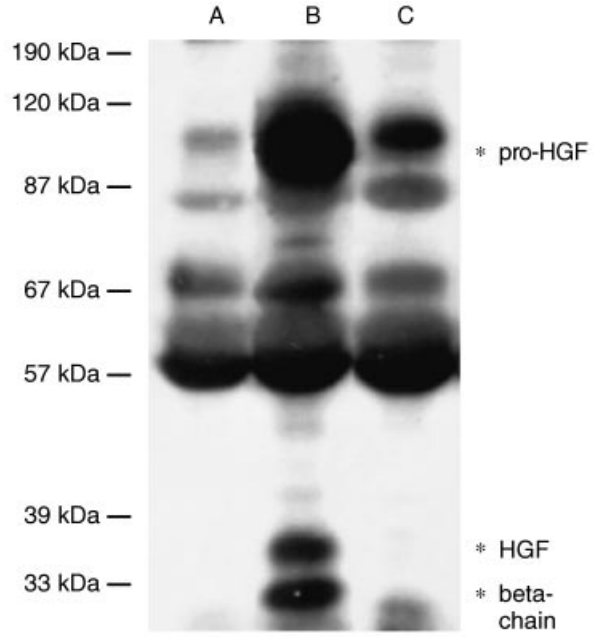

Fig. 3. Western blot analysis of hepatocyte growth factor (HGF). Lane A indicates the basal serum level of HGF; lane B shows the serum level of HGF after 15 minutes of dialytic treatment; lane C shows serum level of HGF after 240 minutes of dialytic treatment.

counted again after 24 and 48 hours. Cell viability (Trypan Blue test) was > 99%.

The ability of serum to induce HGF release was tested on PBMC (see PBMC preparation below) and fibroblasts. Human lung fibroblasts (MRC-5; ATCC) were grown in Eagle's minimum essential medium with 2 mM l-glutamine and Earle's BSS (ATCC) with 1.5 g/liter $NaHCO_3$, 0.1 mM nonessential amino acids and 1 mM sodium pyruvate, supplemented with 10% FCS (ATCC). At passage 19, cells were seeded on 24-well plates (Costar) at a density of 10^5 cells/well and incubated with 10% FCS, or 10% human serum (from normal volunteers and hemodialysis patients). Supernatant was collected for HGF measurement after 48 hours of incubation.

Peripheral blood mononuclear cell preparation and culture

Platelets are a potential source of HGF [1], and we showed in preliminary experiments that platelets contaminate PBMC obtained by gradient centrifugation (120,000 to 160,000 platelets/ml in a final suspension of $2 \cdot 10^6$ PBMC/ml). Therefore, we tested whether platelets, cultured in the same conditions as PBMC, release HGF. Blood (16 ml) was mixed with an ACD anticoagulant solution (4 ml; Baxter) and centrifuged for 10 minutes at 1,200 rpm. The platelet-rich supernatant was washed twice with PBS + EDTA (0.3 mM/liter) and centrifuged for 15 minutes at 1,400 rpm. Platelets, separated in the final pellet, were resuspended in Iscove's medium (800,000 platelets/ml) with 1% fetal calf serum (FCS) or the serum of patients (5%) for 48 hours, and HGF was measured in the supernatant.

We have described methods of PBMC culture in detail previously [14]. Briefly, PBMC were separated by standard Ficoll-Hypaque gradient centrifugation and cultured in suspension ($2 \cdot 10^6$/ml) for 48 hours in Iscove's (Flow Laboratories) containing 100 IU/ml penicillin and 100 μg/ml streptomycin, and 1% decomplemented FCS or 5% serum of patients and controls. Flow cytometry of PBMC yielded 80 to 83% lymphocytes, 11 to 12% monocytes, and 4 to 6% neutrophils. Cell viability was tested by the Trypan Blue exclusion test and yielded 96 to 98% viable cells.

Statistics

Analysis of variance by ANOVA and Newman-Keuls test were used for comparison of the means.

