Effects of Lipoxin A_4 on Proliferation and Apoptosis of K562 and HL-60 Leukemic Cells

Xiao-Yan Zhou[1,2], Ping Wu[2], Zhen-Yu Cai[1], Fang-Yun Xu[1], Hong-Mei Wang[1], Du-Yun Ye[2]*

([1]*Department of Pathophysiology, Medical College of Nanchang University, Nanchang 330006, China;* [2]*Department of Pathophysiology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*)

Abstract Inflammation is a critical component of tumour progression. Lipoxins (LXs) are approved for potent anti-inflammatory properties. However, there are few reports dealing with their effects on cancer. In this study, we tested their effects on HL-60 and K562 leukemia cells. Western blot and real time PCR were used to examine the expression of lipoxin A_4 receptor (ALX). Cellular proliferation and cycle were determined *via* cell counting kit-8 assay (CCK-8) and propidium iodide staining. Apoptosis was assessed by the annexin V assay. We discovered LXs inhibited the proliferation of HL-60 and K562 cells, increased the number of cells in G_0/G_1 phase, decreased that in S phase, and induced the apoptosis of HL-60 and K562 cells. It hinted LXs inhibited the proliferation of HL-60 and K562 cells and the mechanism might be associated with cell cycle arrest in G_0/G_1 phase and induction of apoptosis.

Key words eicosanoids; leukemic cell; proliferation; apoptosis

Although inflammation has long been known as a localized protective reaction of tissue to irritation, injury, or infection, there has been a new realization about its roles in a wide variety of diseases, including cancer. Today, the causal relationships between inflammation and cancer are more widely accepted. In brief, the tumorous microenvironment which is an indispensable participant in the neoplastic process, fostering proliferation, survival, and migration of tumor cells, is largely orchestrated by inflammatory cells and cytokines. The research indicated use of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, could reduce colon cancer risk by 40%–50%, and might be preventative for lung, oesophagus, and stomach cancer [1–3]. Also, lots of scholars have taken much interest in the antileukemie potentiality of NSAIDs constantly. These data manifested NSAIDs were potent antileukemia [4–8].

Aspirin triggers the formation of a series of potent bioactive eicosanoids, 15-epi-lipoxins (15-epi-LXs or aspirin-triggered LXs) [9,10]. Lipoxins (LXs), including lipoxin A_4 (LXA_4), lipoxin B_4 (LXB_4), 15-epi-LXA_4, and 15-epi-LXB_4, are eicosanoids generated during inflammation *via* transcellular biosynthetic routes [9,10]. Three major routes of LXs biosynthesis in human cell types have been established, such as: pathways of peripheral blood platelet-leukocyte interactions, route of initiation at the mucosal surfaces by 15-lipoxygenase (15-LO), and aspirin-triggered lipoxin-generation [9,10]. All LXs show similar activities in many biologic systems, and represent a unique class of lipid mediators which can be regarded as "braking signals" in inflammation. Mainly, LXs prevent acute inflammation by means of potent agonist actions at the G protein-coupled receptor termed lipoxin A_4 receptor (ALX) [11], also known as formyl peptide receptor-like 1 (FPRL1) or FPRL2.

However, whether LXs, important anti-inflammatory and pro-resolving mediators, being triggered by aspirin and sharing some biological effects with aspirin, influence leukemia, remains unresolved——these are the focus of this research. Leukemia cell lines, such as HL-60 and K562 cells, are unique models for screening potent antileukemie agents *in vitro*. In this study, we used the two leukemia cell lines, HL-60 and K562, to

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* Corresponding author. Tel: 86-27-83690562, E-mail: yedy@mails.tjmu.edu.cn

investigate the effects of LXA_4 on the cellular proliferation and apoptosis.

1 Materials and Methods

1.1 Materials

RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO-BRL (Basel, Switzerland). LXA_4 was from Cayman Chemical Company (USA). Cell counting kit-8 (CCK-8), RNase A, and propidium iodide (PI) were purchased from Sigma (Sigma-Aldrich, Denmark). Annexin V-FITC kit was the product of Bender (Bender MedSystems, USA). SYBR Green PCR Master Mix was the product of ABI (Applied Biosystems, USA). Lipoxin A_4 recceptor (ALX) polyclonal antibodies were purchased from Santa Cruz (USA).

