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Leflunomide inhibits cytokine-induced DNA synthesis of rabbit synovial cells in culture

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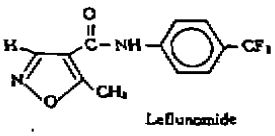
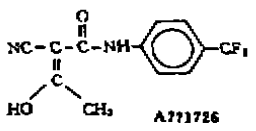
ABSTRACT The effects of interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNFα) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on DNA synthesis of rabbit synovial cells were studied. IL-1β 1000—10 000 U·ml^{-1}, IL-6 10—1000 U·ml^{-1}, TNFα 0.5—50 U·ml^{-1} and GM-CSF 1—100 ng·ml^{-1} concentration-dependently stimulated DNA synthesis in rabbit synovial cells in culture. Leflunomide (LFM) and its metabolite A77 1726 elicited an inhibitory effect on such cytokine-induced DNA synthesis of synovial cells. These results suggested that IL-1β, IL-6, TNFα and GM-CSF play a key role in the pathogenesis of rheumatoid arthritis. Inhibition of cytokine-induced proliferation of synovial cells by LFM may partially explain its antirheumatic activity.

KEY WORDS leflunomide; A77 1726; DNA; synovial membrane; interleukin-1; interleukin-6; tumor necrosis factor; granulocyte-macrophage; colony-stimulating factor; cytokines; cultured cells

Interleukin-1 (IL-1) and tumor necrosis factor (TNF) are key mediators in the pathogenesis of chronic arthritic diseases. They stimulated DNA synthesis in human synovial fibroblasts[1,2]. Interleukin-6 (IL-6) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are also important cytokines in the inflammatory response[3,4]. The present work dealt with effects of IL-1, IL-6, TNF, and GM-CSF on the proliferation of cultured rabbit synovial fibroblasts. Leflunomide (LFM), *N*-(4-trifluoro-methylphenyl)-5-methylisoxazol-4-carboxamide, is very effective in preventing and curing several arthritis models and autoimmune diseases[5,6]. Synovial cell proliferation is one of the earliest histologic alternations in rheumatoid arthritis[7]. We speculated that the antirheumatic effects of LFM and its metabolite A77 1726 (A77), *trans*-2-cyano-3-hydroxy-*N*-(4-trifluoro-methylphenyl)-2-butenamide, may be associated with their inhibition on the proliferation of synovial cells. Effects of LFM and A77 on cytokine-induced DNA synthesis in synovial cells were also studied.





MATERIALS AND METHODS

Regents and drugs Minimum essential medium (MEM) was purchased from Sigma. [^{3}H]TdR (814 TBq·mol^{-1}) was obtained from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. Recombinant human IL-1β (rHu-IL-1β), rHu-IL-6, and rHu-GM-CSF were kind gifts from Steven GILLIS, Immunex Research and Development Corporation, USA. RHu-TNFα was provided by Dr Y Sohmura, Dainippon Pharmaceutical Co, Japan. LFM and A77 were generously provided by Dr Gert GASPRITZ, Hoechst Aktiengesellschaft Werk, Kalle-Albert, Germany. Fetal calf serum (FCS) was supplied by Department of Pathology, Second Military Medical University, Shanghai.

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Cultivation of rabbit synovial cells Synovial membrane was obtained aseptically from the knee joint cavities of normal adult New Zealand rabbits. The tissue was cut into pieces and the inner side was stuck on the flask wall. The cultivation was carried out at 37 ℃ in 5 % CO_2 + 95 % air. The cells were subcultured after 1 wk when the flask was covered with cells and maintained in MEM supplemented with 10 % FCS, penicillin 100 U·ml^{-1}, and streptomycin 100 μg·ml^{-1} at 37 ℃.