RESULTS

Effects of dialysis on serum hepatocyte growth factor

At T_0, HGF averaged 0.24 ± 0.19 SD ng/ml and was not significantly different from the mean value in patients with CRF (0.19 ± 0.08 SD ng/ml) and normal volunteers (0.15 ± 0.10 SD ng/ml). The effects of dialysis on serum concentration of HGF are shown in Figure 1. In patients treated with Cuprophane dialyzers, HGF rose strikingly to 7.44 ng/ml at T_{15} and remained 15 times higher than T_0 at T_{240}. In patients treated with PMMA and CAM HGF levels increased similarly to the patients treated with CU. A marked rise in HGF occurred independently of the use of heparin (Fig. 2); however, the peak serum HGF concentration was significantly higher ($P < 0.05$) in the standard dialysis versus the no-heparin dialysis.

Characterization of hepatocyte growth factor

Figure 3 shows Western blot analysis performed on serum. Results obtained on plasma were overlapping. Before starting dialysis (lane A) a weak band of pro-HGF (92 kDa) is detectable and no β-chain is visible. At T_{15} (lane B) pro-HGF is markedly increased and two intense bands of β-chain (32 and 34 kDa; the two forms are differently glycosilated) are clearly evident. Note the greater amount of the 32 kDa form. At T_{240} (lane C) serum level of pro-HGF is still higher than at T_0, and a weak signal allows detection of only the lower band of the β-chain.

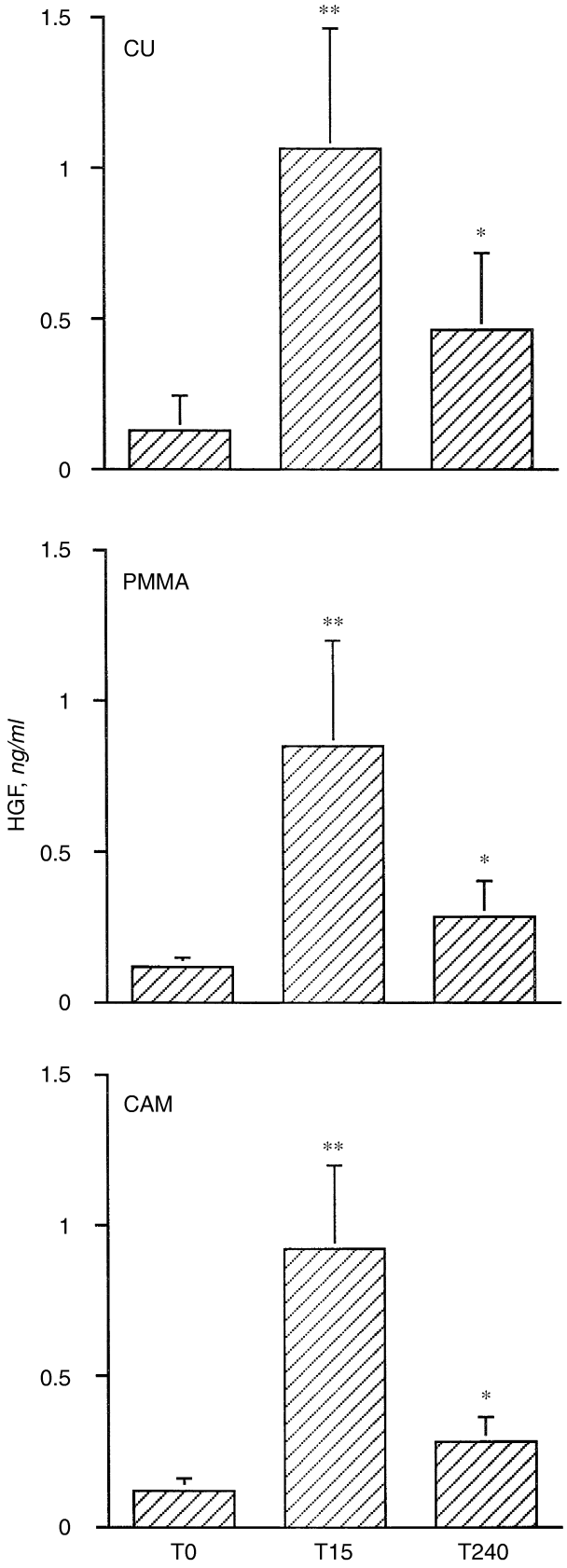

Fig. 4. Hepatocyte growth factor (HGF) release from peripheral blood mononuclear cells (PBMC) of dialysis patients. Columns are means, and bars are SD. Abbreviations are in the legend to Figure 1. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus T_0.

