Original Article

Impaired release of interleukin-6 from human osteoblastic cells in the uraemic milieu

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Abstract

Background. Osteoblast-derived interleukin-6 (IL-6) affects bone metabolism and is linked with a number of pathological states characterized by increased bone resorption, including osteoporosis and renal osteodystrophy. To examine the possibility that uraemia directly influences the release of this cytokine in bone, we have investigated the effect of human uraemic serum on the release of IL-6 from human osteoblast-like cells.

Methods. Individual serum samples collected from healthy male volunteers or male haemodialysis patients prior to and during a dialysis treatment were assayed for IL-6, interleukin-1β (IL-1β) and soluble IL-6 receptor (sIL-6R) using specific enzyme-linked immunoassay assays. MG-63 and SaOS-2 cells were cultured in media containing pooled sera from both groups and alongside matching charcoal-stripped sera. IL-6 concentrations were determined in harvested cell supernatants after 24 h. In further experiments, media containing individual sera obtained from five patients at regular intervals during their haemodialysis treatment were incubated with MG-63 cells to determine the effects of the dialysis process on IL-6 secretion.

Results. Haemodialysis patients had significantly higher (n = 10, P < 0.001) circulating concentrations of IL-6 (7.0 ± 1.6 pg/ml) than normal subjects (0.4 ± 0.1 pg/ml), but there were no significant differences in the concentrations of either IL-1β or sIL-6R. These serum concentrations did not change significantly during 80 min of dialysis. IL-6 release by MG-63 cells incubated with charcoal-stripped serum from normal or from uraemic subjects was similar. Incubation with untreated sera from normal subjects increased IL-6 release by ~6-fold above the charcoal-stripped control, whereas sera from uraemic subjects increased IL-6 release by only ~2- to 3-fold (normal vs uraemic of 678 ± 595 and 257 ± 169 pg/ml, respectively, P < 0.001). Similar results were seen with SaOS-2 cells. Haemodialysis did not restore the capacity of uraemic serum to augment IL-6 release to the same degree as normal serum.

Conclusions. These data show that the augmentation of IL-6 release from human osteoblastic cells after incubation with normal serum is greater than after uraemic serum. This may indicate the presence of an inhibitor of IL-6 release in uraemic serum that is involved in the deranged bone turnover of uraemic patients.

Keywords: bone remodelling; haemodialysis; interleukin-6; osteoblast; renal osteodystrophy; uraemic serum

Introduction

Renal osteodystrophy describes a spectrum of skeletal abnormalities seen in patients with renal disease and encompasses a series of disorders of bone remodelling [1]. Historically, the predominant group of bony abnormalities have been those attributable to hyperparathyroidism, with associated increases in bone turnover resulting in the histological appearances of osteitis fibrosa. However, low turnover bone lesions in the absence of associated aluminium toxicity have been increasingly recognized [2,3]. Several studies in end-stage renal disease (ESRD) patients have identified
normal bone turnover co-existing with moderately elevated levels of parathyroid hormone (PTH) and thus to the recommendation that PTH concentrations are maintained at 2–3 times the upper limit of the normal reference range [4,5]. Recently published K/DOQI guidelines have gone even further, advising a target PTH concentration of 150–300 pg/ml (16.5–33.0 pmol/l) for all those on dialysis or with a glomerular filtration rate of <15 ml/min [i.e. stage five chronic kidney disease] [6]. While the realization that many PTH assays detect potentially antagonistic fragments in addition to intact PTH provides at least a partial explanation of the need for such levels [7], it seems likely that additional factors present in the uraemic milieu may also serve to depress bone cell function.

Since bone remodelling requires coupling of osteoclastic bone resorption and osteoblastic bone formation, disruption of these linked processes may play a role in the aetiology of osteodystrophy. A large number of osteotrophic factors such as bone morphogenetic proteins cytokines and growth factors influence the principal mediators of osteoclastic resorption, namely osteoprotegerin and RANK-L [8]. Amongst these, the pluripotent cytokine interleukin-6 (IL-6) is thought to play an important role in the initiation and maintenance of osteoclastogenesis [9] and has been implicated in the pathogenesis of a wide variety of skeletal pathologies characterized by accelerated bone remodelling and resorption. There is evidence for the involvement of IL-6 in the pathogenesis of renal osteodystrophy [10], and we have demonstrated previously that IL-6 release by osteoblast-like cells can be modulated by vitamin D-like compounds [11].

In the present study, the circulating levels of IL-6, interleukin-1β IL-1β, and soluble IL-6 receptor (sIL-6R) in patients prior to and during a haemodialysis (HD) session are examined and the effect of both normal and uraemic serum on IL-6 release from human osteoblastic cells explored. In addition, the capacity of both charcoal stripping and therapeutic HD to remove those substances potentially interfering with the serum-stimulated release of osteoblastic IL-6 is evaluated.