Measurement of DNA synthesis[8]. When the cells grew to conglomerate they were trypsinized and deposited on a 96-well micro-tissue culture plate (NUNCLON, Denmark) at a density of 20 000 cells/well. The cells were allowed to adhere for 24 h. The medium was replaced by MEM supplemented with 10 % FCS and antibiotics. IL-1β, IL-6, TNFα, GM-CSF, LFM, and A77 together with [^{3}H]TdR 1850 Bq/well, were added. The cells were incubated at 37 ℃ for 24 h. The incorporation of [^{3}H]TdR was determined by scintillation counter (Xi-an 2105, China).

Statistics Each experiment was done 3 times and data from a representative experiment were presented. Differences were evaluated by t test.

RESULTS

Stimulation of DNA synthesis by cytokines A 24-h treatment of confluent monolayers of synovial cells with various concentrations of cytokines resulted in a concentration-dependent increase in [^{3}H]TdR incorporation. The maximal increase was obtained with IL-6 1000 U·ml^{-1}, TNFα 50 U·ml^{-1}, and GM-CSF 100 ng·ml^{-1}. The effects of IL-1β on DNA synthesis appeared to be the weakest (Tab 1).

Inhibition of LFM and A77 on cytokine-induced DNA synthesis Both LFM and its metabolite A77 exhibited inhibitory effects on the DNA synthesis of synovial cells induced by IL-1β 1000 U·ml^{-1}, IL-6 100 U·ml^{-1}, TNFα 5 U·ml^{-1} and GM-CSF 10 ng·ml^{-1}. The inhibition was concentration-dependent, being most effective at 10 μmol·L^{-1} of either LFM or A77 (Tab 2).

Tab 1. Effects of interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor α (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF) on DNA synthesis of rabbit synovial cells in culture. $n = 5$ samples, $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ vs control.

Cytokines		[^{3}H]TdR incorporation/dpm
Control		9 457±1 276
IL-1β/U·ml^{-1}	10	9 689±1 021^a
	100	10 082±2 112^a
	1 000	12 410±889^c
	10 000	11 291±1 187^b
IL-6/U·ml^{-1}	1	11 744±2 067^a
	10	13 304±1 492^c
	100	14 561±2 071^c
	1 000	20 967±3 074^c
TNFα/U·ml^{-1}	0.05	10 952±1 818^a
	0.5	12 270±2 247^b
	5	16 863±2 079^c
	50	21 467±3 324^c
GM-CSF/ng·ml^{-1}	0.1	11 008±2 684^a
	1	12 517±2 236^b
	10	19 884±3 043^c
	100	20 045±2 798^c

DISCUSSION

The 'hyper' proliferation of synovial fibroblasts is thought to be a key manifestation in the pathology of rheumatoid arthritis[9]. IL-1 and TNF, 2 important inflammatory mediators, were reported to be capable of stimulating the human synovial cell proliferation, arachidonic acid release and phospholipid metabolism[1]. Stimulated by IL-1 and TNF, synovial cells can produce GM-CSF and IL-6[3]. This paper provides evidences that IL-1, TNF, IL-6 and GM-CSF significantly stimulated proliferation of rabbit synovial cells in culture. Our data indicated that these cytokine-fibroblast interaction may play a role in the development of proliferation lesion of rheumatoid arthritis.

The results presented herein also demonstrated the ability of LFM and its main

Tab 2. Inhibitory effects of leflunomide and A77 1726 on the DNA synthesis of rabbit synovial cells in culture induced by IL-1β, IL-6, TNFα, and GM-CSF. $n=5$ samples, $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ vs control.