Effects of dialysis on peripheral blood mononuclear cells

Hepatocyte growth factor in the supernatant of PBMC isolated from dialysis patients at T_0 averaged 0.11 ± 0.11 SD ng/ml, a value that was not significantly different from HGF released by PBMC isolated from normal volunteers (0.07 ± 0.06 SD ng/ml, $P > 0.05$). However, as shown in Figure 4, dialysis with all of the tested membranes strongly stimulated PBMC to release HGF. In fact, the HGF concentration in supernatant of PBMC collected at T_{15} and T_{240} was much higher than in the supernatant of PBMC collected at T_0. Hepatocyte growth factor was not released by platelets contaminating PBMC, because the result of EIA performed on supernatant of platelets cultured in the same conditions as PBMC was not different from zero, that is, from that performed on blank culture medium.

Tubular cell proliferation assay

As shown in Figure 5, the addition of recombinant HGF to culture medium of HK-2 cells caused a dose- and time-dependent increase in cell number. An addition of T_{15} serum caused a rise in cell number that was significantly higher than the increase caused by normal serum and T_0 serum; the difference was significant both at 24 and at 48 hours. Note that the HGF concentration in T_{15} serum was 7 ng/ml and that the rise in cell number occurring with this serum was half way between that occurring with 3 and 10 ng/ml of recombinant HGF.

Effects of serum on hepatocyte growth factor release by peripheral blood mononuclear cells and fibroblasts

The release of HGF from PBMC of normal volunteers cultured with serum of normal volunteers averaged 0.06 ± 0.03 SD ng/ml and did not differ from the amount of HGF produced by PBMC cultured with the serum of dialysis patients collected at T_0 (0.11 ± 0.09 SD ng/ml). In contrast, serum collected at T_{15} and T_{240} caused a HGF release. In fact, after subtracting the HGF that was in the serum, the HGF supernatant concentrations were significantly higher in cultures treated with T_{15} and T_{240} serum than in cultures treated with T_0 serum. The phenomenon occurred independently of the type of membrane used for dialysis (Fig. 6).

The effects of serum on fibroblasts are shown in Figure 7. The HGF concentrations in the supernatant of cells conditioned with normal serum and serum collected at T_0 were similar, while it was significantly increased in the supernatant of cells treated with serum collected at T_{15}.

DISCUSSION

The present study shows that hemodialysis causes a prompt and prolonged release of HGF into the circulation, raising HGF serum concentration up to 30 times. The release of HGF takes place even if dialysis is performed without heparin, so that it does not depend on heparin-induced shedding of receptor-bound HGF from peripheral tissues.

Hepatocyte growth factor normally circulates as a pro-HGF that is converted into an active form by zymogens homologous to coagulation factors. Usually this conversion occurs in injured tissues, where the zymogens that transform pro-HGF are in turn activated from inactive precursors. As shown by our Western blot analysis, pro-HGF released during dialysis is rapidly and largely activated. In fact, 15 minutes after starting treatment a large amount of both monomeric and dimeric HGF is present in serum, and the presence of dimeric HGF is associated with a mitogenic