Subjects and methods

Normal serum samples were obtained from 10 healthy male volunteers with no history of febrile illness in the preceding 4 weeks. Uraemic sera were obtained from 10 male ESRD patients treated by HD (renal diagnoses: small kidneys, uncertain cause, 3; hypertensive nephrosclerosis, 2; renovascular disease, 1; membranous glomerulonephritis, 2; focal and segmental glomerulosclerosis, 1; adult polycystic kidney disease, 1). All patients had been on HD for at least 1 year (mean 26 months, range 13–62) and had a mean age of 39 years (range 28–67). All patients underwent three, 4 h dialysis sessions per week using cellulose acetate dialysers and bicarbonate buffering. Venous access in all subjects was by primary arteriovenous fistulae. Blood was sampled from the arterial access port prior to passing through the dialyser at the start of a HD treatment session. Serum was obtained from blood samples taken into plain glass tubes and allowed to stand upright at room temperature for 20 min before separation by centrifugation at 2000 r.p.m., removal of serum with a sterile pipette and immediate storage at −20 °C.

Blood samples were also obtained from five additional patients during an individual dialysis session. The characteristics of these patients are shown in Table 1. Samples were taken prior to dialysis and at 30, 60, 120, 180, 240 and 300 min into their treatment. The first 60 min comprised extra-corpooreal circulation alone, with no dialysate flow and no programmed ultrafiltration (hereafter referred to as sham dialysis). After 60 min, dialysate flow was introduced and continued for the remainder of the dialysis treatment.

Individual normal and uraemic serum samples were assayed for IL-6, sIL-6R and IL-1β. IL-1β is known to increase the release of IL-6 from both MG-63 and SaOS-2 cells [13] and was measured to assess any contribution to the differences in measured IL-6. The sIL-6R is a 50 kDa ligand protein derived from the surface shedding of the gp80 component of the IL-6 receptor. Its ability to bind free IL-6 means it is a potential source of interference with the measured concentration of IL-6. Some aliquots of human sera were ‘inactivated’ by charcoal stripping, a process that removes hydrophobic structures including steroids and growth factors [12]. A 2 g aliquot of activated charcoal was added to 50 ml of serum and mixed at room temperature for 4 h. Serum was recovered by centrifuging for 30 min at 3500 r.p.m. The supernatant was re-centrifuged for a further 20 min at 20 000 r.p.m. at room temperature. Prior to use, the serum was filtered through 0.45 and then 0.2 μm filters (Minisart®, Sartorius AG, Göttingen, Germany). Additional aliquots were heat treated (65 °C for 30 min) as an alternative method of growth factor and hormone inhibition.

Ethical approval for these procedures was obtained from the relevant local body (East London and the City Health Authority, reference P/00/241). Patients provided written informed consent.

Cell culture and experimental design

Two human osteoblast-like cell lines were used in these experiments. MG-63 cells produce relatively low levels of alkaline phosphatase and do not form a mineralized matrix in vitro. They are often considered to be of a relatively immature phenotype. SaOS-2 possess some of the characteristics of a more differentiated osteoblastic cell, producing more constitutive alkaline phosphatase and also possessing the ability to form a mineralized matrix in long-term
cultures. Cells were cultured and maintained in α-minimal essential medium (α-MEM) containing 10% fetal calf serum (FCS) (Gibco Life Technologies Ltd) and antibiotics (benzylpenicillin 100 U/ml, streptomycin 10 μg/ml, amphotericin 2.5 μg/ml) in a humidified 1:19 air/CO₂ environment at 37°C. Cells were seeded into 24-well plates at a density of 200,000 cells/well. They were allowed to adhere for 24 h before aspiration of media, washing with sterile phosphate-buffered saline (PBS) and incubation with α-MEM, antibiotics and 10% charcoal-stripped FCS (CS-FCS) to provide a non-stimulating (growth-arrested) environment for a further 24 h. To examine the effects of human sera on IL-6 secretion, cells were then incubated for 24 h in media supplemented with antibodies and the human sera of interest at a 10% concentration. Negative controls were provided by refreshing cells with 10% CS-FCS, and positive controls (for IL-6) by using 10% CS-FCS to which IL1-β (R&D Systems Europe Ltd, Abingdon, Oxon, UK) had been added (at 100 IU/ml). At the end of the 24 h incubation period, media were collected and stored at −20°C for no longer than 1 month before assay. Cells were washed with PBS and subjected to three freeze/thaw cycles between −70°C and room temperature to allow cell lysis for the measurement of total cellular protein. All samples were assayed in at least quadruplicate and individual experiments repeated three or more times.

