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Leflunomide Inhibits PDK1/Akt Pathway and Induces Apoptosis of Human Mast Cells¹

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Mast cells release many inflammatory mediators that play an important role not only in allergic diseases but also in chronic inflammatory diseases, autoimmune diseases, and others. A lot of mast cells exist in synovium of rheumatoid arthritis, and it is known that synovitis does not occur in mast cell-deficient mice. Thus, it is thought that mast cells play a very important role in rheumatoid arthritis pathogenesis. Leflunomide is a drug used clinically in the treatment of rheumatoid arthritis. We used clinical doses of 2-cyano-3-hydroxy-N-(4-trifluoromethylphenyl)-butenamide (A77 1726), which is an active metabolite of leflunomide, and decreased the number of viable human primary mast cells in a concentration-dependent manner. This decrease was not reversed by uridine. Inhibition of pyrimidine synthesis by dihydro-orotic acid dehydrogenase inhibition, which is the primary mechanism of action of A77 1726, was not involved. A77 1726 dramatically induced apoptosis of human mast cells and inhibited the phosphorylation of Akt, an important survival signal of mast cells, in a concentration-dependent manner. Caspases 3 and 9, downstream molecules of Akt survival pathway, were also fragmented by A77 1726. In addition, it became evident for the first time that the mechanism involved in this result was the concentration-dependent inhibition of PDK1 phosphorylation, which controls the activation of Akt. These results indicate a new way of controlling mast cells and may therefore be the basis for innovative approaches to the treatment of various diseases related to mast cells. *The Journal of Immunology, 2007, 179: 6479 – 6484.

Leflunomide (LEF)³ is a low-molecular-weight compound currently used clinically in the treatment of rheumatoid arthritis (RA). LEF is a prodrug that is rapidly converted in the gastrointestinal tract and plasma to its active, open ring metabolite, the malononitrilamide, 2-cyano-3-hydroxy-N-(4-trifluoromethylphenyl)-butenamide (A77 1726). The main mechanism of antirheumatic action is thought to be inhibition of de novo pyrimidine synthesis, caused by a reduction of dihydro-orotic acid dehydrogenase (DHODH) activity. It has been suggested that this mechanism of action is mainly effective in lymphocytes (1).

Mast cells, due to their degranulation, release a variety of inflammatory mediators such as inflammatory cytokines and chemokines; they are involved not only in allergic diseases such as asthma and atopic dermatitis but also in chronic inflammatory, autoimmune, and fibrotic diseases (2, 3). In addition, mast cell-specific molecules, such as tryptase and chymase, continue to appear (4, 5). Therefore, the role of mast cells in the pathology of numerous intractable diseases is becoming clearer. Mast cells are present in high numbers in the synovial membrane of RA joints and release various inflammatory cytokines, such as TNF-α, that are closely related to RA (6). What’s more, mast cell-deficient mice are innately resistant to arthritis (7). Thus, mast cells are believed to play an important role in this illness (8, 9). However, the effect of A77 1726 on mast cells has never been investigated.

In addition, LEF has been reported to be effective against allergic diseases such as atopic dermatitis and asthma (10 –13). Although mast cells play an important role in these conditions (14, 15), the direct effect of A77 1726 on human mast cells has not been elucidated.

Stimulation of receptors by the c-Kit ligand stem cell factor (SCF) is important in maintaining the survival of mast cells. When the c-Kit ligand is activated, the Akt signal, in turn, is activated via PI3K (16). Akt is a very important survival signal for mast cells (17, 18). We therefore examined the effects of A77 1726 on human mast cells, focusing on the Akt survival signal.

Materials and Methods

Materials

A77 1726, an active metabolite of LEF, was used as LEF. A77 1726 was supplied by Aventis Pharma. A77 1726 was dissolved in DMSO and the same concentration of DMSO used for dissolving 200 μM A77 1726 was added to the A77 1726 control.

Cells and culture conditions

Generation of umbilical cord blood-derived mast cells. We generated human umbilical cord blood-derived mast cells (CBMCs) according to a method established by Saito et al. (19). The umbilical cord blood was provided under informed consent. Umbilical cord blood samples were drawn into heparinized syringes and diluted two times with PBS. They were then separated by density-gradient centrifugation using lymphocyte separation medium (Organon Teknika). CD34⁺ cells were positively selected from cord blood mononuclear cells using a CD34⁺ cell isolation kit.
(Milenyi Biotec). From $10^4$ to $10^5$ CD34+ cord blood cells were suspended in 1-ml aliquots of IMDM (Invitrogen Life Technologies; 12440-053) supplemented with 1% insulin-transferrin-selenium (Invitrogen Life Technologies; 41400-045, 100×), 5 × $10^{-5}$ M 2-ME (Invitrogen Life Technologies; 21985-023), 1% penicillin plus streptomycin (Invitrogen Life Technologies; 15140-122), 2% FCS, SCF (PeproTech; 300-07) at 100 ng/ml and IL-6 at 50 ng/ml. The suspensions were cultured at 37°C in 5% CO₂. Every 5–7 days, half the medium was replaced with fresh medium containing SCF plus IL-6. Mature CBMCs were obtained at 12–14 wk of culture.