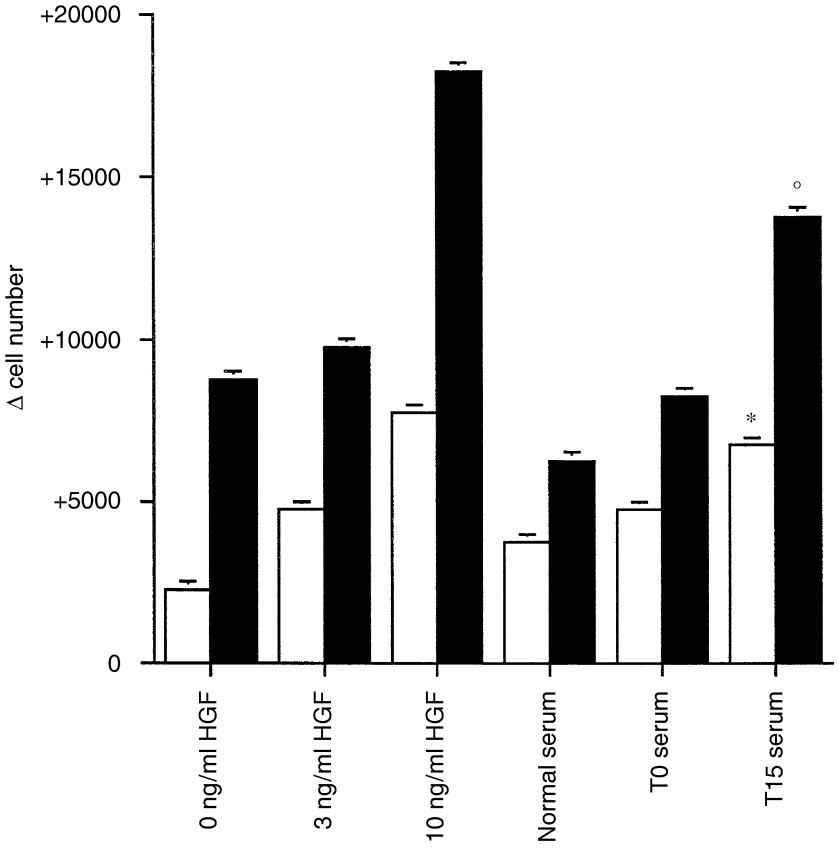

Fig. 5. Effects of recombinant hepatocyte growth factor (HGF) and of serum on proliferation of proximal tubular cells HK-2. Columns represent the average change in cell number/well at (□) 24 hours and (■) 48 hours, and bars are SD. $^{*}P < 0.001$ versus normal serum and T_0 serum at 24-hours of incubation. $^{\circ}P < 0.001$ versus normal serum and T_0 serum at 24- and 48-hours of incubation.

activity of serum on renal tubular cells *in vitro*. At the end of dialysis the amount of pro-HGF is still much greater than in basal conditions, while active HGF is barely detectable. Our study does not clarify whether this decay of active HGF is due to receptor binding and deposition in peripheral tissues, or to reduced serum zymogen activity. However, the increased availability of circulating HGF, even in its inactive form, is biologically relevant since HGF may be activated in injured sites, for example, in the kidney in patients with acute tubular necrosis, or in the liver in patients with hepatitis.

Serum HGF peaks early after the start of extracorporeal circulation, in parallel with dialysis-induced leukocyte activation [18], suggesting that leukocytes participate in releasing HGF. Until now, the ability of circulating leukocytes to produce HGF has not been proven, but HGF is expressed in leukocytes infiltrating inflamed liver, and cells with monocyte/macrophage phenotype produce HGF in culture [19]. These observations prompted us to study the production of HGF by PBMC activated by dialysis. Our *in vitro* experiments show that PBMC collected during dialysis release much more HGF than PBMC collected before starting dialysis, indicating that: (*a*) PBMC are actually able to produce HGF and (*b*) dialysis stimulates HGF production by PBMC. In theory, platelets contaminating PBMC might be the source of HGF measured in PBMC culture supernatant [20]. However, we have cultured highly enriched platelets under the same conditions as PBMC have shown that they do not produce HGF. Therefore, our findings definitely demonstrate that dialysis induces HGF production from PBMC. PBMC stimulation occurs promptly, that is, within 15 minutes of dialysis, and persists throughout the dialysis session.

Experimental models of acute tissue damage have shown that HGF production is induced by substances named "injurins," which are released from injured organs into the circulation. Injurins released from an injured site induce HGF production in distant, undamaged organs (liver, kidney, spleen), which act to accelerate tissue repair [9]. Therefore, we investigated whether injurin-like factors account for HGF production during dialysis. Having shown that PBMC taken during dialysis produce HGF, we used PBMC collected from normal volunteers to test injurin-like activity in the serum of dialysis patients. Our *in vitro* studies clearly indicate that a PBMC stimulating factor appears in serum after 15 minutes of dialysis and is still present at the end of treatment. In fact, serum collected at 15 and 240 minutes of dialysis is effective in inducing HGF production from PBMC, while serum collected before dialysis and from the serum of normal volunteers are not effective. In addition, we tested the ability of serum collected during dialysis to stimulate the release of HGF from fibroblasts, that is, cells normally present in solid organs. Our observation that serum sampled at 15 minutes of dialysis causes HGF production by fibroblasts suggests that, in addition to PBMC, internal organs stimulated by a circulating factor(s) produce HGF during dialysis.

