Effects of TNF inhibitors on human monocytes

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Objective: Our objective was to explore the effects of the tumor necrosis factor (TNF) inhibitors, etanercept and infliximab on the function of human monocytes.

Methods: Monocytes from healthy donors were cultured in the presence of staphylococcal enterotoxin B (SEB) with pharmacologically attainable concentrations of biological agents or control immunoglobulin G (IgG). The expression of interleukin-6 (IL-6) mRNA was determined by real-time quantitative RT-PCR. The expressions of CD80 and CD86 and the induction of apoptosis of monocytes were measured by flow cytometry.

Results: Both etanercept and infliximab promoted apoptosis of SEB-stimulated monocytes. The induction of apoptosis of monocytes by these biological agents were reversed by the addition of IgG but not by IgG F(ab’)2 fragments. Etanercept and infliximab significantly suppressed the expressions of CD80 and CD86 in SEB-stimulated monocytes as well as suppressing the expression of mRNA for IL-6 in SEB-stimulated monocytes.

Conclusions: These results demonstrate that one of the mechanisms of action of TNF inhibitors involves the induction of apoptosis of monocytes, which involves interaction with constant fragment (Fc) receptors on monocytes. Moreover, these data also indicate that TNF inhibitors strongly inhibit IL-6 production of monocytes and concurrently suppress the expression of costimulatory molecules.

Key words: monocytes, TNF inhibitors, apoptosis, mRNA, IL-6
with informed consent by centrifugation of heparinized venous blood over sodium diatrizate-Ficoll gradients. Monocytes were prepared from PBMCs using the Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA, USA). The monocyte population obtained in this manner contained <0.1% CD3+ cells, <0.1% CD19+ cells, and >93% CD14+ cells.

The reagents, infliximab and etanercept were purchased from Mitsubishi Tanabe Pharma (Tokyo) and Takeda Pharmaceutical (Tokyo), respectively. The control human IgG1 was purified from serum of a patient with human IgG1 myeloma using DEAE (diethylaminoethanol)-Sepharose columns. Human IgG-F(ab')2 (Gamma Venin® P) was purchased from Sanofi, Paris, France.

RPMI (Roswell Park Memorial Institute) 1,640 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 (SRH Bio-Sciences, Lenexa, KS, USA) were used for cultures. Purified monocytes (1 × 10⁶/well) were cultured in the presence of SEB (100 pg/ml) (Serva, Heidelberg, Germany) in each well of 24-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) with control IgG (10 μg/ml), or pharmacologically attainable concentrations of etanercept (10 μg/ml) or infliximab (10 μg/ml) for 24 and 48 hours.

**IL-6 measurement**

IL-6 concentrations in the culture supernatants were measured using the Human IL-6 ELISA Development Kit from Peprotech (Rocky Hill, NJ, USA).

**RNA isolation and real-time quantitative RT-PCR**

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 Molony murine leukemia virus (M-MLV) reverse transcriptase (Takara Bio, Otsu, Shiga) with random primers (Takara Bio) and was subjected to analysis with real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) using LightCycler 4.1 (Roche Diagnostics, Lewes, UK). Real-time quantitative RT-PCR of IL-6 and β-actin was performed using SYBR Premix Ex Taq II (Takara Bio) with the following primers: sense, 5'-GGAGACTTGCCTGGTGAAAA-3' and antisense, 5'-GTCAGGGGTGGTTATTGCAT-3' for IL-6 (gene accession No. M14584); sense, 5'-TGGCACCCAGCACAATGAA-3' and antisense, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' for β-actin (gene accession No. NM001101). 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**

Statistical significance was evaluated by Wilcoxon’s signed rank test and paired t test where appropriate.

**Results**

**Effects of TNF inhibitors on Annexin V expression of SEB-stimulated monocytes**

Initial experiments examined the effects of TNF inhibitors on Annexin V expression of SEB-stimulated monocytes to explore their influences on the induction of apoptosis. As shown in Figure 1, TNF inhibitors, etanercept and infliximab significantly increased the expression of Annexin V of monocytes compared with control IgG. These biological agents also increased the numbers of the Annexin V-positive and PI-negative cells significantly. The results, therefore, indicate that TNF inhibitors promote apoptosis of SEB-stimulated monocytes. Moreover, the data confirm that human monocytes are direct targets of TNF inhibitors.

