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A SYNTHETIC CORTICOSTEROID, DEXAMETHASONE REGULATES GENERATION OF SOLUBLE FORM OF INTERLEUKIN-6 RECEPTOR OF HUMAN LYMPHOCYTES, *IN VITRO*

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In contrast to most of the soluble cytokine receptor antagonists properties, the soluble IL-6 receptor (sIL-6R) occurring in various body fluids of healthy persons and patients with various diseases is an agonist. The enhancing effect is due to its ability to form complex with IL-6 and to bind to gp130 making constitutively IL-6 receptor negative cells responsive for IL-6. The generation as well as the functional role of soluble IL-6 receptor is poorly understood. Earlier, we found that the sIL-6R levels in sera of patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) were higher than those of the control group measured by ELISA sandwich technology.

In the present study we detected different levels of sIL-6R in the supernatants of lymphocyte cultures of healthy persons and patients with RA as well as SLE. Moreover, we found, that *in vitro* dexamethasone treatment stimulated generation of sIL-6R in both healthy persons and in active SLE, while it strongly suppressed production of sIL-6R in both RA groups. At mRNA level, we found that in SLE both the IL-6R mRNA encoding the membrane spanning and alternatively spliced (soluble) variants increased. Surprisingly, the strong decrease of sIL6R protein in RA was not found at mRNA level.

Keywords: Soluble interleukin-6 receptor (sIL-6R) – rheumatoid arthritis (RA) – systemic lupus erythematosus (SLE) – dexamethasone

INTRODUCTION

Interleukin-6 (IL-6), plays a central role in the regulation of hepatic acute phase reaction, maturation of B- and plasma cells, immunoglobulin production, that way in mucosal IgA production, B-cell malignomas, development of several autoimmune diseases, and – by osteoclast stimulation – in the pathogenesis of oestrogen deficient osteoporosis [15, 16, 18]. Primary sources of IL-6 are fibroblasts, monocytes, keratinocytes and T-lymphocytes [16]. Activated B-cells of SLE patients also produce IL-6 and express IL-6 receptor (IL-6R) on their surface and by autocrine stimulation IL-6 can upregulate function of B cells [17, 20].

IL-6 binds to its specific receptor (IL-6R), a ligand specific, 80 kDa alpha subunit followed by attachment to gp130 molecule (130 kDa), a molecule shared by other

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cytokine receptors which is, involved in binding of IL-11, oncostatin-M (OM), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and cardiotrophin-1 (CT-1). Upon binding IL-6 to the receptor, gp130 is homodimerized and is subsequently involved in down-stream signal processes binding tyrosine kinases (JAKs) and activating STAT 1 and STAT3 transcriptional factors [8]. Both IL-6R and gp130 occur also in soluble form in biological fluids. Recently we proved by endonuclease digestion and direct nucleotide sequencing [10] that in addition to surface cleavage, alternative splicing is also involved in the generation of sIL-6R. Soluble IL-6R (sIL-6R) makes a complex with IL-6 in fluid phase and this complex can bind to the membrane gp130 provoking more or less similar signal like IL-6 does following binding to the membrane anchored receptor [13]. Since gp130 is expressed on practically all nucleated cells sIL-6R plays considerable physiological importance; it makes cells (even without endogenous IL-6 receptor expression) to be IL-6 responsive [14]. Other consequence of the presence of sIL-6R is that the receptor-ligand complex prolongs the IL-6 serum half-life, and the duration of the IL-6 effects [22].

The correlation between IL-6 and systemic autoimmune syndromes is well documented [5, 7, 12], but there is less information about the relevance of sIL-6R in these diseases. High levels of IL-6 were found in sera of patients with systemic lupus erythematosus (SLE) during acute exacerbation and some weeks before. Effective treatment of SLE patients normalised the elevated IL-6 and soluble IL-2 levels as well [24]. High levels of IL-6 were found in rheumatoid arthritis (RA) patients' synovial fluids, and that correlated with plasma IL-6 levels. The *in vitro* IL-6 production of RA patients' synovial cells was higher than that of osteoarthritic synovial cells [5]. We also described the *in vivo* occurrence of sIL-6R in blood of SLE and RA patients with inactive and active stages as well as in healthy persons [23]. In this work we found *in vitro* generation of sIL-6R by lymphocytes in cell cultures and opposite effect and dissimilar molecular regulation by dexamethasone.

