A translational approach to micro-inflammation in end-stage renal disease: molecular effects of low levels of interleukin-6

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ABSTRACT

Inflammation plays a key role in the progression of cardiovascular disease, the leading cause of mortality in ESRD (end-stage renal disease). Over recent years, inflammation has been greatly reduced with treatment, but mortality remains high. The aim of the present study was to assess whether low (<2 pg/ml) circulating levels of IL-6 (interleukin-6) are necessary and sufficient to activate the transcription factor STAT3 (signal transducer and activator of transcription 3) in human hepatocytes, and if this micro-inflammatory state was associated with changes in gene expression of some acute-phase proteins involved in cardiovascular mortality in ESRD. Human hepatocytes were treated for 24 h in the presence and absence of serum fractions from ESRD patients and healthy subjects with different concentrations of IL-6. The specific role of the cytokine was also evaluated by cell experiments with serum containing blocked IL-6. Furthermore, a comparison of the effects of IL-6 from patient serum and rIL-6 (recombinant IL-6) at increasing concentrations was performed. Confocal microscopy and Western blotting demonstrated that STAT3 activation was associated with IL-6 cell-membrane-bound receptor overexpression only in hepatocytes cultured with 1.8 pg/ml serum IL-6. A linear activation of STAT3 and IL-6 receptor expression was also observed after incubation with rIL-6. Treatment of hepatocytes with 1.8 pg/ml serum IL-6 was also associated with a 31.6-fold up-regulation of hepcidin gene expression and a 8.9-fold down-regulation of fetuin-A gene expression. In conclusion, these results demonstrated that low (<2 pg/ml) circulating levels of IL-6, as present in non-inflamed ESRD patients, are sufficient to activate some inflammatory pathways and can differentially regulate hepcidin and fetuin-A gene expression.

Key words: end-stage renal disease (ESRD), fetuin-A, hepcidin, interleukin-6 (IL-6), micro-inflammation, signal transducer and activator of transcription 3 (STAT3).

Abbreviations: C/EBP, CCAAT/enhancer-binding protein; CRP, C-reactive protein; CVD, cardiovascular disease; DAPI, 4′,6-diamidino-2-phenylindole; ESRD, end-stage renal disease; gp130, glycoprotein 130; gp80, glycoprotein 80; IL, interleukin; IL-6R, IL-6 receptor; JAK, Janus kinase; LVH, left ventricular hypertrophy; NF-κB, nuclear factor κB; rIL-6, recombinant IL-6; RRT, renal replacement therapy; RT-PCR, real-time PCR; sgp130, soluble gp130; sIL-6R, soluble IL-6R; STAT, signal transducer and activator of transcription; p-STAT3, phospho-STAT3; TNF-α, tumour necrosis factor-α.

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INTRODUCTION

CVD (cardiovascular disease) is the leading cause of morbidity and mortality in ESRD (end-stage renal disease): approx. 50% of ESRD patients die from CVD, and cardiovascular mortality is 15–30 times higher than in the age-adjusted general population [1].

In ESRD, in addition to traditional Framingham risk factors, a considerable number of non-classical factors are known to play a role in the CVD progression, such as inflammation [2], vascular calcification [3] and LVH (left ventricular hypertrophy) [4]. The presence of inflammation, mainly induced by the poor biocompatibility of RRT (renal replacement therapy) [5–7], is evidenced by elevated circulating levels of CRP (C-reactive protein) and IL (interleukin)-6, both considered strong predictors of poor outcome [8–10]. Bologa et al. [8], in particular, have reported that the relative risk associated with each 1 pg/ml increase in IL-6 concentration was enhanced by 4.4% in ESRD patients.

IL-6 is a cytokine that provokes a broad range of cellular and physiological responses, including the immune response, inflammation, haematopoiesis and oncogenesis, by regulating cell growth, gene activation, proliferation, survival and differentiation. IL-6 signals through a receptor composed of two different subunits: an α subunit [gp80 (glycoprotein 80) or IL-6R (IL-6 receptor)] that produces ligand specificity, and gp130 (glycoprotein 130), a receptor subunit shared in common with other cytokines in the IL-6 family [11,12]. Binding of IL-6 to its receptor initiates cellular events, including activation of JAKs (Janus kinases) and Ras-mediated signalling. Activated JAKs phosphorylate and activate the STAT (signal transducer and activator of transcription) transcription factors, particularly STAT3 [13]. Phosphorylated STAT3 then forms a dimer and translocates into the nucleus to activate the transcription of genes containing STAT3-response elements [14].