Drugs/μmol·L^{-1}		[^{3}H]TdR incorporation/dpm			
		IL-1β 1000 U·ml^{-1}	IL-6 100 U·ml^{-1}	TNFα 5 U·ml^{-1}	GM-CSF 10 ng·ml^{-1}
Control		7 944±848	8 620±934	11 370±1 497	9 135±766
Leflunomide	0.1	6 691±1 033^a	5 447±556^c	8 250±771^c	6 791±1 250^a
	1	5 152±798^c	4 410±452^c	6 234±878^c	6 287±408^c
	10	4 754±630^c	4 189±803^c	5 031±103^c	4 384±327^c
A77 1726	0.1	5 274±447^c	5 105±900^c	7 913±884^c	6 864±811^c
	1	4 549±815^c	4 774±491^c	5 158±285^c	5 730±433^c
	10	4 335±303^c	4 029±374^c	4 242±373^c	3 081±204^c

metabolite A77 1726 in inhibiting the cytokine-induced rabbit synovial cell DNA synthesis. LFM is a novel anti-phlogistic and immunomoadulaing agent that has been shown to be efficacious in the treatment of immuno-adjuvant arthritis in rats, progressive polyarthritis in mice and rheumatoid arthritis in humans[5,6]. From the data reviewed by Bartlett RR *et al*, this drug possesses properties that separate it from presently known agents used to combat such diseases[10]. Inhibition of synovial cell proliferation by LFM and A77 might partially explain its unique anti-phlogistic activity.

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来氟米特抑制细胞肽诱导的兔滑膜细胞脱氧核糖核酸合成

R965.2

(英文内容见下页)

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摘要 重组人 IL-1β 在1000—10 000 U·ml^{-1}，IL-6在10—1000 U·ml^{-1}，TNFα 在0.5—50 U·ml^{-1}，GM-CSF 在1—1000 ng·ml^{-1}范围内对培养的兔滑膜细胞 DNA 合成呈浓度依赖性刺激作用。来氟米特及其代谢产物 A77 1726对此有明显抑制作用。提示 IL-1，IL-6，TNF 和 GM-CSF 在关节炎的发病中有重要地位，来氟米特抑制滑膜细胞增殖可能与其特异性的抗炎作用有关。

关键词 来氟米特；A77 1726；脱氧核糖核酸；滑膜；白细胞介素-1；白细胞介素-6；肿瘤坏死因子；粒细胞-巨噬细胞集落刺激因子；细胞肽；培养的细胞

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Effects of cyproheptadine on TXB_2 and 6-keto-$PGF_{1\alpha}$ plasma levels in rabbits with hemorrhagic shock

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ABSTRACT Profound hemorrhagic shock was produced in 26 rabbits by exsanguination *via* carotid artery until blood pressure (BP) = 5.3 kPa (40 mmHg) for a period of 90 min. Rabbits were equally divided into a cyproheptadine (Cyp) treated group and a .control group. The blood sampies before and 90 min after shock and 30 min after liquid and blood infusion and administering Cyp (10 mg·kg^{-1}) were collected from the carotid artery. With radioimmunoassay, we measured the thromboxane B_2(TXB_2) and 6-ketoprostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) contents in plasma. The results indicated that the TXB_2 and 6-keto-$PGF_{1\alpha}$ levels during shock (1024±924, 30±32) and after liquid and blood infusion (990±943, 60±54) were higher than those (221±134, 6±4) in normal rabbits ($P<0.01$, $P<0.05$). Cyp reduced obviously the TXA_2 plasma level in rabbit with shock (304±299 *vs* 990±943, $P<0.05$). We conclude that the decrease of TXB_2 content is one of the possible mechanisms of cyproheptadine anti-shock effect.

KEY WORDS cyproheptadine; hemorrhagic shock; thromboxane B_2; 6-ketoprostaglandin F1 alpha

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Our previous study showed that cyproheptadine (Cyp) had a beneficial anti-shock effect[1]. This article is to provide further experimental evidences for anti-shock effect of Cyp through the detections of thromboxane A_2 (TXA_2) and prostacyclin I_2(PGI_2) levels.

MATERIALS AND METHODS

Cyp (Changzhou 4th Pharmaceutical Factory); ^{125}I-TXB_2 and ^{125}I-6-keto-$PGF_{1\alpha}$ radioimmunoassay (RIA) kit (Research Department of Thrombus and Hemostasis, Suzhou Medical College); FMJ-182 gamma counter (Rihuan Apparatus Manufactory, Shang-