MATERIALS AND METHODS

Patients and controls

Thirteen patients with active SLE (13 females), 11 with inactive disease (3 males, 8 females), 8 patients with active RA (3 males, 5 females), 5 with inactive stage of RA (1 male, 4 females) were investigated. All SLE patients met the American College of Rheumatology 1982 revised criteria for the classification of SLE [27], RA patients were also classified according to the American Rheumatism Association-1987 criteria [2]. As a sign of disease activity of SLE patients SLEDAI score [3], erythrocyte sedimentation (ESR), antinuclear antibodies, anti-DNA, C3 and C4 levels, that of RA patients number of swollen and painful joints, ESR, C reactive protein levels and platelet number were used. Ten healthy control persons (2 males, 8 females) with no personal or family anamnesis of autoimmune diseases were also investigated.

The means of age of healthy control persons, active and inactive SLE and RA patients were 43.3, 39.5, 52.4, 52.9 and 51.8 years, respectively. The average disease durations for active and inactive SLE and RA patients were 5.99, 10.02, 7.94 and 17.4 years, respectively.

None of the control (healthy) group members looks drugs, members of both active and inactive SLE group have already got earlier steroid therapy, except 2 patients (one with active and one with inactive SLE), who took nonsteroid antiinflammatory drugs. Beside the above therapy 3 patients of the active SLE group got cyclophosphamide, 5 of them azathioprine, 3 of them hydroxychloroquine medication. Five patients with inactive stage of SLE took chloroquine derivates, 2 patients azathioprine. Five members of the active RA group participated in steroid therapy, 3 patients got nonsteroids. Four patients took methotrexate, 2 patients aurothiomalate as disease modifying drugs. Two patients with inactive RA got nonsteroids, 3 patients low dose steroid, beside that 2 patients got azathioprine.

Samples and in vitro *culture conditions*

Heparinised blood samples were taken and plasma aliquots were kept at –70 °C and thawed only when analysed. Lymphocyte enriched peripheral blood mononuclear cells were isolated from heparinised peripheral blood by Ficoll-Hypaque gradient. The cells were *in vitro* treated in RPMI with 100 pM and 100 μM concentrations of dexamethasone or without dexamethasone for 48 hours at 37 °C in CO_2 thermostat. Viability of cells remained over 95%, as judged by trypan blue exclusion. The supernatans were taken from 48 hours lymphocyte cultures of the same patients. Supernatants of lymphocyte cultures were hold at –70 °C until testing.

ELISA for quantification of sIL-6R levels

Plastic plates (Nunclon, Nunc, Denmark) were coated in +4 °C for 24 hours by monoclonal murine anti-human IL-6R antibody (concentration: 1 μg/ml) (generous gift of dr. Kiyoshi Yasukawa, Tokyo), then incubated with samples of unknown concentrations sIL-6R and with recombinant sIL-6R standards in 37 °C for 1 hour, then in room temperature for 30 min. As a standard supernatants of NIH 3T3 cell line, transfected with plasmid containing IL-6R gene were used (provided by Prof. dr. Stefan Rose-John, Mainz, Germany). Then the plates were incubated with rabbit polyclonal anti-IL6R antibody (SIGMA, Budapest) (1 μg/ml) at room temperature for 30 min. As a second antibody horseradish peroxidase conjugated goat anti-rabbit antibody (SIGMA, Budapest) was used. The reaction was developed by OPD (Reanal, Budapest). All samples were measured in duplicates.

Isolation of RNA and RT-PCR for IL-6 receptor gene expression

Isolation of total RNA from cells was carried out by acid guanidine-phenol method [4]. Reverse transcription and polymerase chain reaction was performed on a Pharmacia Ataq Gene Controller, by using a mixture of 20 pmole/µl sense and antisense primers. RT PCR reagents and reverse transcriptase were purchased from Promega/Eastport and Perkin-Elmer, respectively. The oligonucleotide primers (Pharmacia) for IL-6R spanning the sequence of the cDNA 811. bp- 1195.bp [19] were:

5' – AAG GAC CTC CAG CAT CAC TGT GTC A – 3' as sense, and

5' – CCT TCA GAG CCC GCA GCT TCC ACG T – 3' as antisense.

Glycerolphosphate dehydrogenase (GAPDH) (sense: 5' GGT ATC GTG GAA GGA CTC AT3', antisense: 5' ACC ACC TGG TGC TCA GTG TA3') [1] was used as an internal "house-keeping" control.

Statistical analysis

For comparisons between sIL-6R levels in control group and two diseases groups, and for comparisons between supernatants of dexamethasone treated or untreated groups 2-tailed Student's *t*-probe was applied.

RESULTS

Figure 1 shows the soluble IL-6R levels of supernatants of lymphocyte cultures from healthy controls, SLE and RA patients. Similarly to the earlier findings in plasma [23], we found the highest amount of sIL-6R in culture supernatants of SLE group, exceeding that of in lymphocyte cultures of healthy controls ($p > 0.079$) and that of RA patients ($p < 0.05$). sIL-6R values of lymphocytes of RA patients are significantly lower compared to those of healthy controls.

