Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder leading to joint destruction. The roles of proinflammatory cytokines have been appreciated in the pathogenesis of RA. Among the proinflammatory cytokines, tumor necrosis factor-α (TNF-α) is known to play a central role in RA. In fact, the agents inhibiting TNF-α have brought drastic changes in the treatment of RA, so called “paradigm shift.”

TNF-α is produced from macrophages and lymphocytes by various inflammatory triggers. TNF-α expressed on the cell surfaces is called transmembrane TNF-α, which is cut by metalloproteinases such as TNF-α converting enzyme (TACE) into soluble TNF-α. It is noteworthy that Mitoma et al. demonstrated that infliximab (IFX) and adalimumab exerted antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) to TNF-α-expressing Jurkat T cells.

Certolizumab pegol (CZP) is a novel PEGylated, Fab’ fragment of anti-TNF-α monoclonal antibody. The parent antibody was selected from a panel of hybridomas producing monoclonal antibodies against human TNF-α. The complementarity determining regions from the parent antibody were then inserted into a human Fab’ IgG framework, along with several other framework residues of the variable regions that were essential for maintenance of affinity. The artificial Fab’ was subsequently PEGylated via the site-specific attachment of a 40 kDa polyethylene glycol (PEG) moiety.

Treatment with CZP has been shown to comparable beneficial effects to other TNF inhibitors in the treatment of RA. CZP differs from the other TNF-α inhibitors in its lack of an Fc region, which minimizes potential Fc-mediated effects such as CDC or ADCC. Accordingly,
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CZP did not induce ADCC or CDC, but induced nonapoptotic cell death in transmembrane TNF-α expressing cells.19

We have recently demonstrated that etanercept and IFX promoted apoptosis of human monocytes as well as suppressed their expression of mRNA for IL-6.20 More importantly, the effects of etanercept and IFX on human monocytes were reversed by the addition of immunoglobulin G (IgG), but not by IgG-F(ab’)2 fragments, indicating that Fc structures of etanercept and IFX are important for their actions on human monocytes.20 Since CZP lacks an Fc region, it is possible that the effects of CZP on human monocytes might be different from those of other TNF inhibitors with Fc structures, such as IFX. The current studies were therefore undertaken to disclose the effects of CZP on human monocytes.

Materials and Methods

Cell preparation
Peripheral blood mononuclear cells (PBMCs) were obtained from healthy adult volunteers with informed consent using centrifugation of heparinized venous blood over sodium diatrizorate-Ficoll gradients. Monocytes were prepared from PBMC using Monocyte Isolation Kit II (Miltenyi Biotec). Monocyte population obtained in this manner contained <0.1% CD3+ cells, <0.1% CD19+ cells, and >93% CD14+ cells.

Reagents
CZP was purchased from Astellas, Tokyo. Control human IgG1 was purified from serum from a patient with human IgG1 myeloma using DEAE-Sepharose column. IFX was purchased from Mitsubishi Tanabe Pharma, Osaka. Polyethylene glycol (PEG) (20 kDa) was purchased from Wako, Osaka.

Cell cultures
RPMI 1640 medium (Nikken, Kyoto) supplemented with penicillin G (100 U/ml) (Life Technologies, Grand Island, NY, USA), streptomycin (100 μg/ml) (Life Technologies), L-glutamine (0.3 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA), and 10% fetal bovine serum (Life Technologies) were used for cultures. Purified monocytes (1 × 10⁶/well) from four healthy individuals were cultured in 24-well microtiter plates (Nunc, Roskilde, Denmark) with CZP or IFX at pharmacological attainable concentrations (0.1 to 100 μg/ml). After 24 hours, the supernatants were replaced with fresh culture medium, followed by the addition of lipopolysaccharide (LPS) (0.5 μg/ml) (Sigma-Aldrich). After an additional 24 hours of incubation, the supernatants were harvested and assayed for TNF-α. Purified monocytes (1 × 10⁶/well) from 16 healthy individuals were cultured with control IgG, IFX, CZP, or PEG at 10 μg/ml using the same culture system. The supernatants were harvested and assayed for TNF-α (n = 16) and IL-6 (n = 14). In some experiments, the cells from 6 healthy individuals were harvested after 1 hour into the 2nd 24-hour incubation period for mRNA analysis with PCR. Data are expressed as the ratio of copy numbers of TNF-α mRNA to those of β-actin mRNA.