We have not investigated the nature of the factors circulating during dialysis that cause the HGF release. A specific injurin with molecular mass of 10 to 20 kDa has been purified in rat serum [10], but injurins are a heterogeneous and ill-defined class of

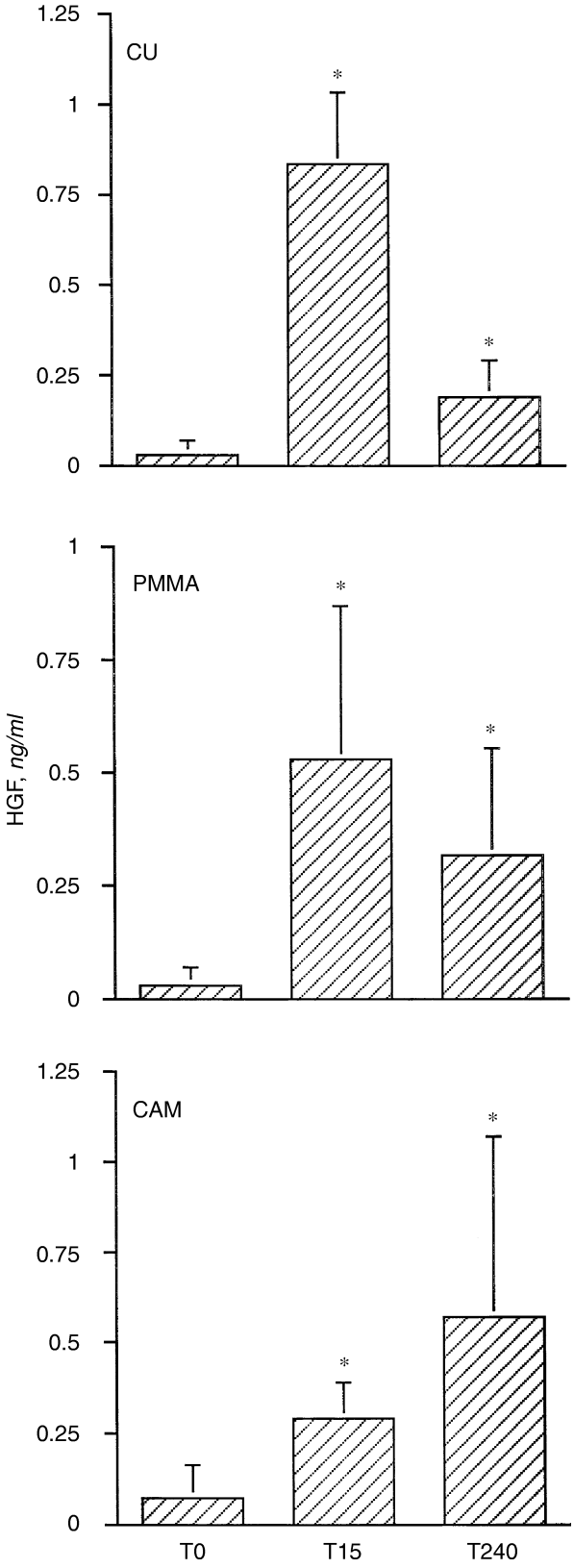

Fig. 6. Hepatocyte growth factor (HGF) release from peripheral blood mononuclear cells (PBMC) of normal volunteers conditioned with serum of dialysis patients. Columns are the average concentration of HGF in supernatant after subtraction of HGF added with serum. Bars are SD. Abbreviations are in the legend to Figure 1. $^*P < 0.01$ versus T_0.

