Gold sodium thiomalate suppresses the differentiation and function of human dendritic cells from peripheral blood monocytes

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Abstract Objective

Gold sodium thiomalate (GST) is a drug commonly used for the treatment of rheumatoid arthritis (RA). To clarify the mechanism of therapeutic effects of GST on RA, we investigated if GST affects the differentiation of dendritic cells (DC), which are thought to play a pivotal role in RA pathogenesis.

Methods

We generated immature DC (iDC) in vitro from PB monocytes during the 5 to 7-day culture in the presence of IL-4 and GM-CSF. Mature DC (mDC) were induced by adding TNFa on day 5 of the 7-day culture with GM-CSF and IL-4. DC capacity of stimulating T cells was examined in allogenic MLR using generated DC as stimulators. IL-12 production from DC was assayed with ELISA.

Results

We found that: 1) mDC generated in the presence of GST showed lower expression of CD1a, CD83, CD80, CD86, HLA-ABC and HLA-DR compared to control mDC on FACS analysis. 2) GST-treated mDC showed reduced capacity of stimulating allogenic T cells in mixed leukocyte reaction. 3) IL-12p70 production after stimulation with SAC or LPS plus IFNy was markedly reduced in GST-treated mDC.

Conclusion

GST suppresses the differentiation and function of DC generated from peripheral blood monocytes. This previously unknown action may explain the in vivo effects of GST in the treatment of RA.

Key words

Co-stimulatory molecules, dendritic cells, gold sodium thiomalate, interleukin-12, rheumatoid arthritis.

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Introduction

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) in immune system (1,2). Recently, peripheral blood monocytes and precursor cells have been shown to differentiate into DC in vitro with combinations of cytokines (3-5). This technique has contributed much to elucidate the maturation process and function of DC. Immature DC capture and process antigens, and become mature DC upon inflammatory stimulation. Mature DC express higher levels of co-stimulatory molecules and efficiently activate antigen-specific T cells (4, 6). DC are now thought to play a central role in immune responses occurring in infectious diseases, autoimmune diseases, and graft rejection (7).

Rheumatoid synovial tissue and fluid are enriched in DC with potent APC function, and recent reports have indicated a role for DC in the pathogenesis of rheumatoid arthritis (RA) (8). Peripheral blood DC (PB-DC) express little or no CD80 and CD86 antigens. The phenotype of synovial fluid DC is similar to that of PB-DC, but synovial fluid DC can stimulate autologous T cells more efficiently than PB-DC or synovial fluid monocytes (9). CD86 has been shown to be more important than CD80 in their capacity of stimulating T cells. Accordingly, synovial tissue DC, localized to perivascular regions and T cell nodules, express low levels of CD80 and high levels of CD86 (10). Therefore, it is suggested that precursor DC in peripheral blood are recruited across the blood vessels to the inflammatory joint where they become mature DC, and play a role in the initiation and perpetuation of the disease by activating self-reactive T cells (11, 12).

The mechanism of actions of gold compounds used in RA treatment is not fully understood, although they have been widely used for many years. Gold sodium thiomalate (GST), water-soluble salt of gold thiol complexes, acts on various types of cells including monocyte, T cell, B cells, synovial cell, and endothelial cells (13). GST has suppressive effects on monocytes as to monokine production and MHC expression (14,15). Recently, DC are regarded as being critically involved in T cell response in RA. Therefore, we anticipated a possible effect of GST on the differentiation and function of DC. To elucidate the mechanism of actions of GST on the differentiation of DC, we used *in vitro* culture system in which DC are differentiated from PB monocytes in the presence of granulocyte-monocyte colony-stimulating factor (GM-CSF), interleukin 4 (IL-4), and tumor necrosis factor-alpha (TNF-

). We found that GST could alter the cell surface expression of differentiation and activation antigens on DC, and also the capacity of DC to stimulate T cell proliferation as well as to produce IL-12p70.

