Effect of glucocorticoids and oestrogen on interleukin-6 production by human thyrocytes from patients with Graves’ disease and toxic multinodular goitre and from HTori3 cells

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Abstract

Interleukin-6 (IL-6) is a cytokine released by thyrocytes and is involved in disease processes such as autoimmune thyroid disease. The secretion of IL-6 can be stimulated by interleukin-1 (IL-1), tumour necrosis factor-α (TNF), serum, TSH and agents which increase intracellular cyclic AMP levels. Antithyroid drugs such as methimazole inhibit IL-6 production by thyrocytes but the effects of glucocorticoids and oestrogen have not been investigated. The effects of dexamethasone and 17β-oestradiol on IL-1-, TNF-, TSH-, forskolin- and phorbol 12-myristate 13-acetate (PMA)-stimulated IL-6 release in serum-free conditions were studied in human thyrocytes derived from patients with Graves’ disease and toxic multinodular goitres, and in the immortalised human thyrocyte cell line, HTori3.

Dexamethasone inhibited IL-6 production under stimulated conditions. In serum-free conditions, no basal release of IL-6 was assayable. In all but one of the primary thyroid cultures, TSH did not stimulate IL-6 release above the lower detectable limit of the assay. In Graves’ and multinodular goitre thyrocytes, inhibition of IL-1 (100 U/ml)-stimulated IL-6 release by dexamethasone (100 nmol/l) was 62.51%±10.43 (s.e.m.), and in HTori3 cells it was 78.35%±3.9. The degree of IL-1 stimulation of IL-6 release and inhibition by dexamethasone was not significantly different in thyrocytes derived from either Graves’ or multinodular glands. 17β-Oestradiol had no effect on IL-1-stimulated IL-6 release in either primary thyroid cell culture or in HTori3 cells.

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Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine which is synthesised and released within the endocrine system under normal and disease conditions (1, 2). Human thyrocytes from patients with Graves’ disease express IL-6 mRNA and secrete IL-6 when cultured (3–7). IL-6 release from these cells can be stimulated by thyrotrophin (TSH), tumour necrosis factor-α (TNF), γ-interferon and interleukin-1 (IL-1) (4, 8). IL-6 is also secreted by normal rat thyrocytes (8), rat FRTL-5 cells (8, 9) and the human thyrocyte cell line HTori3 (10), which comprises thyroid cells immortalised by transfection with simian virus 40 DNA (11). IL-1β and TSH alone enhance IL-6 production from the rat thyrocyte cell line FRTL-5 (8, 9). IL-6 release from HTori3 cells is also stimulated by IL-1α, TNF, γ-interferon, high doses of TSH and by increasing intracellular cyclic AMP levels with forskolin (10). These cells provide a useful tool to study IL-6 release by thyrocytes as they possess no contaminating cells such as lymphocytes, fibroblasts or macrophages which can secrete IL-6 independently (11). The function of IL-6 is not clear but it has been shown to inhibit thyroid hormone secretion and thyroid peroxide gene expression in human thyrocytes (12).

The IL-6 gene promoter has two inhibitory glucocorticoid elements in addition to the stimulatory cyclic AMP and multiresponsive elements which respond to cellular stimulation with IL-1, TNF, serum, phorbol esters and IL-6 (13). Oestradiol in some tissues also inhibits IL-6 gene expression (14–17). We have investigated and compared the effects of glucocorticoids and oestradiol on stimulated IL-6 secretion from thyrocytes derived from patients with Graves’ disease and multinodular goitres, and those of the HTori3 cell line. The experiments were performed in serum-free conditions to eliminate the stimulatory effect of fetal calf serum on IL-6 release. This study has previously been reported in abstract form (18).
**Materials and methods**

**Primary thyroid cell culture**

Dispersed thyrocytes were prepared from human thyroid tissue which had been routinely excised for the treatment of either Graves’ disease (n=5) or toxic multinodular goitre (n=3). All eight patients had received antithyroid drugs prior to surgery to control their hyperthyroidism. The tissue was trimmed of fat and fibrous tissue and minced finely then suspended in PBS, pH 7.4. The suspension was filtered through sterile gauze and then resuspended in PBS with 0.5% dispase (Boehringer Mannheim, Lewes, East Sussex, UK) and incubated for 30 min at 37°C with a magnetic stirrer. The tissue was again filtered through gauze and the supernatant collected. The remaining tissue was further incubated with PBS and 0.5% dispase for 30 min, filtered and the supernatant collected. The two supernatants were centrifuged at 1000 g for 10 min. Cells were suspended in RPMI 1640 and seeded onto 9 cm plates overnight at 37°C. They were then washed to remove any remaining blood cells, the medium was changed and the cells grown to confluence. The cells were then removed from the plate by trypsin and centrifuged at 1000 r.p.m. for 10 min. The pellet was resuspended in RPMI 1640 and the cells counted. The cells were plated in 24-well plates (Costar, High Wycombe, Bucks, UK) at a concentration of 200,000 cells per well in 0.5 ml RPMI 1640 containing 10% (v/v) fetal calf serum (FCS), penicillin (100 mg/ml), streptomycin (100 mg/ml), i-glutamine (2 mmol/l), fungizone (1.23 mg/ml) and neomycin (50 µg/ml) (all of these reagents were from Northumbria Biologicals Ltd, Cramlington, Northumberland, UK). The cells were incubated at 37°C in an atmosphere of 95% air and 5% CO₂.

