Nafamostat Mesilate Induces Production of Interleukin-12 and -18 in Human Peripheral Blood Mononuclear Cells

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Abstract.

Little has been reported on the drugs inducing production of monocyte-derived cytokines like interleukin (IL)-18 and IL-12. We found that nafamostat mesilate elicits IL-12, IL-18, tumor necrosis factor-α and interferon-γ production, and the expression of intercellular adhesion molecules-1, B7.1, B7.2, CD40, and CD40 ligand in human peripheral blood mononuclear cells. The cytokine production and adhesion molecule expression were abolished by anti-IL-12 and IL-18 antibodies. Therefore, IL-18 and IL-12 may play roles in the significant and immediate effects of nafamostat mesilate.

Keywords: nafamostat mesilate, interleukin-18, interleukin-12

Interleukin (IL)-12 is a central immunoregulatory cytokine that promotes Th1 differentiation and cell-mediated immune responses (1). IL-12 acts on T-cells to induce the production of interferon (IFN)-γ (2). It is reported that bacteria, bacterial products, and intracellular parasites are strong inducers of IL-12 expression (3). Human thioredoxin (Trx) also induces secretion of IL-12 from monocytes in peripheral blood mononuclear cells (PBMC) (4). Little is known about drugs inducing IL-12 production. IL-18 induces the proliferation of activated T-cells, activation of NK-cells, and secretion of several cytokines, and it acts together with IL-12 in promoting the generation of IFN-γ-producing Th1 cells (2). IL-12 and IL-18 synergistically enhance IFN-γ production in PBMC (5). Cell-to-cell interactions mediated by engaging intracellular adhesion molecule (ICAM)-1, B7.1, B7.2, CD40, and CD40 ligand (CD40L) on monocytes and their ligands on T-cells play roles in innate immune responses including cytokine production (6). IL-18 elicits the expression of ICAM-1, B7.2, and CD40 ligand on monocytes (5, 6). The combination of IL-18 and IL-12 results in a more potent anti-tumor response (7), and NKT cells activated with this combination are essential and collaborate in natural host immunity against the growth of tumors (8). Furthermore, combination therapy using IL-12 and IL-18 induces tumor regression by inhibiting angiogenesis (9). Little has been reported about clinically available drugs that induce IL-12 and IL-18 production.

In this study, we examined the effect of nafamostat mesilate (6-amidino-2-naphthy-γ-guanidino-benzoate dimethanesulfonate; Torii Pharmaceutical Co., Ltd., Tokyo) on IL-18, IL-12, tumor necrosis factor (TNF)-α, and IFN-γ production in PBMC. Normal human PBMC were obtained from ten human volunteers with their written informed consent. Samples of 50 ml of peripheral blood were withdrawn from a forearm vein, after which PBMC were isolated and suspended at 1×10^6 cells/ml in medium as previously described (6). PBMC were treated with nafamostat mesilate at 0 – 10^-4 M. After 24-h incubation, the cell-free supernatant fractions of PBMC were assayed for IL-18, IL-12, TNF-α, and IFN-γ protein by enzyme-linked immunosorbent assay (ELISA) using commercially available kits such as IL-18 (MBL, Nagoya) and IL-12, TNF-α, and IFN-γ (Quantikine, R&D Systems, Minneapolis, MN, USA) as previously described (6).

All experiments were performed at least in triplicate, and the results were calculated as the means ± S.E.M. of five independent experiments. The statistical signifi-
cance of difference was evaluated by ANOVA followed by Dunnett’s test. A probability value of less than 0.05 was considered to indicate statistical significance.

Nafamostat mesilate induced the production of IL-18, IL-12, TNF-α, and IFN-γ in a concentration-dependent manner (Fig.1A). The ED$_{50}$ values of nafamostat mesilate for enhancing the production of IL-18 and IL-12 were 3 and 2 µM, respectively. The level of IL-18 and IL-12 production reached to about 350 and 2100 pg/ml, respectively, with the stimulation of nafamostat mesilate at $10^{-4}$ M in PBMC.

The effect of nafamostat mesilate on the expression of ICAM-1, B7.1, B7.2, CD40, and CD40L on monocytes was determined as shown in Fig. 1B. For flow cytometric analysis, fluorescein isothiocyanate (FITC)-conjugated mouse IgG$_1$ mAb against ICAM-1/CD54 (6.5B5), phycoerythrin (PE)-conjugated anti-CD14 mAb (DAKO, Glostrup, Denmark); FITC-conjugated mouse IgG$_1$ mAb against B7.1/CD80 (MAB104) (IMMUNOTECH, Marseille, France); FITC-conjugated mouse IgG$_1$ mAb against B7.2/CD86 (2331FUN-1), CD40 (5C3) (Pharmingen, San Diego, CA, USA); FITC-conjugated mouse IgG$_1$ mAb against CD40L/CD154 (Ancel, Bayport, MN, USA); and FITC-conjugated an IgG$_1$ class-matched control (CMC) (Sigma Chemical, St. Louis, MO, USA) were used. Changes in the expression of human leukocyte antigens on monocytes were examined by double-labeling flow cytometry using a combination of anti-CD14 Ab with Abs against ICAM-1, B7.1, B7.2, CD40, or CD40L. PBMC at $1 \times 10^6$ cells/ml were treated with $0 – 10^{-4}$ M nafamostat mesilate. After 24-h incubation, cultured cells at $5 \times 10^5$ cells/ml were prepared as previously described (6). The expression of these antigens was analyzed using a FACScalibur (Becton Dickinson Biosciences, San Jose, CA, USA), for which the data was processed using the CELL QUEST program (Becton Dickinson Biosciences). Nafamostat mesilate up-regulated ICAM-1, B7.1, B7.2, CD40, and CD40L expressions (Fig. 1B).