IL-6 is also the major inducer of acute-phase protein synthesis in liver cells. A typical acute-phase protein pattern in ESRD is represented by lower plasma concentrations of fetuin-A [15] and higher concentrations of hepcidin [16]. Vascular calcification has been associated in ESRD patients with low circulating levels of fetuin-A [15]. Fetuin-A, in fact, is an important circulating inhibitor of calcification in vivo [17]; it is produced by hepatocytes and down-regulated by IL-6 [18]. Low concentrations of this glycoprotein were associated with raised amounts of CRP and enhanced cardiovascular mortality [15]. Hepcidin is a circulating peptide hormone mainly synthesized in the liver, which has recently been proposed as a factor regulating iron homeostasis through the interaction with the main iron export protein ferroportin [19]. When hepcidin concentrations increase, it binds to ferroportin molecules and induces their internalization and degradation, and iron release is decreased. Inflammation causes an increase in hepcidin production, which is, therefore, a potent mediator of anaemia in chronic diseases [19–22]; anaemia, in turn, plays a key role in LVH occurrence and progression [23]. LVH has long been known as an important and independent risk factor for mortality and cardiovascular events, in both dialysis patients and the general population [24].

Over the last few years, a great improvement in the quality of RRT has been achieved, but, despite the reduction in inflammation, cardiovascular mortality still remains high. In ESRD, the concept of microinflammation (i.e. small increases in circulating levels of IL-6 and CRP; with no signs or clinical symptoms of inflammation) still requires more accurate evaluation [25]. In particular, it is not clear what concentration of IL-6 is necessary and sufficient to trigger a micro-inflammatory condition and how it may be more accurately defined. A mean level of 4.4 pg/ml IL-6 was observed in ESRD patients free of inflammatory clinical events, with a large intra-individual variation [25]. In another study, values between 1.6 and 2.5 pg/ml were observed in dialysis patients with no inflammation/malnutrition [26]. These values are markedly different from those found in the healthy population, where circulating levels of approx. 1.0 pg/ml or less are detected [27]. This could suggest that even small increases in circulating IL-6 levels could play a role in enhancing CVD risk. This hypothesis is supported by Ridker et al. [28], who observed that very small increases in IL-6 levels (1.81 compared with 1.46 pg/ml) was associated, in apparently healthy subjects, with an increased risk of future myocardial infarction.

No data are available in ESRD patients demonstrating that small increases in circulating levels of IL-6 are sufficient to activate, in hepatocytes, the gp130/STAT3 cascade, as well as to regulate the gene expression of some acute-phase proteins involved in cardiovascular risk. This issue was the main aim of the present study. In particular, we have investigated whether low (<2 pg/ml) serum concentrations of IL-6, obtained by incubating serum fractions from ESRD patients in the culture medium of primary human hepatocytes, are able to induce significant IL-6-dependent molecular modifications (i.e. gp130 and STAT3 activation) in these cells, as well as to modulate the differential gene expression of fetuin-A and hepcidin.

Part of this work was presented at the Annual Meeting of the American Society of Nephrology, held in Philadelphia, PA, U.S.A. on November 4–9, 2008, and subsequently published in abstract form [28a].

MATERIALS AND METHODS

Patient selection

In order to obtain a sufficient amount of serum, we selected 12 ESRD patients undergoing RDT (regular
dialysis treatment) in our renal unit. All patients were undergoing standard dialysis for at least 1 year before the study. No patient had a fever or any clinical symptoms of inflammation and none were on steroid or immunosuppressive therapy. Healthy age-matched laboratory staff volunteers (n = 6) were also included in the study as the healthy control group.

The study was approved by the local Ethics Committee, and all enrolled subjects (healthy subjects and ESRD patients) gave their informed consent before the study.

The clinical features of both healthy subjects and ESRD patients included in the study are shown in Table 1. The ESRD patients were divided into two subgroups on the basis of circulating CRP levels. Patients with CRP \( \leq 3 \) mg/l (n = 6) were classified as having non-inflamed ESRD, whereas patients with CRP > 3 mg/l (n = 6) were classified as having micro-inflamed ESRD. Despite the small number of patients, the resulting amount of serum necessary to perform our cell experiments was sufficient and adequate. Serum fractions (10 ml from each serum sample) obtained from all subjects in the same group (healthy, non-inflamed ESRD and micro-inflamed ESRD respectively) were pooled in order to obtain larger amounts of serum with the same characteristics (in particular with the same concentrations of IL-6 and its soluble receptors).

