Autocrine down-regulation of glucocorticoid receptors by interleukin-11 in human osteoblast-like cell lines

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Abstract

It has recently been suggested that interleukin (IL)-11 plays a role in the pathogenesis of glucocorticoid (GC)-induced osteoporosis. IL-11 belongs to the gp130 cytokine family, which includes also IL-6. We have previously investigated GC–IL-6 interplay, showing that GC inhibits IL-6 release and IL-6 up-regulates GC receptor (GR) numbers in the human osteoblast-like cell lines Saos-2 and MG-63, which constitutively have an opposite pattern of expression for GR, IL-11, IL-6, alkaline phosphatase and osteoprotegerin (OPG). The aim of this study was to investigate GC–IL-11 interplay in the same two cell lines. First, cells were incubated with cortisol (0·01–1 µM) for 20 h in the presence and in the absence of a known IL-11 secretagogue (IL-1α/β); cell media were assayed for IL-11 by ELISA. Secondly, cells were incubated with IL-11 (0·1–100 ng/ml) or specific anti-IL-11 monoclonal antibody for 20 h, and then assayed for GR by a radioligand binding assay. Similar to IL-6, both constitutive and IL-1α/β-inducible IL-11 release were dose-dependently inhibited by cortisol (P<0·01); at variance with IL-6, exogenous IL-11 dose-dependently decreased GR numbers in MG-63 cells (P<0·05), while anti-IL-11 antibody significantly increased GR numbers in both cell lines (P<0·05). IL-11-induced reduction of GR in MG-63 cells was confirmed by Western blot analysis. While exerting opposite effects on GR numbers, neither IL-6 nor IL-11 significantly modified GC-dependent inhibition of OPG release. Our data indicate that even physiological concentrations of cortisol negatively modulate IL-11 secretion and demonstrate, for the first time, an inhibitory effect of the cytokine on GR. Thus, the concept of autocrine–paracrine loops that modulate GC action and involve gp130 cytokines is corroborated. These loops could have clinical relevance for the dynamics of bone loss in patients given GC and having high concentrations of these cytokines in the bone microenvironment.


Introduction

Interleukin (IL)-11 belongs to the gp130 cytokine family, which also contains IL-6, oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, cardiotrophin 1 (the recently discovered novel neurotrophin-1/cardiotrophin-like cytokine) and Kaposi’s sarcoma-associated herpesvirus IL-6 (Nicholas et al. 1997, Heinrich et al. 1998, Senaldi et al. 1999). The activities of these cytokines are mediated through formation of receptor complexes sharing the signal-transducing glycoprotein gp130; such receptor complexes activate tyrosine kinases of the Janus family and the tyrosine phosphatase SHP-2, which in turn transduce via the STAT family of transcription factors and the MAP kinases pathway respectively (Heinrich et al. 1998).

Glucocorticoid (GC)-induced osteoporosis is the most frequent secondary osteoporosis. Its prominent pathogenetic feature is decreased bone formation; as far as bone resorption is concerned, an early and transient increase is followed by sustained decrease (Canalis & Giustina 2001, Weinstein 2001). IL-11 has been consistently shown to up-regulate osteoclastogenesis (Girasole et al. 1994), mainly through up-regulation of expression of receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) by cells of the osteoblastic lineage (Ragab et al. 2002). In vitro, IL-11 stimulates osteoblast differentiation of mouse mesenchymal progenitors (Taguchi et al. 1998, Suga et al. 2001). In vivo, IL-11 administration is able to recover impaired osteoblastogenesis and osteoclastogenesis seen in the P6 strain of senescence-accelerated mice.
Table 1 Functional phenotype of MG-63 and Saos-2 cells. Data are expressed as means ± s.e. (number of independent experiments)

