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Il Farmaco 58 (2003) 983–987
IL FARMACO
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Effects of leflunomide on human cartilage

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Received 12 November 2002; accepted 15 February 2003

Abstract

Modern therapeutic approach in rheumatoid arthritis (RA) includes early use of disease-modifying anti-rheumatic drugs (DMARDs). DMARDs may influence the course of disease progression, and their introduction in early RA is recommended to limit irreversible joint damage. Among DMARDs, leflunomide and methotrexate are more utilised in pharmacological therapy. In the present work, we considered the effects of leflunomide, in comparison with those of methotrexate and to those of leflunomide–methotrexate combination on human cartilage to verify its effectiveness in arthritic disease, simulated by our experimental model. We measured in vitro the amount of glycosaminoglycans (GAGs) and the production of nitric oxide (NO) released into the culture medium of human articular cartilage treated with interleukin-1β (IL-1β), which promotes the cartilage destruction during articular disease. Leflunomide, in the presence of IL-1β decreased NO production and GAGs release respect IL-1β alone treated samples, in dose-related manner. Our results suggest that leflunomide is able to protect cartilage matrix from degradative factors induced by IL-1β with respect to methotrexate and leflunomide–methotrexate combination.
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Keywords: NO; GAGs; Leflunomide; Human articular cartilage

1. Introduction

Rheumatoid arthritis (RA) is a complex pathology, that evolves from a local inflammatory disease to a chronic process with distinct inflammatory and destructive components. Its pathophysiology involves a cascade of catabolic events that lastly lead to loss of articular cartilage and irreversible resorption of subchondral bone. The deterioration of articular structure typical of RA is caused from a lack of balance between process of enzymatic degradation and regeneration of cartilage tissue. Various pro-inflammatory cytokines are present in the synovial fluid of RA patients and, particularly, interleukin-1β (IL-1β) plays a central catabolic role [1]. It can cause cartilage destruction by stimulating the release of degradative products and it can increase bone resorption by stimulating osteoclast differentiation and activation. Moreover, recent findings have shown that IL-1β is a very potent stimulator of chondrocyte nitric oxide (NO) synthesis, acting by upregulating the level of the inducible form of nitric oxide synthase (iNOS) [2–4], and by strongly supporting the involvement of NO in arthritic conditions. NO has been shown to mediate some of the catabolic effects elicited by IL-1β, as suppression of proteoglycan synthesis, activation of protease gene expression, inhibition of cell proliferation and induction of apoptosis. During the process of cartilage destruction, other key molecules released from the cartilage matrix are glycosaminoglycans (GAGs) [5], which released is promoted by NO presence.

Modern therapeutic approach in RA includes early use of disease-modifying anti-rheumatic drugs (DMARDs). DMARDs may influence the course of

Abbreviations: IL, interleukin 1β at the concentration of 10 ng/ml; Lefl 25, leflunomide at the concentration of 25 μg/ml; Lefl 50, leflunomide at the concentration of 50 μg/ml; Lefl 100, leflunomide at the concentration of 100 μg/ml; Met, methotrexate at the concentration of 0.0454 μg/ml.

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doi:10.1016/S0014-827X(03)00109-5

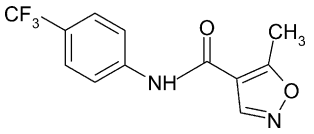

Fig. 1. Chemical structure of leflunomide.

disease progression, and their introduction is made to limit irreversible joint damage. Among DMARDs more utilised in pharmacological treatment, methotrexate and leflunomide have showed a significant behaviour. Leflunomide is a novel DMARD with proven clinical efficacy in active RA. With its chemical name *N*-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxazide and a molecular weight of 270.2 Da ($C_{12}H_9F_3N_2O_2$), it belongs to derivatives of isoxazole (Fig. 1). Leflunomide is rapidly converted to the ring-open form, a malononitrilamide termed A771726 [3-cyano-3-hydroxy-*N*-(4-trifluoromethylphenyl)-crotonamide] [6]. It is a beta-keto amide with the *cis*-enolic hydroxy group to the amidic moiety. It shares no apparent chemical or structural relationship with existing immunomodulating drugs and is distinct in its mechanism of action from any other clinically used DMARD. Leflunomide and its active metabolite A771726 are considered to exert their beneficial effects by the inhibition of dihydrorotate dehydrogenase, an enzyme responsible for de novo pyrimidine biosynthesis, thereby blocking proliferation of a variety of effector cells [7]. The efficacy of leflunomide has been tested in several animal model of RA or in RA-like conditions [8].