Neutralization of TNF-α
Culture medium containing known concentrations of recombinant TNF-α was incubated with CZP, IFX, control IgG, or PEG at 10 μg/ml for 2 hours, after which the concentrations of TNF-α in the mixture were measured (n = 4).

Measurement of TNF-α and IL-6
The concentrations of TNF-α and IL-6 in the culture supernatants were measured using Human TNF-α ELISA Development Kit (Pepro Tech, Rocky Hill, NJ, USA), and Human IL-6 ELISA Development Kit (Pepro Tech).

RNA isolation and real-time quantitative polymerase chain reaction
Total RNA was isolated from cultured cells using ISOGEN (Nippon Gene, Tokyo) according to the manufacturer’s specifications. cDNA was prepared from 1 μg of total RNA using M-MLV reverse transcriptase (Takara Bio, Shiga) with random primers (Takara Bio), and was subjected to analysis with real-time polymerase chain reaction (PCR) using LightCycler 2.0 (Roche Diagnostics, Lewes, UK). Real-time PCR of TNF-α and β-actin was performed using SYBR Premix Ex Taq II (Takara Bio) with the following primers: sense, 5’-TGCTGTTTCCCTCAGCTCTT-3’ and antisense, 5’-CAGAGGGCTGATTAGAGAGGT-3’ for TNF-α (gene accession No. NM_000594.3, position 263-372); sense, 5’-TGGCACCCAGCACAATGAA-3’ and antisense, 5’-CTAAGTCATAGTCCGCCTAGAAGCA-3’ for β-actin (gene accession No. NM_00110.3, position 1043-1204). Amplification was performed according to the standard protocol recommended by the manufacturer. All results were calibrated to the copy number of β-actin obtained from the same cDNA samples.

Statistical analysis
Results are expressed as the median (maximum value, minimum value). Statistical significance was evaluated...
using Wilcoxon’s signed rank test and paired t test. P-values <0.05 were considered statistically significant.

**Results**

*Effect of CZP on the production of proinflammatory cytokines by peripheral blood monocytes*

Initial experiments compared the capacities of CZP and IFX to neutralize soluble TNF-α in culture medium at a pharmacologically attainable concentration. Control IgG and PEG did not influence the concentrations of TNF-α, whereas both CZP and IFX reduced the concentrations (Figure 1). It was noteworthy that CZP was much more potent than was IFX in neutralizing TNF-α in the culture medium.

Subsequent experiments compared the effects of CZP and IFX on the production of TNF-α of peripheral blood monocytes. Because both CZP and IFX have been shown to neutralize soluble TNF-α in culture medium, a 2-step culture system was used. Preincubation of monocytes

**Figure 1.** Neutralization of soluble TNF-α by CZP and IFX

The neutralizing effect of control IgG, PEG, IFX, or CZP on recombinant TNF-α was examined, as described in the "Materials and Methods" section. The median (maximum, minimum) values for TNF-α were: for control IgG, 1096 (1124, 1066) pg/ml; for PEG, 1090 (1116, 1042) pg/ml; for IFX, 488.1 (511.7, 473.4) pg/ml; for CZP, 198.8 (211.1, 166.3) pg/ml.