### Table 1: Clinical features of healthy subjects, and non-inflamed and micro-inflamed ESRD patients included in the present study

Values are expressed as means ± S.D. *P < 0.01 compared with healthy subjects and non-inflammed ESRD patients. ADPKD, adult dominant polycystic kidney disease; BMI, body mass index; GN, glomerulonephritis; HD, haemodialysis; Kt/V, dialysis adequacy; nPCR, normalized protein catabolic rate.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy subjects</th>
<th>Non-inflamed ESRD</th>
<th>Micro-inflamed ESRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (n) (male/female)</td>
<td>6 (4/2)</td>
<td>6 (3/3)</td>
<td>4 (4/2)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48.8 ± 6.4</td>
<td>52 ± 8.2</td>
<td>53.6 ± 4.2</td>
</tr>
<tr>
<td>Time on HD (months)</td>
<td>–</td>
<td>28.64 ± 6.12</td>
<td>26.12 ± 5.34</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>62.8 ± 3.4</td>
<td>61.8 ± 2.8</td>
<td>62.2 ± 3.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.2 ± 0.8</td>
<td>21.8 ± 1.4</td>
<td>22.6 ± 1.3</td>
</tr>
<tr>
<td>Cause of ESRD (n)</td>
<td>–</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>GN</td>
<td>–</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ADPKD</td>
<td>–</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Renal vascular disease/hypertension</td>
<td>–</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Other/unknown</td>
<td>–</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>Smokers (n)</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Kt/V</td>
<td>–</td>
<td>1.36 ± 0.04</td>
<td>1.32 ± 0.04</td>
</tr>
<tr>
<td>nPCR (g · kg⁻¹ · day⁻¹)</td>
<td>–</td>
<td>1.18 ± 0.3</td>
<td>1.22 ± 0.4</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>2.30 ± 0.4</td>
<td>2.60 ± 0.2</td>
<td>6.98 ± 0.6*</td>
</tr>
<tr>
<td>Serum albumin (g/dl)</td>
<td>4.02 ± 0.6</td>
<td>3.96 ± 0.4</td>
<td>3.16 ± 0.2*</td>
</tr>
</tbody>
</table>

In order to confirm the specific role of IL-6 in the present study, we also performed experiments after the neutralization of this cytokine in the serum by using a specific blocking antibody, an anti-(human IL-6) antibody. This antibody produced in goats immunized with purified human rIL-6 (recombinant IL-6) was selected for its ability to neutralize the IL-6 activity of both natural IL-6 and human rIL-6 (AB-206-NA; R&D Systems). In addition, in a direct ELISA this antibody has no cross-reactivity with any of the other cytokines tested. The optimal concentration of the antibody required to neutralize rIL-6 was determined using three samples with different concentrations of rIL-6 prepared in our laboratory: 5, 10 and 20 pg/ml rIL-6. To measure the ability of the antibody to neutralize rIL-6 in these standards, rIL-6 was incubated with various concentrations of the antibody at 4 °C overnight and evaluated at room temperature (20 °C) using the IL-6 ELISA assay described above.
IL-6, sIL-6R and sgp130 concentrations in healthy subjects, and non-inflamed and micro-inflamed ESRD patients

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Healthy subjects</th>
<th>Non-inflamed ESRD</th>
<th>Micro-inflamed ESRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/ml) (whole serum)</td>
<td>0.53</td>
<td>2.97</td>
<td>8.88</td>
</tr>
<tr>
<td>IL-6 (pg/ml) (culture medium)</td>
<td>0.1</td>
<td>0.6</td>
<td>1.8</td>
</tr>
<tr>
<td>sIL-6R (ng/ml) (whole serum)</td>
<td>18.2</td>
<td>50</td>
<td>67</td>
</tr>
<tr>
<td>sgp130 (ng/ml) (whole serum)</td>
<td>306</td>
<td>273</td>
<td>290</td>
</tr>
</tbody>
</table>

**Design of the study on hepatocytes**

We performed our study on human hepatocytes incubated for 24 h with low (<2 pg/ml) IL-6 concentrations obtained by the addition of human serum drawn from both ESRD patients and healthy subjects.

