Inhibition of IL-6 and IL-8 induction from cultured rheumatoid synovial fibroblasts by treatment with aurothioglucose

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Keywords: electron microscopy, NF-κB, redox regulation

Abstract

Gold compounds have long been used in the treatment of rheumatoid arthritis (RA). However, their actions in RA have not been clarified. In this study, we examined the effect of one of the monovalent gold compounds, aurothioglucose (AuTG), on the IL-1-induced production of IL-6, IL-8 and granulocyte macrophage colony stimulating factor (GM-CSF) from rheumatoid synovial fibroblasts (RSF) isolated from three RA patients. IL-6 and IL-8 induction but not GM-CSF induction was inhibited in most of the RSF after pretreatment with AuTG. Since gene expression of these cytokines is known to be under the control of a common transcription factor, NF-κB, the effect of AuTG on the cellular localization of NF-κB (p65 subunit) and on NF-κB–DNA binding was examined. Although AuTG treatment did not prevent NF-κB nuclear translocation, AuTG blocked the DNA-binding activity of NF-κB when examined in vitro. Morphologically, both metal-specific cell staining using p-dimethylaminobenzylidene rhodamine and transmission electron microscopic examinations demonstrated the accumulation of metal gold in the cytoplasm and some organelles (mitochondria and lysosomes) of the AuTG-treated RSF. These results indicate that one of the anti-rheumatic actions of AuTG might be through its inhibitory action on NF-κB.

Introduction

Cytokines that are abundantly produced in the inflamed rheumatoid synovial fluid, such as tumor necrosis factor (TNF)-α, IL-1, IL-6, IL-8 and granulocyte macrophage colony stimulating factor (GM-CSF), play crucial roles in the pathophysiology of rheumatoid arthritis (RA). Among these cytokines, IL-1 and TNF-α are known to be indispensable for RA pathogenesis since they are known to induce IL-6, IL-8, GM-CSF and themselves through activation of a cellular transcription factor NF-κB (1–3). We recently observed that these cytokines are induced from rheumatoid synovial fibroblasts (RSF) upon stimulation with IL-1 or TNF-α (4). Handel et al. (5) and Marok et al. (6) have demonstrated the activation of NF-κB in situ in the synovial lining cells of RA joint tissues.

The efficacy of chrysotherapy in the treatment of RA has been well established since the earlier report by Forestier (7). The previous studies demonstrated a marked reduction of IL-6 and IL-8 production as a result of chrysotherapy (8). Gold compounds, containing the elemental aurous gold cation, Au(I), combined with a sulfur-containing ligand, have been widely used in the treatment of RA, although the precise mechanism of their actions is largely unknown. In our previous report (9) we demonstrated in vitro that gold compounds could block the DNA-binding of NF-κB. When NF-κB, purified from human lymphocytes, was incubated with aurothioglucose (AuTG) or aurothiomalate, the NF-κB DNA-binding activity was inhibited at concentrations >10 µM. It was then considered that Au(I) can oxidize the thiolate anions on the NF-κB molecule into disulfides and thus abrogate the DNA-binding activity based on the higher oxidation potential of Au(I) over Zn2+. These observations led us to examine the effect of AuTG on cytokine induction in RSF.
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Methods

Synovial fibroblasts

Synovial biopsy samples were obtained from six patients with RA, but only three cell cultures were successfully maintained in culture. We used these three cell cultures from three patients (women; ages between 48 and 63 years) who had active RA (case 1: class II, stage 2; case 2: class IV, stage 4; case 3: class II, stage 3), as defined by the clinical criteria of the American Rheumatism Association (10). Synovial tissues were obtained by arthroscopic synovectomy of the knee joints. All experiments were carried out using the cell culture during the third to seventh passages. Cell surface marker studies indicated positive for fibroblast-specific antigen (5B5) and vimentin, whereas negative for CD1a (NA1/34), CD31 (JC/70A) and von Willebrand factor (F8/86). mAb gen (5B5) and vimentin, whereas negative for CD1a (NA1/34, JC/70A and F8/86) were purchased from Dako (Glostrup, Denmark). These characteristics indicated that those cells were of fibroblast origin. RSF thus isolated from the resected tissues were cultured in F-12 medium (HAM) (Gibco/BRL, Grand Island, NY) supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA) and 0.05 µM 2-mercaptoethanol (4).