Materials and methods

Reagents and cytokines

GST was a kind gift from Shionogi Pharmaceutical Co. (Osaka, Japan). GST was dissolved into RPMI1640 (Nissui, Tokyo, Japan) medium, and used at the concentration of 0.2 to 20 µM. Recombinant human IL-4 was kindly provided by Dr. Kazuaki Hama (Ono Pharmaceutical Co. Ltd., Osaka, Japan). Recombinant human GM-CSF and TNF were obtained from Genzyme (Cambridge, MN, USA). Recombinant human inrterferon (IFN) was obtained from Serotec Ltd. (Oxford, UK), Lipopolysaccharide (LPS) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), Staphylococcus aureus cowan I strain (SAC) was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Anti-CD1a, anti-CD14, anti-CD86, anti-HLA-ABC, anti-HLA-DR, and isotype-matched control Abs were purchased from PharMingen (San Diego, CA, USA). Anti-CD80 and anti-CD83 Abs were from Immunotech (Marseille, France), and FITC-anti mouse Ig GAM was from Becton Dickinson (San Jose, CA, USA).

Monocyte preparation

PBMC were isolated from normal healthy donors or patients with RA by density gradient centrifugation on Ficoll-Paque (Pharmacia Biotech, Uppsalla, Sweden). T cells were removed with neuraminidase-treated sheep red

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blood cells (N-SRBC)-rosetting. Non-T cells were incubated on a macrophage-separated plate (JIMRO, Tokyo, Japan) for 1 hour, and adherent cells were harvested after 25 min-incubation in cold phosphate-buffered saline (PBS) containing 5mM EDTA. The harvested cells were positive for CD14 (> 90%), and negative for CD3 (< 2%) and CD19 (< 2-7%), and used as PB monocytes in the following experiments.

Cell culture

Immature DC (iDC) were generated from PB monocytes. As shown in Figure 1-A.1, PB monocytes were cultured at 5×10^5 cells/ml in 12-well tissue culture plates (Costar, Cambridge, MA, USA) for 7 days in RPMI 1640 supplemented with 10% FCS, 0.025 M HEPES, ampicilin, streptomycin, IL-4 (25 ng/ml) and GM-CSF (50 ng/ml). For generating mature DC (mDC) (Fig. 1-A.2), TNF (10 ng/ml) was added at



Fig. 1. Effect of GST on the cell surface antigen expression of immature DC (iDC) and mature DC (mDC) generated in vitro from PB monocytes in both healthy donors (Fig.1-A.1, -A.2 and -A.3) and RA patients (Fig.1-B.). The representative results of five experiments are shown in upper panel. iDC and mDC were generated as shown in lower panel.



Fig. 2. Dose dependent effect of GST on mature DC (mDC) phenotype. mDC were generated as shown in Fig.1-A.2. Results are shown as mean fluorescence intensity (MFI) (mean \pm SD) from five independent experiments. *p < 0.001; #p < 0.05; +p < 0.01 vs. control.

day 5 of the culture stated above. To examine the effects of GST on DC differentiation, GST was added at the beginning (day 0) or day 5 of the culture (Fig. 1-A.3). On day 7, cells were harvested, counted under microscope using Tripan blue excursion and examined for their phenotypes.

FACS analysis

Harvested cells were washed twice with cold PBS and resuspended in PBS with 1%BSA. Cells were aliquotted into 5 x 10^4 cells/sample and stained with the indicated mAbs for 30 min at 4°C, subsequently incubated with FITC-conjugated 2nd Ab for 30 min at 4°C in the dark. Flow cytometry analysis was carried out with an EPICS Profile flow cytometer (Coulter, Miami, FL, USA).

Primary allogenic mixed lymphocyte reaction (allo-MLR)

mDC were generated in the presence or absence of GST (20 μ M) as shown in (Fig. 1-A.2). The generated mDC were pre-treated with mitomycin-C (MMC, 25 μ g/ml) for 30 min at 37°C, washed extensively, and used as stimulator cells. Responder cells were purified T cells by N-SRBC rosetting from different donors. Then, T cells (1 x 10⁵) were cultured with indicated number of MMC-treated DC for 6 days without GST.