Another DMARD approved for RA is methotrexate, a folate antagonist. It is used in the rheumatic therapy by suppressing the blood cells that cause inflammation [9]. Moreover, today there is an increasing interest in combining anti-rheumatic drugs that act on different sites in the pathological cascade of RA to obtain a greater and prolonged efficacy in adverse events.

Starting from the above considerations, we investigate in the present work the in vitro effects of leflunomide, methotrexate and the combination leflunomide–methotrexate on metabolism human cartilage treated or untreated with IL-1β by measuring the amount of GAGs release and NO production into culture media. Moreover, we would consider the eventual synergetic activity of leflunomide–methotrexate combination.

2. Experimental section

2.1. Drugs

Leflunomide was dissolved in DMSO, appropriately diluted in Dulbecco's modified Eagle's medium (DMEM) (Sigma, UK) and dispensed to the wells in 2.5, 5 and 10 μl volumes to give the wanted final concentrations: 25, 50 and 100 μg/ml. Methotrexate, instead, was used at concentration of 0.0454 μg/ml.

2.2. Culture of human articular cartilage

Articular human cartilage was obtained from knee surgery and washed in Hank's balance salt solution containing antibiotics. These explants were sliced in discs (3–4 mm in diameter) and weighed. The samples were placed into 24-well plates containing 1 ml of DMEM, phenol red free, glutamine (10 mM) and penicillin/streptomycin (50 U/ml and 50 mg/ml, respectively), and enriched with 10% heat inactive foetal calf serum. Cartilage pieces were incubated in a 37 °C/5% CO_2/95% air humidified incubator. Controls were incubated with medium alone. The samples were cultured in the presence and absence of human recombinant IL-1β (Peprotech, UK), 10 ng/ml in each well, to mimic conditions in inflammed joints where there might be effects of this cytokine on proteoglycan turnover. In human tissues, with high proteoglycan synthetic rates, this cytokine caused a significant reduction in proteoglycan synthesis.

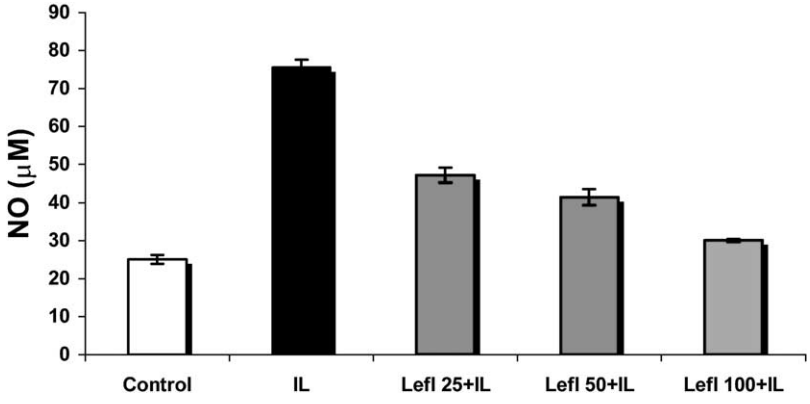

Fig. 2. NO production (mean ± SEM) in the culture medium from human articular cartilage 120 h after the addition of leflunomide at 25, 50 and 100 μg/ml with IL-1β. Values are expressed as μM. (*) Significantly different from IL-1β-treated samples ($P < 0.05$).