Measurement of cytokine levels

The cytokine concentration in RSF culture supernatant was determined using cytokine-specific ELISA kits for IL-6 (Biotrak human IL-6 ELISA system; Amersham, Amersham, UK), IL-8 (IL-8 ELISA kits; Toray Fuji Bionics, Tokyo, Japan) and GM-CSF (Biotrak human GM-CSF ELISA system; Amersham) as recommended by the manufacturers. These cytokine concentrations were measured 12 h after the stimulation with 10 ng/ml IL-1β. The effects of AuTG (Sigma, St Louis, MO) on induction of these cytokines were evaluated at its non-toxic concentrations. The cytotoxic effect of AuTG on RSF was evaluated by MTT assay and Trypan blue staining. These experiments were carried out >3 times. Three replicates were used for each test condition in the three independent cultures. The statistical significances of differences in the mean cytokine production were evaluated by the t-test.

Immunofluorescence

For immunofluorescent studies, RSF were fixed in 4.5% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 and then incubated with rabbit polyclonal antibody against the p65 subunit of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min at 37°C. After washing with PBS, the cells were incubated with FITC-conjugated goat anti-rabbit antibody (Cappel Organon Teknika, Durham, NC) for 20 min at 37°C.

Electrophoretic mobility shift assays (EMSA)

EMSA were performed with the partially purified NF-κB from human primary lymphocytes as reported (9,11,12). The DNA-binding activity was examined with the specific κB DNA probe as described. NF-κB was incubated with various concentrations (0.1–1000 µM) of AuTG on ice for 20 min and DNA-binding activity was examined by EMSA.

Staining of metal gold in the cells

RSF were incubated with various concentrations of AuTG for 12 days. Cells were fixed with 2.5% glutaraldehyde in PBS and stained with toluidine blue (Sigma) or p-dimethylaminobenzylidene rhodamine (Sigma) according to the methods previously described (13).

Transmission electron microscopy

The cultured cells were fixed for 30 min in a fixative containing 2.5% glutaraldehyde and 2% sucrose buffered with 0.05 M sodium cacodylate (pH 7.4). They were washed for 30 min with the same buffer solution and post-fixed for 2 h with a fixative containing 1% osmium tetroxide, 2% sucrose, in 0.05 M sodium cacodylate buffer (pH 7.4). After post-fixation, the samples were dehydrated in a graded series of ethanol for 10 min each, immersed twice in absolute ethanol for 15 min and embedded in epoxy resin (14) without the propylene oxide penetration procedure. Thin sections were obtained, stained with uranyl acetate alone, without using lead citrate in order to visualize metal gold and observed using a Hitachi H-7000 transmission electron microscope.

Results

Effects of AuTG on cytokine production by ELISA

RSF obtained from three different RA cases were stimulated with 10 ng/ml IL-1β in order to examine the induction of cytokines, IL-6, IL-8 and GM-CSF, that are known to be under the control of NF-κB. After preincubation with various concentrations of AuTG, the concentrations of these cytokines in the culture supernatant were measured with or without stimulation with IL-1β for 12 h. As shown in Fig. 1(A), the concentrations of IL-6, IL-8 and GM-CSF produced from RSF were increased in all three cases in response to IL-1β. For example, in case 1, the concentrations of IL-6, IL-8 and GM-CSF were increased with IL-1β stimulation by factors of 48 (from 1.7 to 8.15 ng/ml), 16 (from 3.78 to 61.0 ng/ml) and 127 (from 15.3 to 1950 pg/ml) respectively. These results were consistent with our previous report using different ELISA systems (4).

We then examined the effects of AuTG on cytokine induction (Fig. 1A). At 0.1 mM and 0.02 mM AuTG, induction of IL-6 was significantly blocked in cases 1 and 2, although in case 3 there was no significant effect even at 0.1 mM AuTG. With regard to the IL-8 induction by IL-1β, 0.1 mM AuTG significantly reduced the IL-8 induction in RSF obtained from three different patients with RA. Induction of GM-CSF was not inhibited at either concentration of AuTG. The counter-anion, thiglucose, had no inhibitory effect (data not shown). AuTG was not toxic to RSF at concentrations up to 0.1 mM as observed by MTT assay and Trypan blue staining (data not shown). Therefore, the effect of AuTG on blocking the induction of cytokines was not considered to be due to drug cytotoxicity.