It was previously discovered that etanercept and infliximab were able to induce apoptosis of TNFα expressing Jurkat T cells in the presence of human...
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PBMCs, presumably through antibody dependent cellular cytotoxicity (ADCC), which requires the presence of Fc receptors. It is therefore possible that interactions with Fc receptors on monocytes might be required for the induction of apoptosis of monocytes by etanercept and infliximab. To explore this possibility, we next examined the influences of IgG and IgG-F(ab')2 fragments on the capacities of these biological agents to induce apoptosis of SEB-stimulated monocytes. As shown in Figure 2, addition of IgG, but not IgG-F(ab')2 almost completely reversed the capacities of not only etanercept, but infliximab, to induce the apoptosis of SEB-stimulated monocytes (P < 0.05). These results indicate that Fc receptors on monocytes are involved in the induction of apoptosis of SEB-stimulated monocytes by TNF inhibitors. Moreover, it is also suggested that the outside to inside signal via TNFα alone might not be sufficient for the induction of the apoptosis of human monocytes.

Effects of TNF inhibitors on the expression of CD80 and CD86 on SEB-stimulated monocytes

The following experiments examined the effects of each biological agent on the expression of costimulatory molecules on monocytes stimulated with SEB. Figure 3A shows typical histograms of the expressions of CD80 and CD86 on Annexin V-negative monocytes from a normal healthy donor. Etanercept and infliximab markedly suppressed the expressions of CD80 and CD86, respectively. Accordingly, both etanercept and infliximab significantly suppressed the expressions of CD80 and CD86 on SEB-stimulated monocytes from 9 healthy individuals compared with the control IgG (Figure 3B). The results indicate that TNF inhibitors suppressed the expression of costimulatory molecules on SEB-stimulated monocytes.

Figure 1. Effects of TNF inhibitors on Annexin V expression of SEB-stimulated monocytes

Highly purified monocytes (1 × 10⁶/well) from 8 healthy individuals were cultured in the presence of SEB (100 pg/ml) in each well of 24-well flat-bottomed microtiter plates with etanercept (ETN) (10 μg/ml), infliximab (IFX) (10 μg/ml) or control IgG (10 μg/ml) for 48 hours, after which the cells were stained with FITC-conjugated Annexin V and propidium iodide (PI). The cells were then analyzed by flow cytometry. A. Representative dot plots for staining with FITC-conjugated Annexin V and PI. B. Percentages of total Annexin V-positive cells (upper) or PI-negative Annexin V-positive cells (lower) are shown. Statistical significance was evaluated by Wilcoxon's signed rank test.
monocytes.

**Effects of TNF inhibitors on the IL-6 production of SEB-stimulated monocytes**

Recent studies have revealed that plasma concentration of IL-6, but not TNFα, is correlated with the disease activity and radiographic progression in RA. The remaining experiments, therefore, examined the effects of TNF inhibitors on the production of IL-6 of SEB-stimulated monocytes. As shown in Figure 4A, etanercept and infliximab decreased the concentrations of IL-6 in the culture supernatants of SEB-stimulated monocytes at pharmacologically attainable concentrations. We subsequently examined the effects of these biological agents on the expression of mRNA for IL-6 in SEB-stimulated monocytes. As shown in Figure 4B, etanercept and infliximab suppressed the expression of mRNA for IL-6 in SEB-stimulated monocytes at their pharmacologically attainable concentrations. These results indicate that etanercept and infliximab have direct effects on SEB-stimulated monocytes to suppress their expression of IL-6.

**Discussion**

This study clearly demonstrates that etanercept and...
infliximab induce apoptosis of SEB-stimulated monocytes. Because the addition of normal human IgG, but not IgG-F(ab')2, inhibited the induction of apoptosis by these biological agents, it is suggested that Fc receptors on monocytes are involved in the induction of apoptosis of SEB-stimulated monocytes by TNF inhibitors. A previous study showed that ADCC is one of the mechanisms of the induction of apoptosis in target cells.11 Moreover, we examined the influences of IgG-Fc on the capacities of these biological agents to induce apoptosis of SEB-stimulated monocytes in 2 healthy individuals. The addition of IgG-Fc reversed the capacities of not only etanercept but also of infliximab to induce apoptosis of SEB-stimulated monocytes (data not shown). These results indicate that either IgG1 or IgGs has the influence of inhibiting the induction of apoptosis by these biological agents. Thus, it is strongly suggested that TNF inhibitors might induce apoptosis of human monocytes through ADCC. It is noteworthy that Mitoma et al. demonstrated that infliximab and adalimumab, but not etanercept, induce apoptosis of Jurkat T cells expressing TNFα on the surface through outside to inside signals by membrane bound TNFα.5 Moreover, infliximab and adalimumab exerted much higher complement dependent cytotoxicity to TNFα-expressing Jurkat T cells than did etanercept.5 It should be emphasized, however, that ADCC activities to TNFα-expressing Jurkat T cells of etanercept were comparable to those of infliximab or adalimumab.5 Because the addition of intact IgG almost completely abrogated the capacity of infliximab and etanercept to induce apoptosis of SEB activated monocytes in the present study, strongly suggests that induction of apoptosis of human monocytes by these biological compounds might be mediated through ADCC.