**Human hepatocyte cultures**

Cryopreserved human hepatocytes (BD Bioscience) were thawed and cultured in a batch system. Human hepatocytes were seeded at a final concentration of 2.5 × 10^5 cells/cm^2 on a modified polyetheretherketone (PEEK-WC) semi-permeable membrane. We prepared these membranes with an inverse-phase technique by using the direct immersion-precipitation method [30].

Cell cultures were stimulated with three different low (<2 pg/ml) IL-6 concentrations obtained by adding serum fractions from healthy subjects (medium IL-6 concentration=0.1 pg/ml), non-inflamed ESRD patients (medium IL-6 concentration=0.6 pg/ml) and micro-inflamed ESRD patients (medium IL-6 concentration=1.8 pg/ml). As the cell culture medium cannot contain more than 20% as serum, the IL-6 concentrations in the cultures were five times lower than IL-6 concentrations in whole serum (as shown in Table 2).

It is important to highlight that 1.8 pg/ml is an IL-6 concentration uncommon in inflamed ESRD patients and also lower than in non-inflamed ESRD (Table 2). Therefore, to avoid any confusion, in the rest of the paper we will only report the final IL-6 concentration in the medium (with no indication about the group origin).

In order to evaluate the specific effect of IL-6 contained in serum samples, human hepatocytes were also treated with 20% serum from micro-inflamed ESRD patients (8.88 pg/ml IL-6) and neutralized 12 h before with the anti-(human IL-6) antibody.

Similarly, the effects of IL-6 were differentiated from the effects of other components contained in serum by stimulating hepatocytes with human rIL-6 (Sigma) at the same concentrations in the 20% serum (0.1, 0.6 and 1.8 pg/ml respectively). In addition, we also utilized an rIL-6 concentration of 2.97 pg/ml (corresponding to the IL-6 concentration observed in the whole-serum pool of non-inflamed ESRD patients; Table 2).

Each group of cultures, comprising 12 samples, was stimulated for 24 h and then utilized for the immunostaining of p-STAT3 (phospho-STAT3), gp130 and gp80. Cells were incubated at 37°C in a 5% CO2/20% O2 atmosphere with 95% relative humidity for the duration of the experiments.

**Hepatocyte staining for laser confocal scanning microscopy**

After 24 h of culture, hepatocytes were washed with PBS, fixed for 15 min in 3% paraformaldehyde in PBS at room temperature, and dehydrated for 5 min with ice-cold methanol and 5 min with ice-cold acetone. For double immunostaining of p-STAT3 and gp130, a rabbit polyclonal affinity-purified antibody raised against p-STAT3 of human origin and a mouse monoclonal antibody raised against gp130 of human origin (Santa Cruz Biotechnology) were used. For double immunostaining of gp80 and gp130, a rabbit polyclonal affinity-purified antibody raised against a peptide mapping at the C-terminus of IL-6Ra/gp80 of human origin and a mouse monoclonal antibody raised against gp130 of human origin (Santa Cruz Biotechnology) were used. Primary antibodies were incubated at a 1:200 dilution for 2 h at room temperature. Secondary antibodies were Cy5M2-conjugated AffiniPure donkey anti-(rabbit IgG) and Cy3M3-conjugated AffiniPure donkey anti-(mouse IgG) antibodies (Jackson ImmunoResearch) incubated at a 1:100 dilution for 1.5 h at room temperature. To visualize nucleic acids, the samples were washed twice in PBS and incubated for 20 min with 0.2 μg/ml DAPI (4′,6-diamidino-2-phenylindole; Molecular Probes). Finally, samples were washed, mounted and viewed with a laser confocal scanning microscope (Fluoview FV300; Olympus), as described below.

**Imaging and quantitative analysis**

Imaging of labelled human hepatocytes was obtained using an Olympus Fluoview FV300 laser confocal scanning microscope. Quantitative analysis was performed on different areas of three samples of each investigated treatment using Fluoview 5.0 software (Olympus), and the average intensity/cell compared with the z-axis of acquired images of 0.5 μm optical thickness was calculated. Calibration curves of average fluorescence intensity for p-STAT3, gp130 and gp80 were also obtained by incubating cells with rIL-6 at the same concentrations.
obtained in the 20% serum (0.1, 0.6 and 1.8 pg/ml respectively) and a rIL-6 concentration of 2.97 pg/ml, corresponding to the IL-6 concentration observed in the whole-serum pool from non-inflamed ESRD patients (Table 2).