Cytokine assays

IL-6 was measured in all media and sera by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique (Eurogenetics, Middlesex, UK). IL1-β and sIL-6R were also assayed by commercially available ELISAs (R&D Systems, Abingdon, UK).

Total protein assay

Cellular protein was assayed using the Bradford dye-binding protein assay (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) in a spectrophotometric microtitre-based format. Absorbance was measured at 595 nm and compared with a standard curve prepared using known concentrations of bovine serum albumin.

Statistical analysis

Comparisons between normal and uraemic serum cytokine concentrations were analysed by unpaired t-test, while other results of more than two groups were analysed by one-way or repeated measures ANOVA with Tukey’s post hoc test for inter-group comparisons. *P < 0.05* was considered significant (NS = not significant) and all results are expressed as the mean ± SEM.

Results

Serum cytokine concentrations

The serum concentrations of IL-6 were lower in normal subjects than in serum, taken before dialysis, from stable HD patients (0.4 ± 0.1 vs. 7.0 ± 1.6 pg/ml, respectively, *P = 0.003*). However, no significant differences in the concentration of sIL-6R or IL-1β were seen in sera taken from normal subjects compared with ESRD patients (sIL-6R, 65 ± 26 vs. 96 ± 24 μg/l; NS; IL-1β, 7.6 ± 1.6 vs. 8.7 ± 2.5 pg/ml, NS). In those subjects followed through a dialysis session, no significant changes in the serum levels of IL-6, sIL-6R or IL-1β were found (Table 1).

Serum effects on osteoblast IL-6 release

Significant differences in IL-6 secretion were seen in MG-63 cells when incubated with the various sera (*P < 0.0001* by one-way ANOVA, Figure 1). Serum that had been charcoal stripped, from either normal or uraemic subjects, resulted in similar amounts of basal IL-6 release (1134 ± 127 vs. 853 ± 78 pg/ml, NS). Untreated normal serum augmented IL-6 release by ~6-fold to 6878 ± 595 pg/ml over basal (charcoal-stripped) concentrations. This increase was significantly attenuated with untreated uraemic serum, which increased release by ~2 to 3-fold (to 2579 ± 169 pg/ml) over basal concentrations. The augmentation of IL-6 release by normal sera was significantly greater than that by uraemic sera (6878 ± 595 vs. 2579 ± 169 pg/ml, *P < 0.001*). Heat treatment of uraemic serum had no effect on the release of IL-6 from MG-63 cells (1952 ± 172 heat-treated vs. 1872 ± 165 pg/ml non-heat-treated). IL-1β was by far the most powerful stimulus to IL-6 production (31 802 ± 165 pg/ml), whilst serum-free medium was the weakest (187 ± 33 pg/ml).

Quantitatively similar results were observed with SaOS-2 cells after incubation with human sera. The augmentation of IL-6 release was 6-fold greater with normal serum compared with uraemic serum (374 ± 6 vs. 61 ± 4 pg/ml, respectively).
Effect of haemodialysis on osteoblastic IL-6 release in response to uraemic serum

Having found significant differences in IL-6 production, further experiments were designed to explore the capacity, if any, of HD to restore the ability of uraemic serum to promote IL-6 production to the same degree as normal serum (Figure 2). The initial period of sham dialysis was necessary to explore the possible consequences of both heparinization and extracorporeal circulation of blood for subsequent effects in cell culture. Therapeutic dialysis aided the investigation into the potential removal of mediators of the effect. Although for some patients there was variability in osteoblastic IL-6 secretion at different time points, no significant differences or trends were evident.

**Total cellular protein**

There were no significant differences in total extracted cellular protein as compared with growth-arrested control cells following incubation with any of the sera under investigation (7.22 ± 0.48 mg/ml for uraemic, 8.1 ± 0.32 mg/ml for charcoal-stripped uraemic, 7.64 ± 0.47 mg/ml for normal serum, 7.97 ± 0.32 mg/ml for charcoal-stripped normal serum and 7.97 ± 0.52 mg/ml for pooled post-HD serum).

**Discussion**

These studies demonstrate that the release of IL-6 from human osteoblastic cells is substantially greater during exposure to media containing serum from non-uraemic subjects than during incubation with charcoal-stripped sera from the same individuals. The release of IL-6 is substantially attenuated in the presence of media containing serum from uraemic patients established on maintenance HD, though even uraemic serum significantly augmented IL-6 release in comparison with charcoal-stripped serum. Charcoal-stripped serum from normal and uraemic subjects yielded similar results. These findings suggest that uraemic serum itself might be able to influence bone turnover. In agreement with previous experiments [11,13], the constitutive release of IL-6 from SaOS-2 cells was significantly (~10-fold) lower than from MG-63 cells; however, the effect of both normal and uraemic sera on the SaOS-2 cells paralleled that seen in the MG-63 cells. In both these cell lines, IL-6 release was not augmented with the negative control (CS-FCS) but was strongly augmented (>10-fold) with 100 IU of IL-1β.