T cell proliferation was measured with cell proliferation ELISA according to the manufacture's instruction (Boehringer Mannheim, Mannheim, Germany). Briefly, 5-bromo-2'-deoxyuridine (BrdU) was added in the last 24 hours of the culture, and cells were fixed and stained with anti-BrdU Ab. Color reactions were measured with a micro-plate reader. Each group was performed in triplicate form.

ELISA

To analyze the effect of GST on cytokine production from DC, iDC were generated with or without GST ((Fig. 1-A.1)). Then, generated iDC were treated with SAC (0.01%) or LPS (1 μ g/ml) plus IFN (25 ng/ml) for 48h in 96-well plates at the concentration of 1 \times 10⁶ cells/ml. IL-12p70 and IL-6 in the supernatants were measured by ELISA according to the manufacturer's instruction (PharMingen).

Statistical analysis

Data were shown as the mean \pm standard deviation (SD). Data were analyzed by unpaired t-test when two samples were compared, and by ANOVA with Fisher's PLSD method when samples were more than three samples. P < 0.05 was considered statistically significant.

Results

GST alters the phenotype of DC during differentiation from PB monocytes in both healthy donors and patients with RA

iDC and mDC generated in the absence of GST (Ctl-iDC and Ctl-mDC) had a typical phenotype, CD1a⁺CD83⁻CD14⁻ HLA-DR⁺ and CD1a⁺CD83⁺CD14⁻ HLA-DR⁺, respectively (Fig. 1). When GST was added at the beginning of culture, iDC treated with GST (GST-iDC) showed markedly reduced expression of CD1a (Fig. 1-A.1). mDC induced with GST (GST-mDC) in both healthy donors and patients with RA showed reduced expression of CD1a, CD83 and CD86, a hallmark of mature DC (Fig. 1-A.2 and Fig. 1–B.). These results suggested that GST suppressed the differentiation process of DC.

To examine the effect of GST on DC maturation from iDC to mDC, we added GST together with TNF at day 5 of a 7-day culture (Fig.1-A.3). The CD83 expression on GST-mDC (Fig.1-A.3) was suppressed, even though the suppression was not so complete as that observed in GST-mDC (day 0-7, Fig.1-A.2). GST on day5 to 7 had no effect on CD86 expression (Fig. 1-A.3). These results indicate that GST suppresses the entire process of DC differentiation, especially from monocyte to iDC.

The dose response experiments showed that GST exerted its effects at the concentration of 20 µM, at which the expression of CD1a, CD83, MHC molecules (HLA-ABC, HLA-DR), and that of co-stimulatory molecules (CD-80 and CD86) were significantly decreased in GST-mDC compared to CtlmDC (Fig. 2). Viable cell percentages were more than 90% in the presence of 20 µM GST, and recovered cell counts after 7-day culture were not significantly changed between Ctl-mDC and GST-mDC $(4.3 \pm 1.4 \times 10^5 \text{ vs. } 4.6 \pm 0.6 \pm$ \times 10⁵, from six independent experiments).

GST-mDC have impaired capacity

of stimulating T cells in allo-MLR To further examine the functional difference between Ctl-mDC and GSTmDC, we carried out allo-MLR using Ctl-mDC or GST-mDC as stimulators. T cell stimulating capacity of GSTmDC was significantly lower compared to that of Ctl-mDC (Fig. 3).



Fig. 3. Mature DC (mDC) treated with GST showed a decreased T-cell stimulatory capacity in allogenic MLR. mDC were generated as shown in Fig.1-A.2. BrdU incorporation after allogenic MLR is shown as the optical density (OD, mean \pm SD) from triplicate determinations. Data is representative of three independent experiments. *p < 0.001 vs control of the same stimulator cell numbers.

Table I. IL-12 production was decreased in GST-treated mDC #.

	IL-12p70 (pg/ml)			IL-6 (ng/ml)	
	Control	SAC+IFN	LPS+IFN	Control	LPS+IFN
Ctl-mDC	< 10.0	203.1 ± 29.8 *	355.9 ± 83.8 *	<1.0	41.8 ± 12.1
GST-mDC	< 10.0	14.3 ± 3.6	20.3 ± 12.2	<1.0	37.7 ± 7.8

mDC were acquired by stimulating iDC (Fig.1-A.1, 1×10^6 cells/ml) with SAC (0.01%) or LPS (1 µg/ml) plus IFN (25 ng/ml), and the supernatants were harvested after 48h of incubation. The concentrations of IL-12p70 and IL-6 in the supernatants were detected with ELISA. Results represent the mean ± SD of five different experiments. *p < 0.0001.