A synthetic corticosteroid, dexamethasone strongly influences the *in vitro* generation of soluble IL-6 receptor. Both in healthy controls and in lymphocyte cultures of SLE patients, dexamethasone (100 pM) significantly increases the amount of sIL-6R (Fig. 2A). Oppositely, in RA 100 pM dexamethasone induces a sharp and highly significant suppression of the concentration of sIL-6R. Similar results were found when high concentration of dexamethasone (100 µM) was applied (not shown).

We tried to clarify the molecular background of generation of sIL-6R, we isolated mRNA from lymphocytes of patients with SLE and RA and using reverse transcriptase polymerase chain reaction the actual ratio of mRNAs encoding for cell-bound (385 bp) and soluble IL-6R (appr. 310 bp) was studied. We found (Fig. 2B) that in

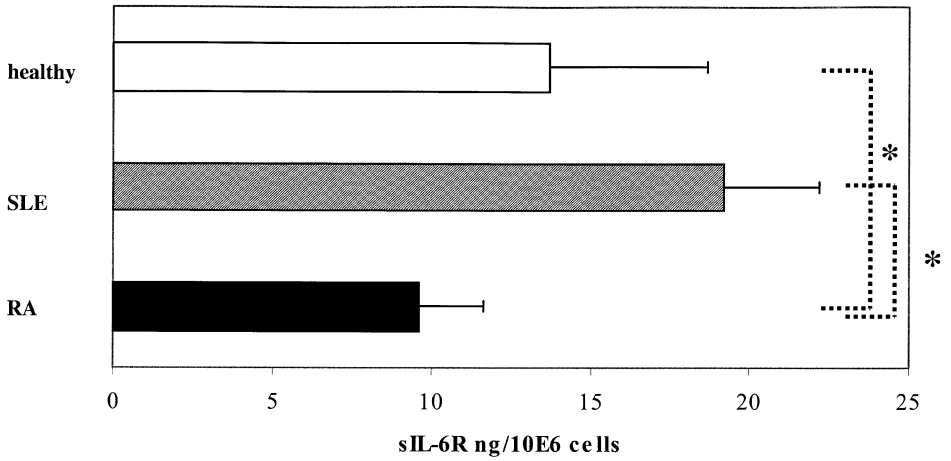

Fig. 1. Soluble interleukin-6 receptor (sIL-6R, ng/10^6 cells/48 hours, mean ± SE) in culture supernatants of lymphocytes separated from the blood samples of healthy donors (n = 9) and of systemic lupus erythematosus (SLE, n = 23) and rheumatoid arthritis (RA n = 13) patients. Quantity of sIL-6R was determined by "sandwich" ELISA method (for details see Materials and Methods). P values (Student *t*-probe) are indicated, *: $p < 0.05$

the representative samples from SLE patients (1 and 2) and a healthy person (not shown) a moderate upregulation of the mRNA moieties of both cell-bound and soluble IL-6R was found, reminding to the tendency found at protein level (Fig. 2A).

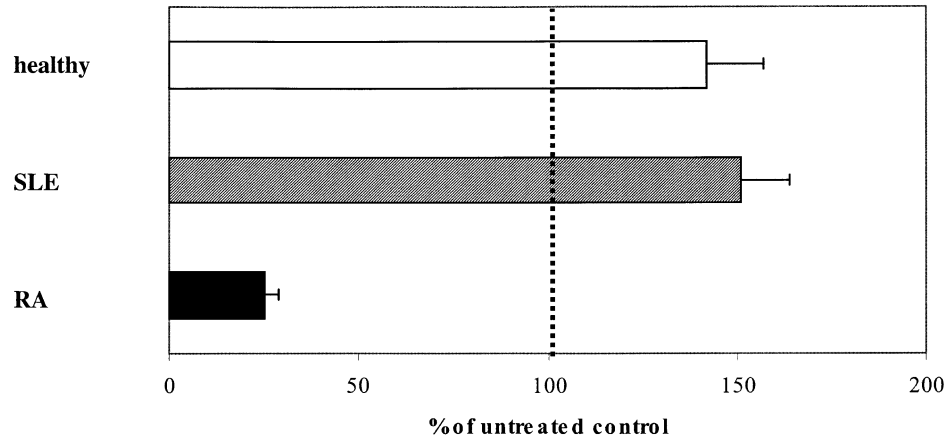

Fig. 2A. Changes in the *in vitro* production of soluble IL-6 receptor by lymphocytes cultivated for 48 hours in the presence of 100 pM dexamethasone. Quantity of sIL-6R was determined by "sandwich" ELISA method (for details see *Materials and Methods*). The columns are representing percent of untreated controls (100%, indicated by the dotted line). All three values are significantly different ($p < 0.01$) from their own control (untreated) values