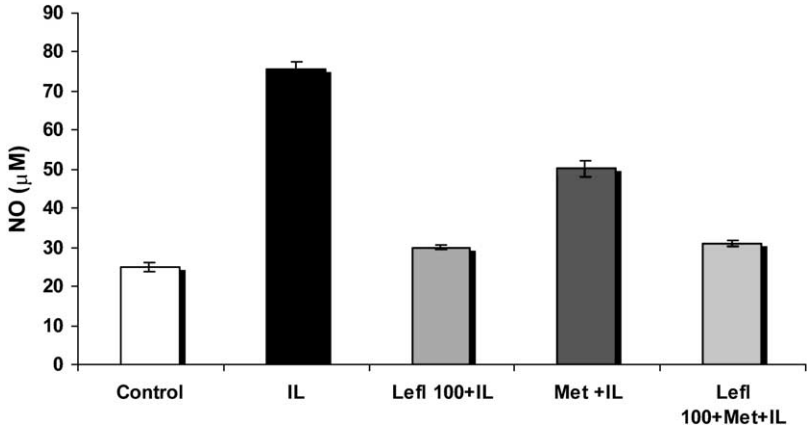

Fig. 3. NO production (mean ± SEM) in the culture medium from human articular cartilage 120 h after the addition of 100 μg/ml leflunomide with IL-1β, 0.0454 μg/ml methotrexate with IL-1β, leflunomide–methotrexate with IL-1β. Values are expressed as μM. (*) Significantly different from IL-1β-treated samples ($P < 0.05$).

2.2.1. Determination of GAGs

GAGs were tested in the culture medium by utilising a spectrophotometric assay for polysulfated GAGs using 1,9-dimethylmethylene blue at a wavelength of 535 nm [10].

Shark chondroitin sulfate was used to construct a standard curve.

2.2.2. Determination of NO

Tissue culture media were assayed for nitrite concentration by using the Griess reaction [11]. The assay involved the addition of equal volumes of sulfanilamide (1%, w/v), *N*-(1-naphthyl)ethylenediamine (1%, w/v) and culture media into 96-well plates. The absorbance was measured at 540 nm, and finally, the nitrite concentration determined from a sodium nitrite standard curve (0–120 μM). The values were expressed as micrometre nitrite released per 100 mg of wet weight cartilage.

2.2.3. Statistical analysis

All the results presented are mean ± SEM of three experiments performed on quadruplicate samples. The Student's *t*-test was used to evaluate the differences between the means of each group.

3. Results and discussion

The aim of our study is to evaluate the effects of leflunomide, methotrexate and the combination leflunomide–methotrexate on cartilage selecting two targets: NO and GAGs, that are known to be involved in various inflammatory processes.

No significant differences on NO production were observed among treatments with leflunomide, methotrexate, combination leflunomide–methotrexate alone and untreated cartilage samples (data not showed). To stimulate NO synthesis, we used IL-1β, a well-known activator of iNOS expression and NO production [12]. A significant rise of 66% (75.5 μM) in nitrite levels on

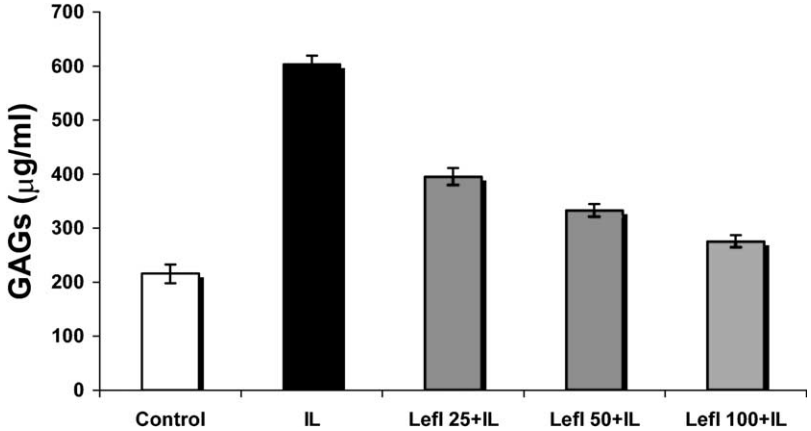

Fig. 4. GAGs release (mean ± SEM) from human articular cartilage 120 h after the addition of leflunomide at 25, 50 and 100 μg/ml with IL-1β. Values are expressed as μg/ml. (*) Significantly different from IL-1β-treated samples ($P < 0.05$).