IL-12p70 production is impaired in GST-treated DC

Since T cell differentiation is deviated to Th1 type by IL-12 and IL-12 level is markedly high in RA joints (16), we tested the effect of GST on IL-12p70 production from DC. We generated iDC (Fig.1-A.1), and stimulated with SAC or LPS plus IFN for inducing mDC and stimulating IL-12 production from DC according to the literatures (17, 18). IL-12p70 production from mDC after both combinations of stimuli was almost completely suppressed when DC were treated with GST, whereas IL-6 production from mDC stimulated with LPS plus IFN was not affected by GST (Table I).

Discussion

GST is one of the most effective disease modifying antirheumatic drugs, and has been analyzed for the mechanism of action on RA. Reports have been described GST's effects on various types of cells such as monocyte and macrophage, T cell, endothelial cell, and synovial fibroblast (19-22). However, there are few studies for GST effects on DC. Recent evidence supporting the role for DC in RA prompted us to examine the effects of GST on the DC differentiation from PB monocytes (2, 10, 11). This is the first report describing the effects of GST on the differentiation of DC in vitro.

We demonstrated that GST altered the phenotypes of generated DC, and sup-

pressed IL-12 production from DC. GST-mDC had lower expression of CD1a and CD83, suggesting that GSTmDC remained at more immature stage than Ctl-mDC. Furthermore, GST suppressed the expression of MHCs (HLA-ABC and HLA-DR) and co-stimulatory molecules (CD80 and CD86), the lack of which during T cell activation is known to result in T cell anergy or hyporesponsiveness (23-25). It is reported that both CD80 and CD86 molecules are expressed on synovial tissue DC, and are up-regulated by cytokines in vitro and in situ, and blocking of CD86 results in a marked decrease in T cell proliferation during allogenic and autologous MLR (7, 10, 11). We demonstrated the lower capacity of GST-mDC to stimulate allogenic T cells in MLR compared with Ctl-mDC. Thus, it is likely that GST-induced suppression of DC function might be the case in vivo because the concentration used in the in vitro experiments, 20 µM, is clinically attainable in patient's peripheral blood (13), and GST is known to be accumulated in inflammatory tissues.

The glucocorticoid, one of the most effective immunosuppressive drugs for RA, is also reported to affect maturation and function of DC in vitro. Glucocorticoid treatment partially reduced IL-12p70 production from DC upon LPS stimulation, whereas DC remained at a more immature stage and showed an impaired capacity to induce MLR (26). Our data showed that GST also has effects on IL-12 production from DC. The IL-12p70 production from GST-mDC was almost completely shut down when GST-mDC were stimulated with the combination of LPS or SAC plus IFN . It is known that synovial helper T cells from RA patients are predominantly Th1, which is promoted by IL-12 production from mDC. Thus, GST may effectively block the role of synovial DC for Th1 induction as well as for antigen presentation on RA joint in vivo.

The cellular response to GST stimulation is not well understood. One hypothesis is that GST might suppress DC maturation via protein kinase C (PKC), as GST is known to inhibit PKC that induces differentiation of human CD-

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34⁺ hematopoietic progenitor cells into DC (27). In addition, gold compounds are reported to suppress DNA binding of NF B (28), which plays major role in IL-6 and IL-8 production. In fact, gold compound is reported to suppress IL1-induced production of IL-6 and IL-8 from synovial cells (29). As PKC is known to activate NF B, PKC-NF B pathway may be the key to be negatively regulated by GST.

In the present report, we demonstrated a new in vitro effect of GST. The strategy of manipulating DC maturation or function will be one of promising treatment in various autoimmune or inflammatory diseases. Our results, showing suppression of DC differentiation and function by GST, might explain one of the *in vivo* effects of GST on RA from the viewpoint of DC manipulation.

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