It was found that infliximab did not result in the increased apoptosis in the RA synovial tissue at 48 hours after initiation of treatment.12 However, 8 weeks of treatment with etanercept or infliximab significantly

Figure 3. Effects of TNF inhibitors on the expressions of CD80 and CD86 on SEB-stimulated monocytes

Highly purified monocytes (1 × 10⁶/well) from 9 healthy individuals were cultured in the presence of SEB (100 pg/ml) in each well of the 24-well flat-bottomed microtiter plates with etanercept (ETN) (10 μg/ml), infliximab (IFX) (10 μg/ml), or control IgG (10 μg/ml) for 48 hours, after which the cells were stained with FITC-conjugated anti-CD80, anti-CD86, or control IgG1, followed by counterstaining with PE-conjugated Annexin V. The cells were then analyzed by flow cytometry. A. Representative histograms of the staining of various molecules on Annexin V-negative monocytes. The percents positive for specific mAb staining are indicated. Stainings with isotype-matched control mAb are indicated by shade. B. Percentages positive for each specific mAb staining of monocytes from nine independent experiments are summarized. Statistical significance was evaluated by Wilcoxon's signed rank test.
increased apoptosis, accompanied by a significant decrease in the synovial macrophage population. Accordingly, etanercept and infliximab induced apoptosis of macrophages and monocytes in synovial fluid in vitro. It should be pointed out that SEB activates monocytes and dendritic cells through the Toll-like receptor (TLR)2. However, previous studies demonstrated that the expression of TLR2 was up regulated in peripheral blood monocytes, synovial macrophages, and synovial tissue of RA, thus, playing an important role in the pathogenesis. Because TNF inhibitors induced apoptosis of SEB-stimulated monocytes, it is possible that they might also induce apoptosis of monocytes in vivo in RA.

Because macrophages and monocytes derived from bone marrow are precursors of type A synoviocytes, the induction of apoptosis of peripheral blood monocytes might also lead to the reduction of synovial macrophages. These results in the present study further confirm that one of the important mechanisms of action of biological agents involves the induction of apoptosis of macrophages and monocytes in RA.

We have previously disclosed that infliximab as well as etanercept, but not tocilizumab, inhibited the proliferation and the interferon-γ production of SEB-stimulated T cells. Neither infliximab nor etanercept inhibited the T cell activation by immobilized anti-CD3 in the absence of monocytes. Because the activation of T cells by SEB requires the presence of antigen-presenting cells, the inhibition of SEB-stimulated T cells by infliximab or etanercept is considered to be a result of the inhibition of monocytes. Accordingly, both infliximab and etanercept suppressed the expression of HLA-DR on monocytes. Moreover, the data in the present study have shown that TNF inhibitors suppress the expression of CD80 and CD86 on SEB-stimulated monocytes. It is, therefore, likely that the suppression of SEB-mediated T cell activation by TNF inhibitors could be due to the inhibition of expression of HLA-DR as well as the costimulatory molecules, CD80 and CD86. The mechanisms of suppression of expression of these molecules by TNF inhibitors remain unknown. Further studies to elucidate the influences of TNF inhibitors on the expression of mRNA for CD80, CD86, and HLA-DR, and on the cleavages of these molecules from the membranes, are warranted to better understand the mechanisms of action.

IL-6 has been found to play an important role in the pathogenesis of RA. Successful treatment with methotrexate or with TNF inhibitors results in the
reduction of plasma IL-6. However, it has also been shown that in patients with chronic progressive neuro-Behcet’s disease, who showed sustained elevation of cerebrospinal fluid IL-6, infliximab markedly decreased those levels on the next day of the first infusion.24 The results in the current studies, together with those of the present study, demonstrate that TNF inhibitors suppressed the production and the expression of mRNA for IL-6 in SEB-stimulated monocytes, confirming that TNF inhibitors inhibit the expression of IL-6 in the sites of inflammation in vivo.

In summary, the collective results in the current studies, including those of this study, have delineated the influences of TNF inhibitors on human monocytes. The common features of TNF inhibitors include the induction of apoptosis and the inhibition of the expression of mRNA for IL-6. The differential capacities of TNF inhibitors on T cell activation appear to be a result of their capacities to inhibit the expression of HLA-DR and costimulatory molecules. Further studies are warranted to elucidate the precise mechanisms by which TNF inhibitors regulate the expressions of HLA-DR, CD80, and CD86.

Aknowledgments
This work was supported by Parents’ Association Grant from Kitasato University School of Medicine and grants from Mitsubishi Tanabe Pharma, Tokyo; Takeda Pharmaceutical, Tokyo; and Chugai Pharmaceutical, Tokyo. These grants had no influence on the study design, the collection, analysis, or interpretation of the data, the writing of the report, or in the decision to submit the manuscript for publication.

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