**Cell lines.** The Jurkat cell line and the Ramos cell line (Burkitt’s lymphoma) were maintained in RPMI 1640 medium supplemented with 10% FCS. Human mast cell line HMC-1 was cultured in IMDM with 10% FCS at 37°C in 5% CO₂.

**Flow microfluorometry**

Staining and flow cytometric analyses of cells were performed using FACSscan (BD Pharmingen) and standard procedures as described elsewhere (20). Cells (2 × $10^3$) were incubated with the negative control of mAb thy-1.2, anti-c-Kit mAb PE-conjugated (Chemicon International) in FACS medium consisting of HBSS (Nissui Pharmaceutical), 0.5% human serum albumin (Yoshitomi), and 0.2% NaN₃ (Sigma-Aldrich) for 30 min at 4°C. The cytoplasmic Ag of cells, which was pretreated with a cell permeabilization kit (Caltag Laboratories), was stained with anti-tryptase mAb (LAB vision) in FACS medium for 30 min at 4°C. After washing the cells three times with FACS medium, they were further incubated with FITC-conjugated goat anti-mouse IgG Ab for 30 min at 4°C. Staining of cells with mAbs was detected using FACSScan.

**Detection of viable mast cell number by TetraColor One**

Cells seeded and incubated on 96-well flat-bottom plates in IMDM containing 2% FCS and SCF at 100 ng/ml were cultured for 24 or 48 h at 37°C in 5% CO₂ with or without 1–100 μM A77 1726, and with or without the addition of uridine 50 μM. Cells were stained with TetraColor One kit including a tetrazolium and electron carrier mixture (Seikagaku Kogyo) for 1 h at 37°C and the OD value of each well was measured at 450 nm by a microplate reader.

**Apoptosis assay**

Apoptosis was evaluated by flow cytometry using cellular annexin V binding (BD Biosciences; annexin V, FITC apoptosis detection kit I). In brief, cells were incubated with or without A77 1726 at concentrations of 10–200 μM for 24 h in IMDM containing 2% FCS and SCF at 100 ng/ml. Cells were then stained with annexin V and propidium iodide (PI) according to the manufacturer’s instructions, then sorted using a FACSscan flow cytometry (BD Biosciences). All PI-negative cells were considered as dead. PI-negative and annexin V-positive cells were considered early apoptotic cells, and the remaining double negative cells were considered viable.

**Cell extraction**

In the signal transduction assay, cells were incubated with or without 10–200 μM A77 1726 for 12 h in IMDM containing 2% FCS (SCF free) at 37°C in 5% CO₂ before stimulation with SCF (100 ng/ml) for 10 min at 37°C. In the caspase assay, cells were incubated with or without 10–200 μM A77 1726 for 12 h in IMDM containing 2% FCS and SCF (100 ng/ml) at 37°C in 5% CO₂. After washing the cells with PBS, cells were lysed in sample buffer (1% SDS, 100 mM DTT, 60 mM Tris (pH 6.8), 0.001% bromphenol blue).

**Western blot analysis**

Cell extracts and Western blots were performed according to the protocol provided by the manufacturer (Cell Signaling Technology). Proteins were visualized using the ECL method (Amersham Biosciences). All Abs for the Western blot (PDK1, Akt, glycogen synthase kinase 3β (GSK3β), phospho-PDK1, phospho-Akt, phospho-GSK3β, caspase 3, caspase 9, cleaved caspase 3, and cleaved caspase 9) were purchased from Cell Signaling Technology. The targets for each phospho-specific Ab were: pAkt (Thr308), pPDK1(Ser241), and pGSK3β(Ser4), respectively.

**Statistical analysis**

Data are expressed as mean ± SD of indicated cultures. Differences from the control were examined for statistical significance by the Mann-Whitney U test. A value of $p < 0.01$ denoted the presence of a statistically significant difference.