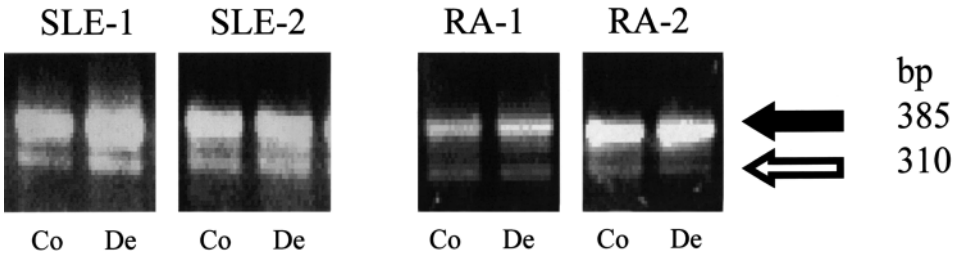

Fig. 2B. Reverse transcriptase polymerase chain reaction (RT-PCR) of IL-6 receptor from lymphocytes of patients with SLE and RA patients. The cells were incubated with medium (Co) or with 100 pM dexamethasone (De) for 48 hours. Positions of IL-6 receptor mRNA corresponding to cell-bound receptor and soluble IL-6R are shown by black and white arrows, respectively. For details see Materials and Methods

However, the sharp decrease of sIL-6R observed at protein level of lymphocytes of RA patients (Fig. 2A) was not reflected by closely similar IL-6R mRNA levels of RA patients (Fig. 2B). Glycerolphosphate dehydrogenase (GAPDH) expression showed no differences (not shown).

DISCUSSION

Soluble interleukin-6 receptor levels known to be markedly elevated in chronic inflammatory diseases, such as HIV-infection [11], myeloma multiplex [6] and interstitial pulmonary diseases [28]. Corresponding these data elevated sIL-6R levels were expected as reported for soluble TNF alpha receptor and soluble IL-2 receptor in patients with RA, SLE or psoriatic arthritis [21, 25].

Earlier we examined patients with various autoimmune diseases and we failed to find any correlation between IL-6 and sIL-6R levels in blood samples of patients with different autoimmune diseases [9]. As far as the source of the circulating sIL-6R is concerned (regardless its significance to estimate disease activity), one can speculate, that lymphocytes are not the only cells, but represent a major part of cell fraction with capacity to shed or secrete their IL-6 receptors.

The therapeutically potent corticosteroid, dexamethasone, like other glucocorticosteroids markedly inhibits transcription of IL-6 (and many other cytokines) [16, 26] however, simultaneously enhances the expression of IL-6R on the cell membrane. Our experiments do not provide reliable molecular explanation, why dexamethasone acts so differently (upregulation in SLE, and strong inhibition in RA) on the generation of sIL-6R in these two disease groups. Our present data suggest opposite *in vitro* action of dexamethasone on the production of sIL-6R in SLE and RA and one may speculate that the molecular events of *in vitro* shedding or alternative splicing in the lymphocytes of SLE or RA patients is dissimilarly regulated. One cannot exclude the possibility that the TH1/TH2 ratio is dissimilar in peripheral blood of SLE and RA patients, and the amount of sIL-6R level is somehow related to this difference.

The alteration of shedding rate of sIL6R cannot be explained by dissimilar previous medication of patients. By the analysis of mRNA specimen corresponding to the membrane spanning 80 kDa and also the *truncated* forms, further complexities became obvious. Only a mild increase has been detected in the amount of both mRNAs in SLE, but no visible decrease of it in RA. It is assumed that the expression of IL-6R gene and its alternatively spliced form could be somehow elevated by dexamethasone in SLE. Oppositely, mRNA level of IL-6 receptors in lymphocytes from RA patients does not follow the major suppression of sIL-6R at protein level. The presence of mRNA for "truncated" sIL-6R but sharp decrease in sIL-6R protein may also reflect a posttranscriptional (translational or secretory) block in lymphocytes from RA. The idea, that dexamethasone perhaps inhibits proteolytic enzymes hat causing a decrease in sIL-6R in culture supernatants requires further comparative studies in lymphocytes of SLE and RA.

We failed to find any effect of IL-6 and IL-1 in generation of sIL-6R *in vitro* (unpublished observations). Another reason for difference between *in vitro* production of sIL-6R by lymphocytes of RA and SLE patients, may be the dissimilar number of corticosteroid receptors in these diseases. Further studies are in progress to test this possibility.

These data may provide further points to understand the regulation of IL-6 related local events in systemic autoimmune diseases, such as SLE and RA. Considering corticosteroid therapy, the pathophysiological relevance of increased (SLE) or decreased (RA) sIL-6R levels may differently affect the consequences, i.e. elevated biological plasma "half-lifetime" of IL-6 and agonist action of sIL-6R-IL-6 complexes.

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