**Cell line**

The HTori3 cell line has been described previously (20). Cells were grown to confluence in 75 cm² flasks in RPMI 1640 as described above. The cells were trypsinised and plated onto 24-well plates at a density of 2x10⁵ cells/ml in RPMI 1640 with 10% FCS (0.5 ml/well).

**Experiments**

Cells were grown to confluence over 24–48 h, washed with Hank’s balanced salt solution and test substances were added in 0.5 ml RPMI 1640 with 0.1% (w/v) BSA (serum-free conditions). We have previously found no significant differences in our experiments between cell number or [³H] thymidine uptake of thyroid cells and the relative position of the wells (peripheral vs central). Dexamethasone was initially dissolved in absolute alcohol but after dilution with medium to the concentrations used, the final concentration of ethanol was 0.001%. Toxic effects are unlikely at this concentration. After 48 h the medium was aspirated and stored at −20°C until assayed. The effects of TNF, forskolin and phorbol 12-myristate 13-acetate (PMA) were not studied in primary cultures because of insufficient cell numbers. Statistical analysis was by analysis of variance.

**IL-6 enzyme-linked immunosorbent assay**

Flexible 96-well Costar plates (Costar) were coated with 50 µl sheep polyclonal antibody to IL-6 (Eurogenetics UK Ltd, Hampton, UK) at 2 µg/ml in a 0.05 mol/l sodium carbonate buffer solution, pH 9.6, and incubated at 37°C for 2 h. Non-specific binding sites were blocked with 5% BSA in Tris-buffered saline (TBS) (150 µl) overnight at 4°C. Plates were washed three times with TBS and 0.02% Tween 20 between each of the following steps. Samples and standards (diluted in appropriate culture media) were added and incubated for 2 h at 37°C. After washing, monoclonal antibody to human IL-6 (1 µg/ml) (Eurogenetics UK Ltd) was added for 1 h at 37°C. Biotinylated anti-mouse IgG (Amer sham International plc, Amersham, Bucks, UK) was added for 30 min and then streptavidin conjugated to alkaline phosphatase at 1/1000 dilution for a further 30 min at 37°C. Alkaline buffer solution (1.5 mol/l, 2-amino-2-methyl-1-propanol (Sigma Chemical Co., Poole, Dorset, UK), pH 10.3) was added, 50 µl to each well, followed by 50 µl phosphatase substrate (Sigma) prepared at 10 mg/ml in distilled water. Plates were incubated at 37°C until fully developed. Fifty microlitres NaOH (0.1 mol/l) were added to stop the reaction and the absorbance was read at 414 nm on a Dynatech MR500. The reaction was then decolourised with 50 µl 4 mol/l HCl per well and the absorbance subtracted from the first to give the absorbance due to the specific enzyme reaction. The detection limit (X₁) of the assay was 4 U/ml, which was calculated using the equation

\[ X_1 = X_{bl} + K \cdot S_{bl} \]

where \( X_{bl} \) is the mean of blank measurements, \( S_{bl} \) is the standard deviation of the blank measurements, and \( K \) is a numerical factor chosen based on the confidence level desired (value 2). This obtains a detection limit that has a probability of 95%. The coefficient of variation for the assay was 8.3%.

**Chemicals**

Dexamethasone, 17β-oestradiol, forskolin and PMA were all purchased from Sigma. Recombinant human IL-1α and TNFα were gifts from Hoffman-La Roche Inc., Nutley, NJ, USA, and Dr G R Adolph, Ernst-Boehringer Institut für Arzneimittelforschung, Vienna, Austria respectively. TSH (bovine) was provided by the National Institute of Biological Standards and Control, Potters Bar, UK.
Results

Dexamethasone inhibited IL-1-stimulated IL-6 production in all of the primary thyroid cultures (Fig. 1). There were no significant differences in this effect between cultures derived from either Graves’ or toxic multinodular glands. The degree of inhibition did, however, differ between cultures from different Graves’ and toxic multinodular goitres, but it is known that there is significant variability between cells derived from different thyroid glands (19). Two of the cultures, one from a patient with Graves’ disease and one from a patient with a multinodular goitre, were relatively resistant to dexamethasone compared with the other thyroid cultures. Cumulated data in this study showed that dexamethasone (100 nmol/l) inhibited IL-1 (100 U/ml)-stimulated IL-6 release by 62.51%±10.43 S.E.M. (n=8). Dexamethasone also inhibited IL-1-stimulated IL-6 release from HTori3 cells with dexamethasone (100 nmol/l) inhibiting IL-1 (100 U/ml)-stimulated IL-6 release by 78.35%±3.9 (n=6 experiments).