**Western blot analysis**

The results obtained with Western blot analysis were also compared with Western blot analysis. After 24 h of stimulation with serum IL-6 (0.6 or 1.8 pg/ml) and under serum-free conditions (control), human hepatocytes were washed once with ice-cold PBS and then pelleted by centrifugation. The cell pellets were resuspended in ice-cold lysis buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl and 1% Triton X-100] supplemented with protease and phosphatase inhibitor cocktails, vortex-mixed and incubated for 40 min at 4°C. During incubation, the samples were sonicated for 30 s and centrifuged at 1320 g for 20 min at 4°C. The supernatants were transferred to new tubes and the protein concentration was determined using a QBIT fluorimeter (Invitrogen).

Western blot analysis was performed as described previously [31]. Equal amounts of protein (30 μg) were boiled for 5 min, separated under denaturing conditions by SDS/PAGE on 6% (w/v) polyacrylamide gel. After transfer to nitrocellulose membrane, non-specific sites were blocked with 5% non-fat dried milk in TBS-T [10 mM Tris/glycine (pH 7.5), 100 mM NaCl and 0.1% Tween 20] for 1 h at room temperature. The membrane was then incubated overnight with anti-(human IL-6Rα/gp80) (1:200 dilution), anti-(human gp130) (1:200 dilution), anti-(human β-actin) (1:500 dilution) primary antibodies (Santa Cruz Biotechnology). The antigen–antibody complex was detected by incubation of the membranes for 1 h at room temperature with a peroxidase-coupled anti-IgG antibody. As shown in Figure 1, rIL-6 (10 pg/ml) and phosphatase inhibitor cocktails, vortex-mixed and incubated for 40 min at 4°C. During incubation, the samples were sonicated for 30 s and centrifuged at 1320 g for 20 min at 4°C. The supernatants were transferred to new tubes and the protein concentration was determined using a QBIT fluorimeter (Invitrogen).

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**Statistical analysis**

Statistical analysis was performed using ANOVA (followed by the Bonferroni post-hoc test) and linear regression analysis. Results are expressed as means ± S.D., and statistical significance was defined as *P* < 0.05.

**RESULTS**

**Serum cytokine concentrations**

As shown in Table 2, the whole-serum pool obtained from micro-inflamed ESRD patients had higher circulating levels of IL-6 (8.88 pg/ml) than the pool from non-inflamed ESRD patients (2.97 pg/ml); the latter, in turn, had higher levels than that from healthy subjects (0.53 pg/ml). These findings confirm previous results from our group and others [25–28,32,33].

Similar differences were obtained with the concentrations of ‘agonistic’ sIL-6R. On the other hand no difference was observed among the concentrations of ‘antagonistic’ IL-6R (sgp130) (Table 2).

Human hepatocytes were incubated with serum fractions pooled according to the three groups of enrolled subjects; since hepatocyte culture medium contained only 20% serum, IL-6 culture concentrations were 5-fold lower than whole-serum pools (0.1, 0.6 and 1.8 pg/ml of IL-6 respectively, as reported in Table 2).

The specific effects of IL-6 on hepatocytes were also evaluated by treatment with serum containing blocked IL-6. The concentration of antibody required to neutralize rIL-6 activity was evaluated using different concentrations of the anti-(human IL-6) antibody.

As shown in Figure 1, rIL-6 (10 pg/ml) was completely neutralized with 2.00 μg/ml antibody. This concentration was therefore chosen to ensure the complete neutralization of IL-6 contained in the serum from micro-inflamed ESRD patients.
Decrease in human rIL-6 activity at different concentrations of anti-(human IL-6) antibody

Effects of low levels of IL-6 on gp130, gp80 and STAT3 activation

We explored, by confocal microscopy, the expression of gp130 and gp80 and the activation of the STAT3 (occurring via phosphorylation at Tyr705) on human hepatocytes cultured for 24 h.