Charcoal stripping is a commonly utilized means of removing steroid hormones, including androgen and oestradiol metabolites, cortisol and thyroid hormones from serum [12]. The process is also likely to remove other growth factors and cytokines, although its exact quantitative effect in this regard is ill defined. IL-6 production after exposure to uraemic serum was enhanced with respect to exposure to charcoal-stripped normal or uraemic sera (Figure 1). We were not able to document any consistent effect of HD on the capacity of uraemic serum to augment IL-6 release (Figure 2). Sequential samples taken from five patients during sham dialysis for 60 min, followed by conventional HD for 240 min, evoked a similar and near constant level
of augmentation. These results imply that, in contrast to charcoal stripping, the factor(s) in uraemic serum affecting IL-6 release were not removed by haemodialysis. This may be a clue to inherent properties of the molecules involved, as charcoal stripping is able to remove larger, often more complex, molecules than HD across standard membranes.

The difference in IL-6 concentration in media harvested from MG-63 cells cultured with the various sera is not due to pre-existing circulating IL-6. Even though a disparity in the concentration of IL-6 in normal and uraemic sera was present (similar to those differences reported by Herbelin et al. [14] in patients with chronic renal failure ± dialysis), these were trivial compared with those in the supernatants at the completion of the experiments. Furthermore, the uraemic and normal serum were diluted 10-fold in media before use. There was no effect of HD itself on circulating IL-6 concentrations (also reported by Herbelin et al. [14]). IL-6, in the presence of sIL-6R, is known to induce its own synthesis in osteoblasts [15] and, if an effect from pre-existing IL-6 in the sera were present, then it might be anticipated that uraemic, rather than normal, serum would increase osteoblast IL-6 secretion.

IL-1β is known to increase the release of IL-6 from the cell lines studied [13] and, indeed, addition of IL-1β to the media as a positive control led to a dramatic increase in IL-6 secretion. There were no significant differences in the concentration of IL-1β in any of the sera used in the incubation experiments and, as HD had no effect on the serum concentrations of IL-1β (Table 2), it seems unlikely that this cytokine played a significant role. The ability of dialysis membranes to influence IL-6 release has been reported previously. Both circulating IL-1β and IL-1β release from harvested peripheral blood monocytes have been shown to be elevated by dialysis involving bioincompatible (cuprophane) membranes [16], though not by cellulose acetate-based membranes akin to those used in this study [17]. Thus, not only is IL-1β unlikely to have contributed to differences in the release of IL-6, but it is also unlikely to have had any tonic effects; the final concentrations that the MG-63 cells were exposed to were all less than those that we have reported previously as having an effect on IL-6 release from this cell line [11].

sIL-6R is a 50 kDa ligand-binding protein derived from surface shedding of the gp80 IL-6 receptor [18] and might be expected to interfere with measured IL-6 levels by binding to free IL-6, possibly through interference with immunoassay detection. Artefacts of this kind seem unlikely as we found no significant differences in the concentrations of sIL-6R in any of the sera from patients or control subjects and dialysis did not influence the circulating concentrations of sIL-6R in the uraemic patients.

Table 2. Effect of haemodialysis on serum cytokines in eight subjects followed for 80 min during a single haemodialysis session

<table>
<thead>
<tr>
<th></th>
<th>Pre-dialysis (n=8)</th>
<th>40 min (n=8)</th>
<th>80 min (n=8)</th>
<th>Normal subjects (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/ml)</td>
<td>6.2±0.9</td>
<td>6.3±0.8</td>
<td>7.0±1.0</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>sIL-6R (µg/l)</td>
<td>82±17</td>
<td>84±18</td>
<td>79±12.3</td>
<td>65±26</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>8.7±2.4</td>
<td>8.9±2.5</td>
<td>8.9±2.1</td>
<td>7.6±1.6</td>
</tr>
</tbody>
</table>

sIL-6R = soluble IL-6 receptor.
Results are expressed as mean ± SEM.
therapeutic dialysis. This suggests there may be a common factor(s) in uraemic serum, apparently not influenced by primary renal diagnosis, causing reduced stimulation of IL-6 release from osteoblasts and therefore potentially involved in the aberrant local signalling in uraemic bone.

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Conflict of interest statement. None declared.

References


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