**Results**

**Confirmation of mast cells derived from human umbilical blood**

We succeeded in obtaining a large amount of mast cells derived from human umbilical cord blood by the method of Saito et al. (19). The cells contained granules as seen macroscopically and were shaped like mast cells. The mast cells that we obtained were confirmed to express c-Kit by flow cytometry. In addition, tryptase is a major production protein of mast cells that comparatively and specifically produces mast cells. It is often used to identify mast cells in histological stains (4). We confirmed by flow cytometry that the mast cells we obtained express tryptase (Fig. 1).

**Effect of A77 1726 on viable CBMCs number**

First, we measured the effect of A77 1726 on viable CBMC number by measuring OD450 absorbance with TetraColor One (Fig. 2A). After 24 h, the absorbance decreased significantly in a manner that was dependent on the concentration of A77 1726 (1–100 μM). After 48 h, the absorbance decreased significantly with the 10 and 100 μM concentrations. Notably, with the 100 μM concentration, there was no increase in absorbance between 24 and 48 h. This showed that A77 1726 decreased viable CBMC number in a concentration-dependent manner.

Next, we investigated whether this effect occurred via the de novo pyrimidine synthesis inhibition effect due to DHODH inhibition, which is one of the known mechanisms of action of A77 1726 (Fig. 2B). This effect of A77 1726 is known to recover when uridine is added (21–24). When A77 1726 and uridine 50 μM were simultaneously added to CBMCs, the absorbance increased significantly in the control, A77 1726 1 μM and 10 μM treated cells after 48 h. However, such increase did not occur in the A77 1726 100 μM treated cells. This demonstrated that A77 1726...
has mechanisms of action other than pyrimidine synthesis inhibition at the 100 μM concentration.

**Induction of apoptosis of CBMCs, Jurkat cells, Ramos cells, and HMC-1 cells by A77 1726**

We investigated whether the concentration-dependent decrease in viable CBMC number due to A77 1726 was by induction of CBMC apoptosis. CBMCs were stained with anti-annexin V-PI stain; 24 h later, those cells that had undergone apoptosis were detected by flow cytometry. Cells that were annexin V positive and PI negative were considered to have undergone early apoptosis, and those that were positive for both annexin V and PI had undergone late apoptosis. The means ± SD of the total apoptotic cells in CBMC and cell lines derived from the results of five different experiments using five independent donors that were similarly obtained. The same experiment was performed under the same conditions in Ramos, Jurkat, and HMC-1 cells, and the means ± SD of the total apoptotic cells in these cultures was determined. A77 1726 at concentrations of 100–200 μM induced a statistically significant increase in the number of apoptotic cells in the CBMC and HMC-1 cells, but not in the Ramos and Jurkat cells. * Significant difference (p < 0.01 in the Mann-Whitney U test).

**Inhibition of PDK1/Akt/GSK3β phosphorylation by A77 1726**

The effect of A77 1726 on the survival signals in CBMCs was investigated to determine how A77 1726 induces apoptosis in CBMCs. The Akt pathway is known as an important cell survival signal pathway whereby cells can withstand apoptosis (18). The activation of Akt has been pointed out in CBMCs also (16). Akt activation requires phosphorylation of the Thr308 site by PDK1 (25). So, we investigated the effect of A77 1726 on Akt phosphorylation (Thr 308) by Western blotting. As shown in Materials and Methods, all the results were obtained in the presence of SCF (100 ng/ml), whereas the e-Kit receptors were in the stimulated state. First, as shown in Fig. 5A, the anti-Akt Ab band was seen in the 60 kDa region, showing that many Akt molecules were expressed in apoptosis in CBMCs and HMC-1 cell lines in a concentration-dependent manner and that there is a statistically significant increase in apoptotic cells in these cultures.
A77 1726 fragmented caspases 3 and 9 in CBMCs in a concentration-dependent manner. Various concentrations of A77 1726 were added to CBMCs to detect fragmentation of various Abs by Western blotting. A. When anti-caspase 3 Ab was used, the intensity of the full length caspase 3 band (35 kDa) was decreased in a concentration-dependent manner, and that of the fragmented caspase 3 band (17, 19 kDa) was increased in a concentration-dependent manner. Even when anti-cleaved caspase 3 Ab was used, the intensities of the fragmented caspase 3 bands (17, 19 kDa) were increased in a concentration-dependent manner. B. Similarly, when anti-caspase 9 Ab was used, the intensity of the full length caspase 9 band (47 kDa) was decreased in a concentration-dependent manner. In contrast, those (37 kDa) of the fragmented caspase 9 bands were increased in a concentration-dependent manner. The data is one representative result among four results.