<table>
<thead>
<tr>
<th></th>
<th>MG-63</th>
<th>Saos-2</th>
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<tbody>
<tr>
<td>ALP (nmol/min per 10⁶ cells)</td>
<td>&lt;1 (n=3)</td>
<td>1058 ± 170 (n=6)</td>
</tr>
<tr>
<td>IL-6 (pg/10⁶ cells)</td>
<td>1874 ± 729 (n=10)</td>
<td>46 ± 1·2 (n=3)</td>
</tr>
<tr>
<td>IL-11 (pg/10⁶ cells)</td>
<td>43 ± 20 (n=6)</td>
<td>572 ± 35 (n=6)</td>
</tr>
<tr>
<td>OPG (pg/10⁶ cells)</td>
<td>39 273 ± 10 912 (n=8)</td>
<td>824 ± 98 (n=4)</td>
</tr>
<tr>
<td>GR number (binding sites/cell)</td>
<td>110 380 ± 4499 (n=11)</td>
<td>31 783 ± 1847 (n=12)</td>
</tr>
<tr>
<td>affinity (nM)</td>
<td>2·8 ± 0·3 (n=11)</td>
<td>3·5 ± 0·4 (n=12)</td>
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ALP, alkaline phosphatase activity.

(Kodama et al. 1998). Transgenic mice overexpressing IL-11 show increased cortical thickness and are protected from age-related cortical bone loss, without any apparent alteration of osteoclastogenesis and bone resorption (Takeuchi et al. 2002). Most importantly, IL-11 has been shown to prevent, at least partially, dexamethasone (Dex)-induced apoptosis of osteoblasts (Kido et al. 2002). Taken together with the well-known inhibitory effect of GC upon IL-11 synthesis (Kim et al. 1999), these data have been interpreted as compatible with a role for IL-11 as a mediator of GC effects on bone.

We have recently investigated GC–IL-6 interplay in human osteoblast-like cells: we have confirmed that physiological concentrations of the naturally occurring GC cortisol dose-dependently decrease IL-6 secretion (Dovio et al. 2001a), and demonstrated for the first time that IL-6 modulates GC receptor (GR) numbers in both an autocrine and a paracrine way (Dovio et al. 2001b). GR number is a key determinant of GC sensitivity both in vitro and in vivo (Reichardt et al. 2000, Laudet & Gronemeyer 2002). Indeed, while the effects of GC on gp130-mediated cytokines secretion and activity have been widely investigated, little attention has been paid to these cytokines as potential modulators of the tissue sensitivity to GC. In the present study, we have investigated GC–IL-6 interplay in the two widely used and well-characterized human osteosarcoma cell lines, Saos-2 and MG-63, which constitutively have a divergent pattern of expression for IL-6, IL-11, alkaline phosphatase and GR. We report that, in contrast to IL-6, IL-11 exerts an autocrine-paracrine down-regulation of GR in Saos-2 and MG-63 cells. We also found that neither IL-6-induced nor IL-11-induced changes of GR number were paralleled by significant increase and decrease respectively, of a typical GR-mediated effect, i.e. inhibition of osteoprotegerin (OPG) release.

Materials and Methods

Materials

Recombinant human (h) IL-11, hIL-6 and anti-hIL-11 and anti-hIL-6 monoclonal antibodies (mouse IgG2b, clone #22616 and mouse IgG1, clone #6708 respectively) were purchased from R&D Systems (Abingdon, Oxon, UK); they were reconstituted using sterile PBS containing 1% BSA, stored at −20 °C, and diluted to the appropriate concentrations immediately before experiments.

Dex and cortisol (Sigma-Aldrich, Milan, Italy) were initially dissolved in 95% ethanol and then diluted in PBS to give a 1 mM stock solution, which was stored at −20 °C. The steroids were promptly diluted to final concentrations before each experiment and binding assay.

[1,2,4,6,7-3H]Dex was purchased from Amersham International (Amersham, Bucks, UK); before each binding assay, solvent was removed under a nitrogen stream and labeled steroid was then dissolved to final concentrations in appropriate medium. Scintillation fluid and scintillation vials were purchased from Packard Instruments Company (Meriden, CT, USA).

Bradford protein assay kit and horseradish peroxidase-labeled protein A were purchased from Biorad Laboratories (Hercules, CA, USA); all other reagents for Western blotting were purchased from Sigma-Aldrich.