Figure 2 shows gp130 (red) and p-STAT3 (yellow) staining, detected by immunofluorescence after 24 h of culture with serum fractions; gp130 and p-STAT3 fluorescence signals were similar in control untreated cells and in hepatocytes incubated with an IL-6 concentration of 0.1 or 0.6 pg/ml. No remarkable differences were found between the groups. On the contrary, stimulation of hepatocytes with a serum IL-6 concentration of 1.8 pg/ml resulted in a noticeable increase in gp130 expression and in a strong activation of STAT3. Hepatocytes treated with serum containing neutralized IL-6 had a fluorescence signal similar to the untreated cells and to hepatocytes incubated with serum containing 0.1 pg/ml IL-6. Similar results were obtained after 3 h of incubation (results not shown).

In addition to the gp130 membrane receptor, we also explored the expression of the gp80 and its localization on the cell membrane. Figure 3 shows the co-localization of gp80 (or IL-6Rα; green) and gp130 (red) on the hepatocyte membrane and their differential expression after 24 h of the treatments. The fluorescence signal was only high for both receptors in the samples treated with an IL-6 concentration of 1.8 pg/ml. Conversely, gp80 and gp130 expression decreased to a large extent in the other cultures and in the treatment with serum containing blocked IL-6.

To confirm these observations, we also performed a quantitative analysis of the average fluorescence intensity for immunostained gp80, gp130 and p-STAT3 (Figure 4). In control untreated cells, in hepatocytes treated with serum IL-6 at concentrations of 0.1 and 0.6 pg/ml, and in hepatocytes treated with serum containing neutralized IL-6, the fluorescence signals for gp80, gp130 and p-STAT3 were similar.

On the other hand, a strong STAT3 activation was found in hepatocytes treated with a serum IL-6 concentration of 1.8 pg/ml; the average fluorescence intensity in this group reached a peak value of 8463 ± 3335, which was significantly ($P < 0.05$) higher compared with the other cultures. In addition, the average fluorescence intensity of gp80 and gp130 (11955 ± 4670 and 10061 ± 3710 respectively) were significantly higher ($P < 0.05$) than those found in the other cultures. A decrease in cytokine concentration from 1.8 pg/ml to 0.6 pg/ml resulted in a decrease in the average fluorescence intensity of both IL-6 membrane-bound receptors and p-STAT3. Blocking IL-6 abrogated the induction of STAT3 and IL-6 membrane-bound receptors, as shown from the average fluorescence intensity values.

In order to confirm the role of serum IL-6 in STAT3 activation, we also cultured human hepatocytes with rIL-6 at the same concentrations in the serum fractions. Figure 5 shows the linear increase in the average fluorescence intensity of gp80, gp130 and p-STAT3 with increasing concentrations of rIL-6 from 0 to 2.97 pg/ml (the last concentration was identical with the IL-6 concentration found in the whole serum of non-inflamed ESRD patients): the $R^2$ values were 0.975 for gp80, 0.988 for gp130 and 0.975 for p-STAT3 respectively. In addition, in this case, the stimulation of cells with rIL-6 produced an increase in the expression of IL-6 membrane-bound receptors with the consequent strong activation of p-STAT3 at higher concentrations of rIL-6.

However, it is interesting to note the different effect of rIL-6 with respect to serum IL-6 from patients. Figure 6 shows a comparison of the average fluorescence intensity values of gp80, gp130 and p-STAT3 between control untreated cells, cells treated with rIL-6 and cells treated with the same cytokine concentration from patients’ serum. A significant difference in the average fluorescence intensity values from cells treated with serum IL-6 (0.6 pg/ml) was only found for gp80 ($P < 0.05$). No differences were observed between the groups treated with rIL-6 and the control (Figure 6a). On the other hand, striking differences were found at increasing concentrations of serum IL-6: all of the average fluorescence intensity values of the immunostained proteins for hepatocytes treated with serum IL-6 at 1.8 pg/ml were significantly higher ($P < 0.01$) compared with those incubated with rIL-6 at the same concentration (Figure 6b). Finally, when hepatocytes were incubated with rIL-6 at a concentration of 2.97 pg/ml, a value found in the serum of non-inflamed ESRD patients, a further increase in the fluorescence signal for gp80, gp130 and p-STAT3 was observed (results not shown).