Discussion

A77 1726 inhibited the number of viable mast cells in a concentration-dependent manner after 24 and 48 h. That effect was particularly marked with the 100 μM concentration, at which the viable cell number failed to recover even when uridine was added. Inhibition of pyrimidine synthesis by DHODH inhibition, which is a typical mechanism of action of A77 1726, recovers with 50 μM uridine (21–24). This suggested that, apart from the inhibition of pyrimidine synthesis, other mechanisms were involved in this effect.

The DHODH inhibitory effect is reported to occur at a comparatively low concentration of A77 1726 (<5 μM) (1, 27). The effect on pyrimidine synthesis is marked in lymphocytes, particularly...
active T lymphocytes, which are involved in the rate-limiting step of nucleic acid synthesis (28–35). Another mechanism of action of A77 1726 is the inhibition of tyrosine kinase; however, a comparatively high concentration of A77 1726 (>50 μM) is required to produce it. Therefore it is of relatively minor importance by comparison to the previously mentioned DHODH inhibition (1, 27, 33, 36). There is also the question of what actually happens to the concentration of A77 1726 after it is administered to patients with RA. According to the LEF (Arava) prescribing information issued by Aventis (37), the usual oral maintenance dose of LEF in RA therapy is 10–20 mg. However, the plasma concentration of A77 1726 in RA patients who reached a steady state following repeated daily administration of 25 mg was ~233 μM (63 ± 36 μg/ml) (37). The concentration of 100–200 μM that we used in our experiment is one that would likely reach the clinical level. This suggests that we need to focus more on actions of A77 1726 other than its DHODH inhibitory effect.

We sought to discover whether the mechanism whereby A77 1726 inhibits the proliferation of viable mast cells is by inducing mast cell apoptosis and, as shown in Fig. 3, did in fact induce such apoptosis. In addition, as shown in Fig. 5, we found that caspases 3 and 9, both factors that induce apoptosis, were activated.

Thus, we focused on the Akt signal, which is important for the survival of mast cells (17, 18). When c-Kit receptors are activated by mast cells, the PI3K/Akt signal is activated (16). Our results also demonstrated Akt phosphorylation following the addition of SCF. As shown in Fig. 4, this Akt phosphorylation was inhibited by A77 1726 in a concentration-dependent manner. Furthermore, we confirmed that decreased phosphorylation of GSK3β, which is a downstream molecule of Akt, induced the fragmentation of caspase 9 and decreased the activation of the Akt molecule.

Furthermore, we elucidated that PDK1, which controls the activation of Akt, is inhibited by A77 1726. At present, the mechanism whereby PDK1 is phosphorylated remains unclear. However, it has been suggested to be by autophosphorylation (38, 39). The site of PDK1 phosphorylation, Ser 241, is very important in PDK1 activation (38). However, as shown in Fig. 4, A77 1726 inhibited Ser241 phosphorylation of PDK1 in a concentration-dependent manner. Thus, we report for the first time that A77 1726 is one of PDK1 inhibitors. This inhibition of the activation of the PDK1/Akt molecule, which is an important signal for cell survival (18), is considered useful for mast cell-targeting therapy.

In clinical use, LEF has been shown to be effective in treating the symptoms of RA. However, many mast cells are present in the synovial sites of RA (6) and play an important role in the production of rheumatoid symptomatology (7–9). LEF has also been reported to be effective in the treatment of atopic dermatitis and asthma, diseases in which mast cells also play an important role (10–13). Having elucidated the ability of A77 1726 to induce apoptosis in mast cells, we believe that it may become part of the clinical treatment of these diseases. Moreover, imatinib mesylate (ST1571), a medication that is used in the treatment of chronic myeloid leukemia, acts on mast cells by inhibiting tyrosine kinase at the c-Kit receptor, thereby also showing efficacy in RA and asthma (40–43). Inhibition of mast cell activity is likely to lead to a decrease in overall immunity. However, mast cells play an important role in maintaining the excessive inflammation characteristic of chronic inflammatory disease; therefore, mast cell inhibition is an important factor in reducing this inflammation. We therefore suggest that such mast cell-targeting therapy will become a new therapeutic concept in the treatment of mast cell-related diseases.

Our study has demonstrated that A77 1726 induced apoptosis in mast cells. In addition, we elucidated for the first time the mechanism of A77 1726’s effect, which is the inhibition of PDK1 and Akt. This newly discovered mechanism of action of A77 1726 is expected to facilitate the development of cures for numerous conditions involving mast cells, including allergic, chronic inflammatory, and autoimmune diseases.

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Disclosures
The authors have no financial conflict of interest.

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