Cells and culture conditions

The human osteosarcoma cell lines Saos-2 and MG-63 are two widely used experimental models for investigating osteoblast function. Saos-2 cells were kindly provided by Prof. M I Brandi, University of Florence; MG-63 cells were purchased from Interlab Cell Line Collection (National Institute for Cancer Research, Genoa, Italy). Saos-2 cells constitutively express alkaline phosphatase activity, as measured by a colorimetric method, while MG-63 cells do not (Table 1; results are expressed as nmol p-nitrophenol/min per 10⁶ cells). Saos-2 and MG-63 cell lines were cultured in McCoy medium (Sigma-Aldrich) and DMEM (Euroclone, Wetherby, UK) respectively, enriched with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM l-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich), in a humidified atmosphere of 95% air/5% CO₂ at 37 °C.

For experiments, cells were plated into six-well plates and treated when subconfluent. First, constitutive and
IL-1β-inducible IL-11 release and modulatory effects of cortisol were evaluated: both cell lines were treated with IL-1β (10 ng/ml), in the presence or absence of cortisol (0·01–1 μM), for 20 h. Cells were also incubated with IL-6 (0·1–2 ng/ml) or with an anti-hIL-6 antibody (100 ng/ml) for 20 h. After treatment, cell culture media were harvested and assayed for IL-11.

To test the effects of IL-11 on GR concentration and affinity, both cell lines were treated with increasing concentrations of IL-11 (0·1–100 ng/ml) or anti-IL-11 antibody (Saos-2: 1 μg/ml; MG-63: 0·5 μg/ml) for 20 h; in the absence of previous data on IL-11 action on GR, this incubation time was chosen according to the results of previous studies investigating the effects of IL-6 on GR (Rakasz et al. 1993, Dovio et al. 2001b). After treatment, cell culture media and cells were assayed for IL-6 and GR respectively.

Finally, to study the effects of cytokines on GC sensitivity, both cell lines were incubated with vehicle, IL-6 (2 ng/ml) or IL-11 (100 ng/ml) for 20 h; then cell culture supernatants were harvested and the same cells were further incubated for 20 h with either vehicle, or cortisol and Dex (0·01–1 μM). After treatment, cell culture media were assayed for OPG.

**Cell count and viability**

Cells were counted and evaluated for viability by trypan blue stain. Briefly, 50 µl of cell suspension were incubated in a dark tube with 50 µl of 0·4% (w/v) trypan blue solution, mixed thoroughly and allowed to stand for 5 min. Thereafter, the trypan blue–cell suspension mixture was transferred to both chambers of a hemocytometer, and cell viability was calculated as the ratio between total viable cells (unstained) and total cells (stained+unstained).

**IL-6, IL-11, OPG and soluble RANKL (sRANKL) ELISA**

After treatment, cell culture media were collected, centrifuged for 10 min at 2500 g at 4 °C, and the supernatants frozen at −20 °C. Supernatants were diluted when necessary before assay. IL-6, IL-11, OPG and sRANKL were measured in duplicate by sensitive ELISAs using commercially available kits (IL-6 and IL-11: R&D Systems; OPG and sRANKL: Immundiagnostik, Bensheim, Germany). Detection limits were less than 0·7, 8·27 and 80 pg/ml respectively; intra- and inter-assay coefficient of variation (CV) values were <10% – samples with CV values >10% were re-assayed. According to the manufacturer’s information, OPG ELISA detects all forms of OPG (i.e. monomeric and dimeric forms, free and complexed with sRANKL). sRANKL ELISA exploits recombinant OPG–precoated wells, and thus detects free sRANKL only (i.e. not OPG–complexed sRANKL). Results are expressed as pg/10⁶ cells.