In Fig. 1(B), the cumulative effects of AuTG are demonstrated. RSF obtained from case 1 were pretreated with 0.1 or 0.01 mM AuTG for up to 12 days. Induction of IL-6 and IL-8 was inhibited by 0.1 mM AuTG and the extent of the inhibition depended on the dose of AuTG and the duration of
Fig. 1. Induction of cytokine production in the culture supernatant of RSF stimulated by IL-1β and effects of AuTG. (A) Induction of IL-6, IL-8 and GM-CSF from RSF with IL-1β and the effects of preincubation with AuTG. RSF, during the third to seventh passage after being obtained from three RA patients, were pretreated with 0.02 or 0.1 mM AuTG for 12 days, and medium was changed every 3 days and AuTG was added each time of medium change. RSF were then stimulated with IL-1β (10 ng/ml) for 12 h and the cell culture supernatant were measured for each cytokine level. The concentrations of cytokines IL-6, IL-8 and GM-CSF were determined by ELISA as described in Methods. These experiments were repeated at least 3 times for each RSF preparation. Representative results are demonstrated. (B) Cumulative effects of AuTG. RSF (obtained from case 1) were pretreated with 0.1 or 0.01 mM AuTG for 3, 6, 9 or 12 days. After stimulation with IL-1β (10 ng/ml) for 12 h, the concentrations of cytokines were measured. Closed squares, 0.01 mM AuTG. Closed circles, 0.1 mM AuTG. Each value indicates the mean of three replicate cultures. Error bars show SD values. Statistical significances are indicated: *P < 0.05, **P < 0.01.
Effects of AuTG on the IL-1-induced nuclear translocation of NF-κB

We examined whether AuTG could inhibit nuclear translocation of NF-κB by immunofluorescence. In Fig. 2, RSF were incubated with or without 0.01 mM or 0.1 mM AuTG for 12 days and then 10 ng/ml IL-1β was used for NF-κB stimulation. Cells were fixed after 30 min of IL-1β stimulation and stained with antibody to the p65 subunit of NF-κB. As demonstrated here, the nuclear translocation of NF-κB was not inhibited even when RSF were pretreated with 0.1 mM AuTG for 12 days.

Effects of AuTG on the nuclear translocation of NF-κB

We then examined the effect of AuTG on the DNA-binding activity of NF-κB in vitro using partially purified NF-κB. In Fig.

3, NF-κB was preincubated with various concentrations of AuTG for 20 min at 4°C and the DNA-binding activity was examined. AuTG inhibited the NF-κB DNA-binding activity at concentrations >100 µM (Fig. 3).

Accumulation of metal gold in RSF treated with AuTG

It was indicated from the in vitro DNA-binding experiments (9) (Fig. 3) that Au(I) should be converted into metal gold after oxidation of (i.e. withdrawing electrons from) the thiolate anions on the cellular proteins including NF-κB. Furthermore, a cumulative nature of the effects of AuTG on IL-6/IL-8 induction (Fig. 1B) suggested accumulation of metal gold in the treated RSF. We thus attempted to visualize metal gold in the RSF treated with AuTG. As shown in Fig. 4, toluidine blue and specific metal gold staining revealed the accumulation of metal gold in the treated cells. In the toluidine blue staining, a number of metachromatic bodies were found in the cytoplasm. Staining with p-dimethylaminobenzylidene rhodamine demonstrated accumulation of rare metal, most likely gold, in the cytoplasm.

Transmission electron microscopic examination

The cultured RSF with or without AuTG treatment were subjected to transmission electron microscopic examination. In order to visualize metal gold in the cells, the cell sections were stained with uranyl acetate alone. As shown in Fig. 5 (inset), the AuTG-treated cells revealed enlarged mitochondrion with distorted cristae and small electron dense granules in the matrix. These electron dense inclusions were also observed in the cytoplasm and had no limiting membrane which was usually observed with cell organelle. In such mitochondrion, the cristae showed an irregular arrangement...
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Fig. 4. Histochemical detection of metal gold in the AuTG-treated RSF. RSF were treated with various concentrations of AuTG for 12 days and examined by the indicated staining. Note that light brown staining in the cytoplasm of the AuTG-treated cells in the lower panel. Original magnifications: toluidine blue staining, ×400; metal gold staining using p-dimethylaminobenzylidene rhodamine, ×200.

and were expanded. It was also noted that lysosomes were filled with electron dense materials, most likely gold metal, which were consistent with previous reports by others (15,16). In contrast, the control RSF did not show any morphological changes in mitochondrion or accumulation of such electron dense granules.