**Figure 2.** Dose response effect of CZP and IFX on the production of TNF-α of monocytes

The effect of CZP (A) and IFX (B) on the production of TNF-α of monocytes with various concentrations was examined by using a 2-step culture system, as described in the "Materials and Methods" section. The median (maximum, minimum) values for TNF-α were: for CZP 0.1 μg/ml, 845.6 (1071, 627.4) pg/ml; for CZP 1 μg/ml, 481.8 (740.6, 344.7) pg/ml; for CZP 10 μg/ml, 236.3 (357.5, 97.44) pg/ml; for CZP 100 μg/ml, 131.3 (213.0, 90.26) pg/ml; for IFX 0.1 μg/ml, 813.1 (878.0, 718.0) pg/ml; for IFX 1 μg/ml, 519.2 (658.8, 456.6) pg/ml; for IFX 10 μg/ml, 327.1 (421.8, 260.2) pg/ml; for IFX 100 μg/ml, 265.1 (278.4, 168.0) pg/ml. Each line on the graph is representative of the same monocyte preparation from the same donor.
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Figure 3. Effect of CZP and IFX on the production of TNF-α and IL-6 of monocytes

The effect of control IgG, IFX, CZP, and PEG on the production of (A) TNF-α and (B) IL-6 of monocytes, at a pharmacologically attainable concentration, was examined using a 2-step culture system, as described in the "Materials and Methods" section. The median (maximum, minimum) values for TNF-α and IL-6 were: for control IgG, 1487 (2085, 789.2) pg/ml; for IFX, 341.5 (1281, 17.48) pg/ml; for CZP, 339.9 (966.0, 31.54) pg/ml; for PEG, 1044 (1788, 770.6) pg/ml; for control IgG, 897.8 (1819, 478.9) pg/ml; for IFX, 439.3 (664.2, 180.2) pg/ml; for CZP, 464.3 (796.1, 251.0) pg/ml; for PEG, 562.6 (1366, 312.8) pg/ml. Each line on the graph is representative of the same monocyte preparation from the same donor.

Figure 4. Effects of CZP on the expression of mRNA for TNF-α

The effects of CZP and PEG on the expression of mRNA for TNF-α at a pharmacologically attainable concentration were examined using a 2-step culture system, as described in the "Materials and Methods" section. The median (maximum, minimum) values for the ratio of copy numbers of TNF-α mRNA to those of β-actin mRNA were: for PEG, 1.026 (17.99, 0.2620); for CZP, 0.0834 (0.9511, 0.0442). Each line on the graph is representative of the same monocyte preparation from the same donor.
with CZP or IFX reduced the production of TNF-\(\alpha\) in subsequent cultures stimulated by LPS in a dose-dependent manner (Figure 2). The effects of CZP and IFX on the production of TNF-\(\alpha\) and IL-6 by human monocytes at 10 \(\mu\)/ml, a pharmacologically attainable concentration, were then examined. Pretreatment of monocytes with IFX or CZP significantly inhibited the production of TNF-\(\alpha\) compared with pretreatment with control IgG or PEG, respectively (control IgG 1487 (2085, 789.2) pg/ml; IFX 341.5 (1281, 17.48) pg/ml, \(P = 0.0001\), CZP 339.9 (966.0, 31.54) pg/ml; PEG 1044 (1788, 770.6) pg/ml, \(P = 0.0001\)), whereas no significant differences was observed between CZP and IFX (\(P = 0.5436\)) (Figure 3A). Moreover, pretreatment of monocytes with IFX or CZP also significantly reduced the production of IL-6 (control IgG 897.8 (1819, 478.9) pg/ml; IFX 439.3 (664.2, 180.2) pg/ml, \(P = 0.0005\), CZP 464.3 (796.1, 251.0) pg/ml; PEG 562.6 (1366, 312.8) pg/ml, \(P = 0.0005\)) (Figure 3B).

**Effects of CZP on the expression of mRNA for TNF-\(\alpha\)**
To further confirm the direct influence of CZP on human monocytes, the subsequent experiments examined the effects of CZP on the expression of mRNA for TNF-\(\alpha\). Preincubation with CZP significantly suppressed the expression of mRNA for TNF-\(\alpha\) of monocytes in subsequent cultures stimulated LPS compared with PEG (PEG 1.026 (17.99, 0.2620); CZP 0.0834 (0.9511, 0.0442), \(P = 0.031\)) (Figure 4).