**Graph binding assay**

After treatment, cells were washed twice with PBS and incubated with fresh phenol red-free medium containing 10% charcoal-stripped FBS for 1 h; subsequently, medium was removed and cells were incubated for 1 h with binding solutions containing high specific activity (82 Ci/mM) [1,2,4,6,7-3H]Dex at six progressively decreasing concentrations (46–2·25 nM), in the presence or absence of 200-fold molar excess of unlabeled Dex, to determine non-specific and total binding respectively. After incubation, cells were washed five times with ice-cold PBS buffer, scraped into 1 M NaOH and transferred to scintillation vials with scintillation fluid. Activity was counted by a scintillation β-counter. Six tubes with labeled Dex alone were assessed for total activity. Specific binding was calculated, at each GC concentration, as the difference between the totally bound reactivity and the non-specifically bound reactivity. Non-specific binding was calculated from the aliquots containing the 200-fold molar excess of non-radioactive Dex, assuming that non-specific binding was non-saturable and linearly related to the concentration of free GC. GC concentration and equilibrium dissociation constant (K_d) were determined by the Scatchard method (Fig. 1).

**Western blot analysis**

MG-63 cells were harvested and resuspended in lysis buffer (0·1 M Tris–HCl, pH 6·8, 2·5% SDS, 0·02 mM phenylmethylsulfonyl fluoride). Cell lysates were then sonicated and immediately frozen at −20 °C. A sample was taken for protein estimation by the Bradford protein assay.

One hundred micrograms of protein were resolved by electrophoresis through 8% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose in Tris–glycine buffer (25 mM Tris–HCl, pH 8·3, 250 mM glycine, 0·05% SDS, 20% methanol). Membranes were
stained with Ponceau S (0·2% in 5% trichloroacetic acid) to evaluate loading equivalency and transfer efficiency, and blocked with PBS containing 0·5% non-fat milk and 0·1% Tween 20. The blot was then washed with PBS and incubated overnight at 4 °C with an anti-GRα polyclonal antibody (rabbit polyclonal IgG, P-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, membranes were washed with PBS containing Tween 20 0·5%, reacted for 1 h at room temperature with horseradish peroxidase-labeled protein A, washed with PBS containing Tween 20 0·5%, reacted with chemiluminescent reagents (Amersham) and then processed for autoradiography. Autoradiographic films were analyzed by densitometric scanning using the Kodak Image Station 440 System, supported by Kodak 1D Image Analysis Software. Results were expressed as arbitrary units (AU)/µg total protein.

Statistics
Statistical analysis of data was performed with Statistica 6·0 (Statsoft Inc., Tulsa, OK, USA). When the effects of GC, IL-11 or IL-6 were studied, a multiple-measures ANOVA followed by the Newman–Keuls post-hoc comparison test was used. When the differences in GC effects in the cell lines were investigated, two-factor ANOVA was performed with one between-groups factor (cell type) and one within-subjects factor (cortisol concentration). P<0·05 was considered to represent statistical significance.

Results
The functional phenotype assessed in our laboratory for these cell lines is presented in Table 1. The capacity for releasing IL-6 and IL-11 was clearly different for Saos-2 vs MG-63 cells: the IL-6/IL-11 ratios in the medium were opposite, i.e. very low and very high respectively. Exposure of both cell lines to IL-1β resulted in a remarkable increase of cytokine release. Expression of GR was also very different in Saos-2 vs MG-63 cells, being approximately 3-fold higher in the latter. Neither cortisol nor IL-11 and IL-6 exerted any significant effect on cell count and viability in our experimental conditions. Cortisol at physiological and supraphysiological concentrations consistently inhibited both constitutive and inducible cytokine release (Fig. 2). Cortisol inhibition expressed as percent change vs control was more pronounced in MG-63 cells than in Saos-2 cells (P<0·02).

Divergent effects of IL-11 and IL-6 on GR numbers
MG-63 cells constitutively display a very high number of GRs; their production of IL-11 is poor in comparison to the abundance of that of IL-6. Exposure to increasing concentrations of IL-11 resulted in a parallel decrease of GR numbers. More than 40% reduction was attained with the maximal concentration of 100 ng/ml (Fig. 3). The effects of IL-11 were clearly divergent with respect to those of IL-6, which consistently led in the present and previous experiments to a dose-dependent increase of GR numbers.