Western blot analysis confirmed the significant increase in STAT3 activation, and gp80 and gp130 expression in the presence of serum containing 1.8 pg/ml IL-6, as observed by confocal microscopy. Marked expression of p-STAT3, gp80 and gp130 was observed in the
Effects of low levels of IL-6 in end-stage renal disease

Figure 2  Confocal images of hepatocytes immunostained for p-STAT3 and gp130 after 24 h of stimulation with serum containing different concentrations of IL-6, serum with blocked IL-6 and under IL-6-free conditions (control).

Hepatocytes were double immunostained for p-STAT3 (yellow) and gp130 (red). Merge represents the co-localization of the two fluorescence signals. DAPI counterstaining was used to detect nuclei (blue). The images are representative of 12 experiments.

hepatocyte samples treated with 20% serum from micro-inflamed ESRD patients (1.8 pg/ml IL-6) compared with those treated with 20% serum from non-inflamed ESRD patients (0.6 pg/ml IL-6) and controls (Figure 7).

Effects of inflammation on fetuin-A and hepcidin gene expression

Figure 8 shows the RT-PCR analysis of both hepcidin and fetuin-A gene expression in human hepatocytes treated with 20% serum containing IL-6 at concentrations of 0.1, 0.6 and 1.8 pg/ml, 20% serum with blocked IL-6 and under serum-free conditions (control). It is evident that the treatment of cells with serum containing 1.8 pg/ml IL-6 up-regulated (31.6-fold) hepcidin gene expression and down-regulated the fetuin-A gene expression (8.9-fold) compared with untreated cultures. As a result, fetuin-A gene expression was modulated to a lesser extent compared with hepcidin. The treatment of cells with serum containing blocked IL-6 abrogated the up- and down-regulation of hepcidin and fetuin-A gene expression respectively, reaching values similar to untreated cultures (control).
**DISCUSSION**

The results of the present study clearly demonstrate that low concentrations of IL-6 (1.8 pg/ml) in the culture medium of primary human hepatocytes and arising from the addition of serum fractions from micro-inflamed ESRD patients were necessary and sufficient to activate, in these cells, gp80, gp130 and STAT3; this phenomenon, observed by confocal microscopy and Western blot analysis after 24 h of incubation (Figures 2, 4 and 7), was associated with a noticeable (over 30-fold) up-regulation of hepcidin gene expression (Figure 8). Lower effects on activation of STAT3 and IL-6Rs were obtained with the same concentrations of rIL-6 (Figures 5 and 6). No significant effect was observed when incubating human hepatocytes with 0.1 or 0.6 pg/ml IL-6. Importantly, when IL-6 activity was completely blocked in the serum containing 1.8 pg/ml of this cytokine, no effect was observed on STAT3 activation.

It is important to underline that these inflammatory effects were obtained by incubating hepatocyte cultures...
with IL-6 concentrations 5-fold lower than those circulating in apparently healthy ESRD patients. On the basis of these results, therefore, we can speculate that circulating levels of IL-6, even in non-inflamed ESRD patients (serum IL-6 = 2.97 pg/ml; CRP ≤ 3.0 mg/l) are sufficient to activate the acute-phase response in human hepatocytes. Therefore our present findings suggest that the control of inflammation in our patients still represents a significant problem, with the target value of 1 pg/ml IL-6, considered as normal, being quite difficult to obtain, despite the evident improvement in RRT quality.

STAT3, the major signal transducer downstream of gp130-like receptors, was first described as a DNA-binding activity from IL-6-stimulated hepatocytes, able to selectively interact with an enhancer element in the promoter of acute-phase genes [14]. Previous studies have demonstrated that IL-6 induced hepcidin expression through STAT3 activation and subsequent hepcidin promoter binding, and that STAT3 was necessary and sufficient for the IL-6 responsiveness of the hepcidin promoter [22,34]. In our present study, a significant activation of STAT3, associated with gp130 overexpression, was observed after 24 h of incubation with 1.8 pg/ml serum IL-6 in the medium.

The results of our present study also demonstrate, although to a lesser extent, a serum-induced down-regulation of fetuin-A gene expression. This phenomenon is not secondary to STAT3 activation, as it is well known that the gene encoding fetuin-A is controlled by a series of binding sites for different C/EBP (CCAAT/enhancer-binding protein) isoforms [35,36].