**Discussion**
Both CZP and IFX are TNF-\(\alpha\) inhibitors that display comparable beneficial effects in the treatment of RA. The current studies have disclosed various different characteristics of CZP from those of IFX. First, the ability of CZP to neutralize soluble TNF-\(\alpha\) was more potent than that of IFX at the same concentration. Because the molecular weights of IFX and CZP are about 144 kDa and 90 kDa, respectively, CZP contains fewer numbers of TNF-\(\alpha\) binding sites than does IFX in the same weights. It is, therefore, suggested that CZP might have stronger binding activities than does IFX. Plasma trough level of CZP of patients taking 200 mg injection as monotherapy for every 2 weeks in the maintenance period is 21.0 \(\mu\)/ml.\(^{21}\) By contrast, plasma trough level of IFX of patients being given 3 mg/kg infusion for every 8 weeks in the maintenance period is 0.5 \(\mu\)/ml.\(^{22}\) In fact, it has been demonstrated that PEGylation significantly increases the circulating half-life of Fab’ molecules by inhibiting proteolysis.\(^{23}\) It is, therefore, likely that CZP might be superior to IFX in the capacity to neutralize TNF-\(\alpha\).

Biofluorescence imaging in arthritic mice has shown that CZP preferentially penetrates inflamed tissue compared to non-inflamed tissue and does so to a greater extent than do either adalimumab or IFX.\(^{24}\) Furthermore, the persistence of CZP in the inflamed tissue was more prolonged compared to either adalimumab or IFX.\(^{24}\) These phenomena are most likely due to the PEGylation of the molecule, although the persistence in the tissue could also be due to the lack of Fc receptor-mediated recycling. Even though these phenomena have not been demonstrated in humans to date, it is possible that the ability of CZP to neutralize TNF-\(\alpha\) is more potent than that of infliximab in inflammatory synovial tissues of RA patients.

The concentration of TNF-\(\alpha\) in the supernatants after stimulation with LPS of monocytes treated with CZP was significantly lower than that with PEG. Because CZP was almost completely removed after the pretreatment, the reduction of TNF-\(\alpha\) in the culture supernatants could not be accounted for by the neutralization of TNF-\(\alpha\) by CZP. Rather, it is strongly suggested that both CZP and IFX inhibited the production of TNF-\(\alpha\) by directly acting on monocytes. CZP and IFX also suppressed the expression of another proinflammatory cytokine IL-6. Furthermore, since CZP significantly suppressed the expression of mRNA for TNF-\(\alpha\) in LPS-stimulated monocytes. The results, therefore, confirm that CZP as well as IFX has direct effects on monocytes to suppress the production of proinflammatory cytokines. Accordingly, previous studies disclosed that CZP suppress the production of IL-1-\(\beta\) by human monocytes.\(^{25}\)

Previous studies have demonstrated that IFX suppresses the production of proinflammatory cytokines of human monocytes.\(^{20}\) More importantly, Fc receptors on human monocytes are involved in the suppressive effects of IFX on monocytes.\(^{20}\) Thus, human IgG, but not IgG-F(ab’)_2, almost completely reversed the suppressive effects of IFX on monocytes.\(^{20}\) CZP is a PEGylated Fc-free TNF inhibitor. It is therefore necessary to examine the effect of CZP on human monocyte in the presence of excessive IgG or IgG-F(ab’)_2.

The precise mechanisms of suppression of human monocytes by CZP remain to be elucidated. It is likely that Fab’ alone would not be enough to suppress the function of human monocytes. On the other hand, PEG has been found to interact with monocytes.\(^{26,27}\) Thus, PEG has been shown to stimulate the function of monocytes in some circumstances.\(^{26}\) In other conditions, PEG has been shown to promote apoptosis of human monocytes.
blood monocyte-derived macrophages. It is therefore likely that CZP might influence the functions of monocytes through interactions with membrane-bound TNF-α and PEG-mediated adhesions. Further studies are necessary to delineate the whole sequelae of the effects of CZP on human monocytes.

In summary, the results in the current studies have disclosed the suppressive influences of CZP on human monocytes in vitro. The prominent features of CZP include the potent neutralizing effects of TNF-α as well as the inhibition of the expression of mRNA for TNF-α. Further studies to delineate the role of PEG are warranted to elucidate the precise mechanisms of action of CZP.

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References