Discussion

In this study we have demonstrated that AuTG inhibited the induction of IL-6 and IL-8 by IL-1β using cultured synovial fibroblasts obtained from joint tissues of RA patients (RSF). These observations confirmed previous findings of the effects of gold compounds on the IL-6 concentration in sera of RA patients receiving chrysotherapy (17,18) and its production from peripheral blood monocytes in culture (19). Similarly, gold compounds were also effective in blocking IL-8 production in the synovial fluid (20) and in the cultured rheumatoid synovial cells (8) or endothelial cells (21). We also found that preincubation, at least for 3 days at 0.1 mM AuTG and for 6 days at 0.01 mM AuTG (Fig. 1B), of RSF with AuTG was necessary to obtain the inhibitory effect, which coincided with the necessity of induction period in chrysotherapy (22,23). Since the serum gold concentrations were reported to reach a peak of 0.01–0.05 mM in the patient taking biweekly injection of 50 mg gold compounds (24), the AuTG concentration used in this study was considered to be within a clinically attainable range.

Since IL-6 and IL-8 gene expression is known to be under the control of NF-κB (1,3 and also see 25 for review), we assumed that one of the actions of AuTG could be through NF-κB. In support of these findings, Williams et al. (26) demonstrated using a transient CAT assay that AuTG could block the NF-κB-dependent gene expression. In this study, the induction of GM-CSF was not inhibited by AuTG (Fig. 1A). Since inducible GM-CSF production was not ascribable solely to NF-κB (2) and other transcription factors, such as those binding to the conserved lymphokine element (27) and another
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upstream element (28), were shown to be involved, inhibition of NF-xB activation was probably not sufficient to block GM-CSF induction.

Interestingly, the effect of AuTG was not evident in blocking the nuclear translocation of NF-xB. In concordance with these observations, we have demonstrated that cellular glutathione (GSH) levels were not changed by AuTG treatment (29), whereas GSH levels were up-regulated by the treatment with one of the anti-NF-xB reagents, α-lipoic acid, that is known to block formation of radical oxygen intermediates and thus blocking ixB degradation followed by NF-xB nuclear translocation (see 30 and the references therein).

However, we have demonstrated that AuTG could block the DNA-binding activity of NF-xB (Fig. 3). We previously demonstrated that this inhibitory activity was specific to the monovalent gold ion [Au(I)] and proposed a model in which Au(I) oxidizes the redox-sensitive thiolate anions (cysteine residues) on the NF-xB molecule (11,12,25) into the disulfide form to abrogate the DNA-binding activity (9). It is possible that oxidation of NF-xB by Au(I) within the cell abrogates the DNA-binding activity irrespective of its intracellular location since the NF-xB–IxB complex upon treatment with chemical oxidants such as diamide lost the DNA-binding activity even before the dissociation of IxB in vitro (11). It was then postulated that the reduced form of Au(I), gold metal, should be accumulated in the cell. We thus performed in this study the experiments shown in Figs 4 and 5, which demonstrated metal gold accumulation in the AuTG-treated cells. The fine electron-dense granules in the cytoplasm of the treated cells were detected by electron microscope even without using lead citrate for staining, thus we assumed that they were most likely metal gold deposits. We assume that the apparent accumulation of these granules in mitochondrion (morphologically, the mitochondrion revealed enlargement and irregular arrangement of cristae) and lysosomal particles is probably the result of intra-cytoplasmic formation and accumulation of metal gold. These findings suggested that Au(I) might be converted into metal gold and subsequently accumulated in the cell after reacting with cellular thiol compounds including the redox-sensitive cysteine residues on the NF-xB molecule.

The anti-NF-xB actions of anti-rheumatic drugs were also reported for other compounds. For example, aspirin (31) and glucocorticoids (32,33) were shown to block the activation of NF-xB by increasing the steady-state level of IxBα either by preventing IxBα degradation (31) or by inducing its gene expression (32,33). Considering the pathogenetic roles of various genes under the control of NF-xB such as cytokines and cell adhesion molecules (25), together with recent
observations of NF-κB in blocking apoptotic cell death (34–36). NF-κB is considered to be an efficient and feasible therapeutic target of RA. As demonstrated in this study and previous reports (31–33), actions of major anti-rheumatic drugs may be mediated by their effects on NF-κB. It is thus conceivable that other compounds known to block the NF-κB activation pathway may also have anti-rheumatic action.

Acknowledgements

This work was supported by grants-in-aid from the Ministry of Health and Welfare, the Ministry of Education, Science and Culture of Japan and from the Human Science Foundation. J.-P. Y. is a research fellow of the Japan Society for the Promotion of Science.

Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AuTG</td>
<td>aurothioglucose</td>
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<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
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<td>RA</td>
<td>rheumatoid arthritis</td>
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<td>RSF</td>
<td>rheumatoid synovial fibroblast</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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References

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