IL-1, IL-6 and TNF-α (tumour necrosis factor-α) activate, through specific cell receptors, three major transcription factor families: NF-κB (nuclear factor κB), C/EBPs and STATs [37]. These factors differently regulate acute-phase protein gene expression at the transcriptional level. IL-1 and TNF-α activate NF-κB, whereas IL-6 specifically induces the tyrosine phosphorylation and DNA-binding activity of STAT3. C/EBPs are a family of six proteins, the most widely expressed, and most well studied, being the C/EBPα and C/EBPβ isoforms. These isoforms, regulated by both IL-1 and IL-6, display contrasting functions in gene activation and cell proliferation [38,39]. Our results, therefore, suggest that low circulating levels of IL-6 may also play a significant role in activating the C/EBP transcription factors.
Figure 6  Comparison of average fluorescence intensity of gp80, gp130 and p-STAT3 expression after 24 h of stimulation with rIL-6 and serum with the same IL-6 concentration
(a) Stimulation with rIL-6, serum with the same IL-6 concentration (0.6 pg/ml) and under serum-free conditions (control). (b) Stimulation with rIL-6, serum with the same IL-6 concentration (1.8 pg/ml) and under serum-free conditions (control). Values are expressed in arbitrary units as means ± S.D. of 12 experiments. gp80, solid black bar; gp130, open bar; p-STAT3, hatched bar. * P < 0.05 compared with gp80 under rIL-6 (0.6 pg/ml) stimulation; § P < 0.001 compared with rIL-6 (1.8 pg/ml).

The second important finding of our present study is the significant difference between the effects obtained in human hepatocytes incubated with serum fractions with 1.8 pg/ml IL-6 compared with those observed with the same concentration of rIL-6 (Figure 6). This serum enhancement of IL-6 effects could be explained by at least two different factors: (i) the presence in the inflamed serum of other pro-inflammatory cytokines (i.e. IL-1 and TNF-α) and factors, and (ii) the presence in the serum of sIL-6R. The first factor is less reasonable, as the gp130/STAT3 axis is specifically and exclusively activated by IL-6-type cytokines (mainly IL-6) [14,40], whereas IL-1-type cytokines (such as IL-1 and TNF-α) stimulate the acute-phase response by NF-κB and/or C/EPB activation [40,41]. In addition, the specific neutralization of IL-6 completely abolished any effect induced by the serum.

Besides the membrane-bound gp80 (or IL-6R), a naturally occurring soluble form, named sIL-6R, is generated by two independent mechanisms: limited proteolysis of the membrane protein and translation from an alternatively spliced mRNA [33,34,42]. Interestingly, sIL-6R together with IL-6 stimulate cells that only express gp130, a process named trans-signalling [12]. This process implies that cells which were originally unresponsive to IL-6 (because of the lack of membrane-bound IL-6R) now become responsive via the sIL-6R/IL-6 complex. Approx. 70% of circulating IL-6 forms a complex with sIL-6R in blood and binds directly to membrane gp130. The other 30% is supposed to have only a transient existence in the blood or binds to the...
membrane-bound receptor (IL-6R). sIL-6R functions, therefore, as a carrier protein for its ligand, thereby markedly prolonging the plasma half-life of IL-6 and indicating that IL-6 signalling is increased by sIL-6R [12]. sIL-6R is largely present in our serum samples, particularly in the serum from micro-inflamed ESRD patients (Table 2).

Taken together, the results of the present study allow us to define micro-inflammation more accurately and demonstrate that even small amounts of IL-6 (< 2 pg/ml), in the presence of sufficient concentrations of sIL-6R, are able to effectively modulate (up- and down-regulate respectively) different genes encoding acute-phase proteins, such as hepcidin and fetuin-A, which are emerging as contributors to cardiovascular mortality in ESRD patients [43,44]. The specific role of IL-6 in the activation of the STAT3 pathway and in the modulation of hepcidin and fetuin-A gene expression was shown by blocking IL-6 present in the serum, which, as expected, did not induce any increase in STAT3 activation and IL-6 cell-membrane receptor expression. These results are consistent with those obtained by Rüdker et al. [28], who observed that an increased risk of future myocardial infarction was associated with a small increase in circulating IL-6 levels (1.81 compared with 1.46 pg/ml) in apparently healthy subjects.

In conclusion, our present results demonstrate for the first time, at a molecular level, the chain of events underlying the incidence of some risk factors involved in cardiovascular events in apparently healthy ESRD patients and open a new scenario in the study and relevance of micro-inflammation.

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