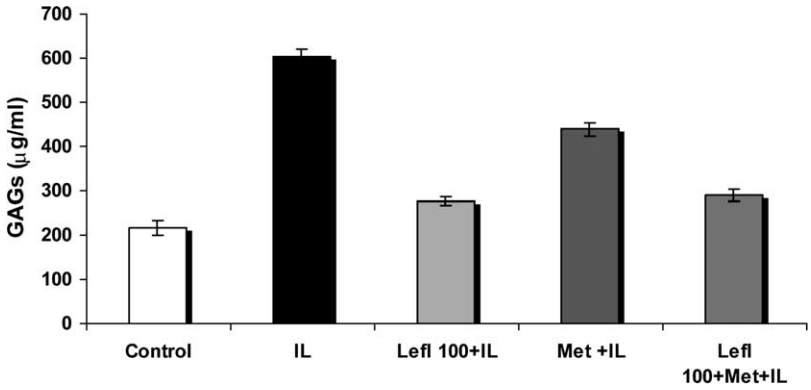

Fig. 5. GAGs release (mean ± SEM) from human articular cartilage 120 h after the addition of 100 μg/ml leflunomide with IL-1β, 0.0454 μg/ml methotrexate with IL-1β, leflunomide–methotrexate with IL-1β. Values are expressed as μg/ml. (*) Significantly different from IL-1β-treated samples ($P < 0.05$).

supernatant of culture cartilage samples treated with cytokine pro-inflammatory was observed in comparison with the low basal values for control samples (25.2 μM) (Fig. 2). The treatment with leflunomide at 25, 50 and 100 μg/ml caused a dose-dependent decrease in nitrite accumulation in culture media of IL-1β stimulated cartilage, showing values of 47.2 ± 1, 41.4 ± 1.4 and 30 ± 0.8 μM, respectively (Fig. 2). These data led us to utilise for combination and comparison with methotrexate, 100 μg/ml concentration of leflunomide. Methotrexate decreases the nitrite level to 50 ± 1.6 μM (33.8% of decrease) with respect the cytokine, but this effect was less significant compared to that of leflunomide (60.3%). The leflunomide–methotrexate combination showed good inhibitory effect (31.1 ± 2 μM, 58.8% of decrease), slightly lesser than that of leflunomide (60.3% of decrease; Fig. 3).

Concerning GAGs determination, we observed a basal release of 215 μg/ml in untreated cartilage. This release was highly increased of 64% (603 μg/ml) by the addition of pro-inflammatory cytokine in the culture media. IL-1β, in fact, increased the degradation of this macromolecule and had an inhibitory effect on GAGs synthesis. Leflunomide used at 25, 50 and 100 μg/ml showed a remarkable capacity to decrease the release of these metabolites in a dose-related manner with 395.6 ± 15 μg/ml (34.5%), 332.9 ± 12 μg/ml (44.1%), 275.7 ± 10 μg/ml (54.4%) values, respectively (Fig. 4). These results led us to utilise 100 μg/ml concentration of leflunomide to determine the effects of methotrexate or leflunomide–methotrexate combination on GAGs release. Methotrexate treatment determined a smaller inhibitory effect (438.7 ± 14 μg/ml; 34.5%) on GAGs release with respect to leflunomide (Fig. 5). Leflunomide–methotrexate combination was slightly less effective than leflunomide (Fig. 5).

In conclusion, we can summarize that the culture of human cartilage stimulated by IL-1β has been established as an in vitro experimental model to reproduce the mechanisms involved in arthritic diseases and this model was used to examine the effects of leflunomide, methotrexate and leflunomide–methotrexate combination. Besides, we have been carried out a further investigation about possible synergetic effect of both DMARDs. The results of the present work suggest that these drugs might be able to inhibit, in different manner, the activity of two pleiotropic pro-inflammatory mediators: NO and GAGs. Leflunomide–methotrexate combination showed a similar effect as leflunomide alone (so it doesn't appear suitable for this experimental model). Methotrexate reduced NO production and GAGs release, but its inhibitory action was not comparable with leflunomide's. Leflunomide decreased NO production and GAGs release more than methotrexate and to combination of the two drugs.

Leflunomide inhibiting NO production and GAGs release showed the better and more significant capacity to slow cartilage destruction during the inflammatory process, simulated with the addition of IL-1β.

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