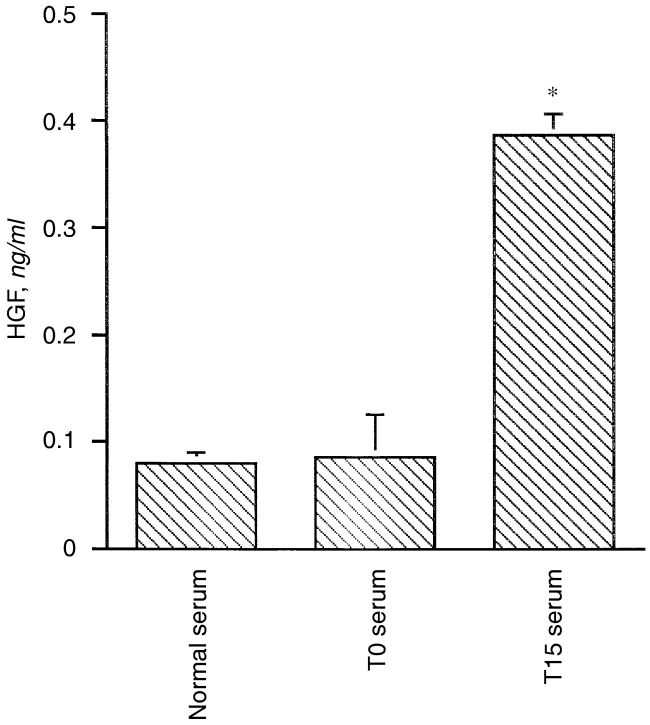

Fig. 7. Effects of serum on hepatocyte growth factor (HGF) release by fibroblasts in culture. Columns represent the average concentration of HGF in supernatant after subtraction of HGF added with serum. Bars are SD. $^*P < 0.001$ versus normal serum and T_0 serum.

substances, possibly different in distinct pathophysiological conditions. Since cytokines like interleukin-1 (IL-1) and tumor necrosis factor (TNF) have been shown to induce HGF production, and hemodialysis causes the release of these and other cytokines from activated leukocytes [14, 21], we suggest that cytokines may act as injurins in the serum of dialysis patients. Should cytokines released by PBMC behave as injurins, during dialysis leukocytes would play a double role as both direct producers of HGF and inducers of its production in tissues. In conclusion, we have shown that hemodialysis causes the release of factors that stimulate HGF production from PBMC, and possibly solid organs. PBMC activated by dialysis are a possible source of these factors (cytokines), but our study findings do not permit any conclusions about their origin and exact nature, nor information on the mechanisms that cause their release. Interestingly, activation of the HGF-releasing factor(s)/HGF system is not peculiar to cellulosic membranes. It also occurs with so-called biocompatible membranes, which implies that it does not depend on phenomena causing dialysis-associated leukopenia, that is, complement activation and increased expression of leukocyte adhesion molecules [22].

The increased HGF production caused by dialysis deserves consideration for several reasons. First, HGF is a pleiotropic substance that causes the proliferation of several cell types including fibroblasts, scattering of cells, angiogenesis, arrangement of cells in complex structures (for example, tubular), and participates in inflammatory phenomena. Although many of HGF's actions concur to tissue regeneration and repair, adverse effects of continued HGF stimulation are possible, for example, in

the mobilization and proliferation of tumoral cells or induction of exaggerated fibrosis in inflamed tissues. Thus, the production of HGF should be taken into account in evaluating the biocompatibility of dialysis. Second, hemodialyis is often required to treat patients with toxic-ischemic acute renal failure (ARF), and experimental and clinical observations indicate that HGF plays an important role in recovery from this disease [19, 23]. Thus, dialysis techniques or schedules may be tailored to sustain HGF production in patients with ARF. Third, experimental and clinical studies suggest that HGF is a protective factor in patients with viral hepatitis [24]. In view of the high prevalence of viral hepatic infections in dialysis patients, the regular attainment of "pharmacological" serum HGF levels in these patients is a model to study the effect on hepatitis of HGF and to test its therapeutic efficacy. Finally, hemodialysis represents a repetitive and reproducible condition of activation of HGF and may be a useful model to further investigate its pathophysiology.

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APPENDIX

Abbreviations used in this article are: ARF, acute renal failure; CAM, cuprammonium; CRF, patients with chronic renal failure who are not on regular dialysis treatment; CU, cuprophane; FCS, fetal calf serum; HGF, hepatocyte growth factor; HK-2 cells, proximal tubular cell line; MRC-5, human lung fibroblasts; N, healthy subjects; PBMC, peripheral blood mononuclear cells; PMMA, polymethylmetacrilate; RDT, regular dialysis treatment.

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