To investigate the involvement of IL-12 and IL-18 production in the expression of ICAM-1, B7.1, B7.2, CD40, and CD40L and the production of IL-18, IL-12, TNF-α, and IFN-γ in PBMC treated with nafamostat mesilate, PBMC at $1 \times 10^6$ cells/ml were treated with anti-IL-12 (Pharmingen) and IL-18 (MBL) Abs between 0 – 10 ng/ml in the presence of nafamostat mesilate at $10^{-4}$ M. After 24-h incubation, the production of IL-18, IL-12, TNF-α, and IFN-γ was determined by ELISA.

Fig. 1. Effect of nafamostat mesilate on cytokine production (A) and adhesion molecule expression (B). PBMC at $1 \times 10^6$ cells/ml were cultured with increasing concentrations of nafamostat mesilate for 24 h. A: At the end of culturing, the levels of IL-18, IL-12, TNF-α, and IFN-γ in the conditioned media were determined by ELISA. B: At the end of the culture, antigen expressions on monocytes were analyzed by double-stained flow cytometry using ICAM-1, B7.1, B7.2, CD40, and CD40L (black bars) Abs or CMC (white bars). *$P<0.05$, **$P<0.01$, compared with the value for medium alone.
(Fig. 2A), and the expression of ICAM-1, B7.1, B7.2, CD40, and CD40L was determined by double-staining-labeling flow cytometry (Fig. 2B). Anti-IL-12 Ab, which at 1 ng/ml is enough to abolish the production of IL-12, inhibited the nafamostat mesilate-initiated productions of IL-18, TNF-α, and IFN-γ in concentration-dependent manners, while anti-IL-18 Ab, which at 1 ng/ml is enough to abolish the production of IL-18, inhibited those of IL-12, TNF-α, and IFN-γ. Moreover, anti-IL-12 Ab inhibited the nafamostat mesilate-induced production of IL-18 up to 90%, whereas anti-IL-18 Ab suppressed that of IL-12 up to 35%. Anti-IL-12 and IL-18 Abs suppressed all adhesion molecule expression induced by nafamostat mesilate. The IC_{50} values of anti-IL-12 and IL-18 Abs that suppressed the production of IFN-γ and the expression of ICAM-1, respectively, were each 0.5 ng/ml. These results suggested that IL-12 and IL-18 were located upstream of the nafamostat mesilate-initiated cytokine cascade and adhesion molecules expression. In the presence of IL-12 or IL-18, nafamostat mesilate had no effect on the expression of ICAM-1, B7.2, or CD40 and the production of TNF-α and IFN-γ (data not shown), suggesting that the serine proteinase inhibitor might not abolish the effect of endogenous IL-18 and IL-12 production. Whereas IL-18 production does not depend on the cell-to-cell interaction between monocytes and T-cells, IL-12 production requires the engagement of CD40 and CD40L on monocytes and T-cells (10). Therefore, the nafamostat mesilate-induced production of IL-12 might depend on the engagement of CD40 and CD40L.

Nafamostat mesilate was found to be a potent inhibitor of human tryptase (11). On the other hand, nafamostat mesilate, a serine protease inhibitor, is

![Fig. 2. Effect of anti-IL-12 and IL-18 Abs on nafamostat mesilate-induced cytokine production (A) and adhesion molecule expression (B) in PBMC. PBMC at 1 x 10^6 cells/ml were incubated with different concentrations of anti-IL-12 (open circles) and IL-18 (filled circles) Abs in the presence of nafamostat mesilate at 10^{-4} M for 24 h. A: At the end of culturing, the levels of IL-18, IL-12, TNF-α, and IFN-γ in the conditioned media were determined by ELISA. B: At the end of the culture, antigen expressions on monocytes were analyzed by double-stained flow cytometry using ICAM-1, B7.1, B7.2, CD40, CD40L Abs or CMC. *P<0.05, **P<0.01, compared with the value for nafamostat mesilate alone.](image-url)
known to be a potent inhibitor of thrombin. Thrombin down-regulates IL-12 production at both protein and mRNA levels in human PBMC (12). The inhibition of IL-12 production was accompanied by an enhanced release of IL-10, since the addition of an anti-IL-10 monoclonal antibody to thrombin-treated PBMC resulted in a partial restoration of IL-12 production. The concentration of thrombin in the conditioned medium was not detected in the present study. Recently, the protease-activated receptor (PAR) family was identified as one branch of the G-protein-coupled receptor superfamily (13). Serine proteinases cleave the N-terminal extracellular portion of PAR, producing the new N-terminal sequence. The newly exposed N-terminal sequence binds to a yet undefined binding site of the receptor, leading to G protein coupled signal transduction. However, we found that the N-terminal sequence, activating peptides for PAR-1 and -2, could not induce IL-12 and IL-18 production in PBMC (data not shown). Therefore, tryptase, thrombin, and PAR were not involved in the effect of nafamostat mesilate on cytokine production. Further study on nafamostat mesilate-initiated intracellular signal transduction for promoting IL-18 and IL-12 production should be carried out.

The plasma half life (t1/2 beta) of nafamostat mesilate is about 23 min (14), and an in vivo pharmacokinetics study in patients with DIC revealed that the concentration of nafamostat mesilate in the blood can reach between 90 – 240 nM when continuously infused at the dose of 0.2 mg/kg per hour (15). If such concentrations can be achieved, they correspond to the micromolar range. Thus, it is likely that nafamostat mesilate modulates immune responses in vivo. In conclusion, we found for the first time that nafamostat mesilate induced the production of IL-18 and IL-12 in PBMC. Nafamostat mesilate therefore has a therapeutic potential for cancer patients.

References


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