Saos-2 cells constitutively display a relatively low number of GRs; their production of IL-11 is remarkably higher than that of IL-6. Again, clearly different effects of the exposure to IL-11 and IL-6 were noticed: while IL-6 was effective in enhancing GR numbers, no significant effect was apparent for IL-11.

In both cell lines, IL-11 deprivation by a monoclonal anti–hIL-11 antibody significantly increased GR numbers (Fig. 3). Neither exogenous IL-11 nor anti-hIL-11 antibody modified GR affinity expressed as $K_d$ (data not shown).
The effects of IL-11 (10–100 ng/ml) on GR in MG-63 cells were confirmed by Western blot analysis using a specific anti-GRα antibody (Fig. 4).

Since reciprocal regulation of IL-6 and IL-11 has been reported (Trepicchio et al. 1996, Nakchbandi et al. 2001), we also measured IL-6 and IL-11 concentrations in the medium of cells exposed to IL-11, IL-6, anti-hIL-11 and anti-hIL-6 antibodies. No effect of IL-11 on IL-6 release, and vice versa, was observed (n=3, data not shown).

**Discussion**

IL-11 is a gp130-mediated cytokine which is expressed in bone microenvironment and acts on both osteoblasts and osteoclasts. Consistent with previous reports, we have shown that the two human osteoblast-like cell lines MG-63 and Saos-2 both release IL-11, although at different levels (Lu et al. 1994), and that IL-1β stimulates release of IL-11 (Elias et al. 1995). Moreover, we have shown that...
the human endogenous GC cortisol is able even at physiological concentrations to inhibit both the constitutive and IL-1β-inducible IL-11 secretion. Our data complement previous observations that have dealt with the use of synthetic derivatives (Kim et al. 1999) and are compatible with the view that IL-11 is involved in the pathogenesis of osteoporosis due to both endogenous and exogenous GC excess (Kido et al. 2002).

The main finding of the present study, however, is the down-regulation of GR by IL-11. In the last decade, modulation of GR by cytokines has been demonstrated for IL-2, IL-4, tumor-necrosis factor-α, IL-10, IL-1α and -β in different GC-responsive cells (Kam et al. 1993, Verheggen et al. 1996, Sartori et al. 1998, Franchimont et al. 1999, Webster et al. 2001). With regard to IL-6, this cytokine has been shown to up-regulate GR in cells of Kaposi’s sarcoma (Guo et al. 1996), lymphoid (Rakasz et al. 1993) and osteoblast-like cells (Dovio et al. 2001b). To the best of our knowledge, the present report is the first dealing with a modulatory effect of IL-11 on GR. In our experimental conditions, IL-11 significantly decreased GR numbers in MG-63 but not in Saos-2 cells. This could be attributable to the different constitutive expression of IL-11, which is much higher in Saos-2 than in MG-63 cells. Data obtained with the use of a highly specific anti-hIL-11 antibody are consistent with an autocrine down-modulatory effect. It is reasonable to think that in Saos-2 cells the constitutive production is enough to attain the effect at the best of its potentiality; consequently, only the deprivation (and not the addition) of the cytokine could result effectively in changing GR numbers. On the other hand, in MG-63 cells the constitutive production of IL-11 is much lower. This offers an explanation for the fact that exogenous enrichment of the signal significantly decreases the same binding. Taken together, our previous and present findings point to an opposite regulatory role of IL-6 and IL-11 upon GR numbers in osteoblast-like cells, positive and negative respectively.