In HTori3 cells, IL-6 production stimulated by (a) TNF 100 U/ml alone was 32 ± 4 S.D. U/ml and TNF with dexamethasone 100 nmol/l 6.2±0.2 U/ml (P<0.005); (b) forskolin 10 μmol/l was 10.1 ± 0.4 U/ml with dexamethasone 100 nmol/l <4 U/ml and (c) PMA 100 nmol/l 37 ± 2 U/ml, with dexamethasone 100 nmol/l 6 ± 0.7 U/ml (P<0.005). These substances were only tested in HTori3 cells as insufficient cell numbers of primary thyrocytes were available. TSH (5000 μU/ml) had no significant stimulatory effect on basal IL-6 secretion under serum-free culture conditions except in one multinodular goitre where TSH stimulated IL-6 release to 10.1±0.1 U/ml which was suppressed to <4 U/ml in the presence of dexamethasone (100 nmol/l). 17β-Oestradiol had no effect on IL-1-stimulated IL-6 secretion in thyrocytes derived from Graves’ or toxic multinodular glands, or from HTori3 cells.

Discussion

We have demonstrated that glucocorticoids are very effective inhibitors of stimulated IL-6 production from human thyrocytes both in primary cultures and in the HTori3 cell line. Glucocorticoids are known to repress strongly IL-6 gene expression (14) in a variety of cell types, and it is likely that they have a similar action in thyrocytes, specifically at the inhibitory glucocorticoid element of the IL-6 promoter. HTori3 cells are more sensitive to dexamethasone than either thyrocytes from Graves’ or multinodular glands. A possible explanation for this finding is that primary cultures are to a certain extent, contaminated with other cell types such as fibroblasts, macrophages and lymphocytes, which potentially may produce other factors which affect IL-6 production.

There were differences in the degree of glucocorticoid-induced IL-6 inhibition between individual primary cultures of either Graves’ or multinodular thyrocytes but not between the two different pathological types. This is interesting in that in an autoimmune disease the cells may be expected to have a different cytokine response compared with a condition which is thought to be non-autoimmune. The reason why Graves’ thyrocytes have different sensitivities to dexamethasone is not clear. All of the patients had been treated with antithyroid drugs prior to surgery and potentially these agents could have influenced the cytokine response.

We found no effect of 17β-oestradiol on IL-6 production by thyrocytes. The phorbol ester (PMA) stimulated IL-6 secretion by the thyrocytes. This effect, although known for other cell types, has not previously been demonstrated in thyrocytes. We did not demonstrate any significant stimulation of IL-6 release by TSH in the absence of FCS (with the exception of one primary culture). This finding is in contrast to the stimulatory

Figure 1 Effect of dexamethasone on IL-1-stimulated IL-6 secretion from thyrocytes from five patients with Graves’ disease and toxic multinodular goitre (MNG) (representative examples) (data expressed as means±S.D. for triplicate wells), and from HTori3 cells (cumulated from three experiments) (data expressed as means±S.E.M.). *P<0.05, **P<0.005 compared with IL-1-stimulated IL-6 secretion (analysis of variance).
effect observed in other studies in the presence of FCS (4, 9, 10).

The source of IL-6 in primary cultures of thyroid cells appears to be mainly from the thyrocytes, as the level of IL-6 release is not significantly affected by reduction of contaminating cells (3), and phytohaemagglutinin, which stimulates IL-6 secretion specifically from peripheral mononuclear cells, has no effect on IL-6 release in thyroid cell cultures (4). The antithyroid drugs, methimazole and propylthiouracil, have been shown to block the IL-6 response to sublethal complement attack in thyrocytes from Graves’ glands (20). Antithyroid drugs may, in part, be responsible for the reduction in lymphocytic infiltrate and thyroid auto-antibodies observed after such therapy. In addition, glucocorticoids but not oestrogens can regulate the stimulated production of IL-6 by human thyrocytes. This effect may have a role in the normal regulation of thyrocyte function but, more importantly, it may have a therapeutic action in autoimmune disease. The local production of IL-6 in the immediate cellular microenvironment may be part of a local homeostatic mechanism and/or cellular alarm system (1) regulating thyrocyte function.

References