It could seem surprising that IL-6 and IL-11, which belong to the same cytokine family and transduce through the same signaling molecule, are able to exert divergent effects in the same cell type. Indeed, IL-6 and IL-11 have different roles in vivo, as suggested by knock-out models (Poli et al. 1994, Sims et al. 2002). It is not clear whether such differences result from different expression
and regulation of specific receptor subunits in the target cells, or from different patterns of activation of intracellular pathways also. In this respect, it has been reported that ternary complexes of IL-11–IL-11 receptor–gp130 and IL-6–IL-6 receptor–gp130 have a very similar overall geometry and activate the same Jak-STAT pathway, but different biological responses in the same cell type could depend on additional receptor components or differences in the kinetics of ligand–receptor complex formation (Dahmen et al. 1998). As a matter of fact, in murine peritoneal macrophages, IL-11 and IL-6 transduction pathways have been demonstrated to be different, in that both IL-6 and IL-11 activated STAT3, whereas IL-6 but not IL-11 activated STAT1 (Trepicchio & Dorner 1998). Differences in other gp130-activated pathways, such as the SHP-2/MAP kinases pathway, could also be relevant to this point.

Since IL-11 has been shown to down-regulate lipopolysaccharide-induced IL-6 release by macrophages (Trepicchio et al. 1996), and IL-6 has been found to down-regulate parathyroid hormone-induced IL-11 release by osteoblasts (Nakchbandi et al. 2001), reciprocal regulation could be involved in the observed divergent effects on GR. An additional support to this interpretation could come from the inverse constitutive expression of these cytokines in our cell lines. Nonetheless, it seems not to be the case, since neither exposure to IL-11 or anti-hIL-11 antibody modified IL-6 release, nor exposure to IL-6 or anti-hIL-6 antibody modulated IL-11 release in both cell lines. Our data do not exclude the possibility that reciprocal inhibitory effects may occur when cytokines release is induced by appropriate secretagogues; however, at least under basal conditions, the mechanisms by which IL-11 down-regulates GR are likely to be independent of IL-6, and need further investigation. Since IL-11 has been found to up-regulate IkBα (Trepicchio et al. 1997), the recent description of an NF-κB response element in the promotor region of GR gene could be of relevance (Webster et al. 2001).

Consistent with previous reports, we have shown that both Saos-2 and MG-63 cells express OPG, although at quite different levels (Vidal et al. 1998a, Kinpara et al. 2000). On the other hand, we could not detect appreciable levels of free sRANKL in our media. This could be due to intrinsic limitations of currently available ELISAs, which utilize OPG as an immobilized capture component and therefore may be interfered with by OPG content in the sample (Kinpara et al. 2000). RANKL/OPG ratio is admittedly a major determinant of osteoclastogenesis, osteoclast activity and apoptosis, and therefore of bone resorption (Hofbauer & Heufelder 2000, Horowitz et al. 2001). We have confirmed a strong inhibitory effect of both cortisol (at physiological and supraphysiological concentrations) and Dex on OPG release. GC-induced inhibition of OPG expression is likely to be due to genomic mechanisms, since it has been reported to take some hours (Vidal et al. 1998a); moreover, our data suggest that such an inhibition is GR- rather than mineralocorticoid receptor-mediated, since similar effects were elicited by cortisol and Dex as well.

Treatment with IL-6 and IL-11 slightly increased OPG release in our cells. Data on the effects on OPG expression of gp130 cytokines are still conflicting (Horwood et al. 1998, Vidal et al. 1998b, Aubin & Bonnelye 2000, Yano et al. 2001, Ragab et al. 2002). It is widely accepted, however, that cells of the osteoblastic lineage exposed to these cytokines up-regulate osteoclastogenesis. Pertinently, it has been demonstrated that IL-11 up-regulates both RANKL and OPG mRNAs; yet the net balance is an increased RANKL/OPG ratio, hence a pro-resorptive signal (Horwood et al. 1998).
Limitations of our study should be noted. First, it deals with osteosarcoma cell lines, and not with primary cultures of normal osteoblasts. Secondly, we failed to demonstrate any change in GC sensitivity. In fact, we have found that while modulating GR number, neither IL-6 nor IL-11 significantly modified the inhibitory effects of GCs on OPG release. An explanation could be seen in the redundancy of GR for the genomic regulation of OPG while modulating GR number, neither IL-6 nor IL-11 significantly modified GC sensitivity of the osteoblast-like cell lines with different constitutive IL-6 expression. Cytokine 47–52.


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Received 5 December 2002
Accepted 8 January 2003