1.2 Cell culture

HL-60 and K562 cell lines (obtained from the China Center for Type Culture Collection, CCTCC) were both cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). The cells were incubated in a humidified incubator containing 5% CO_2 at 37 ℃. The peripheral blood mononuclear cells (PBMCs) were isolated from human peripheral blood. The peripheral venous blood was drawn from healthy volunteers. Blood samples were collected into sterile tubes containing 25 μl of citrate-phosphate dextrose anticoagulant each and diluted to 1 ∶ 2 with phosphate buffer saline (PBS). The PBMCs were separated through density gradient centrifugation. The isolated PBMCs were washed twice, counted by Trypan blue exclusion and suspended in RPMI-1640 growth medium supplied with 15% FBS. The cells were incubated in a humidified incubator containing 5% CO_2 at 37 ℃.

1.3 RNA extraction and quantitative real time polymerase chain reaction

Total RNA extraction and reverse transcription were performed. Then quantitative PCR (qPCR) was performed with SYBR green PCR Master Mix according to the protocols. The following primers were used to amplify ALX cDNA: forward primer, 5'-AACCCCATGC TTTACGTCTTTGTG-3', and reverse primer, 5'-ATTGGCAGCCGTGTCATTAGTTG-3'. Optimal reaction conditions were 40 cycles of a two stage PCR (denaturation at 95 ℃ for 15 s, and annealing/extension at 60 ℃ for 1 min) after an initial denaturation step (95 ℃ for 10 min). The melting curve was carried out at the end of the reaction to confirm the specificity of PCR products. To ensure the comparability between the 2 PCR assays, the efficiency of each assay was determined by measuring serial dilutions of cDNA in triplicate. For the relative comparison, the data of real time PCR were analyzed with $\Delta\Delta C_t$ method [12,13].

1.4 Western blot analysis

Whole-cell lysates (40 μg) were run on 10% polyacrylamide gels and electrotransferred to nitrocellulose membranes. The membranes were blocked at room temperature in Tris-buffered saline containing 5% milk plus 0.5% Tween 20 and incubated overnight at 4 ℃ with primary antibodies, then with anti-rabbit horseradish peroxidase-coupled secondary antibody at 37 ℃ for 2 h. Detection was carried out by enhanced chemiluminescence according to the manufacturer's protocol (Pierce, USA).

1.5 Detection cell proliferation by CCK-8 assay

CCK-8 assay was performed as previously reported [14]. Cell suspension (100 μl) was incubated in a 96-well plate. When grew to about 80% confluence, the cells were incubated in pure RPMI-1640 overnight to synchronize cells at the G_0 period. 10 μl of CCK-8 solution was added into each well of the plate after different drug sera were allowed to work for predicted time. Then the plate was incubated at 37 ℃ for 4 h and the absorbance at 450 nm wavelength was measured with a microplate reader.

1.6 Cell cycle distribution analysis

Cells were cultured for 2 days in RPMI-1640 medium with LXA_4 (0–400 nmol/L). Then each group of cells were harvested by centrifugalization, washed in PBS twice, and fixed with 75% cold ethanol at −20 ℃ overnight. Prior to analysis, the cells were washed and stained with 0.5 ml of propidium iodide (PI) solution (50 μg/ml PI, 50 μg/ml RNase A, 0.1 mmol/L Na_2EDTA, 0.1% Triton-X 100 in PBS) at 4 ℃ for 0.5 h in darkness. Cell cycle distribution was determined *via* a FACScan Flow Cytometer.

1.7 Apoptosis analysis

The assay was performed according to the manufacturer's recommendations (Bender MedSystems, CA). Briefly, the cells were washed in PBS, resuspended in a binding buffer, and incubated with annexin-FITC for 10 min in darkness at room temperature. Then, propidium iodide was added prior to flow cytometry analysis.

1.8 Statistical analysis

All results were subjected to statistical analysis using one-way ANOVA. Data are expressed as mean±SD and differences were taken statistically significant at a value of $P<0.05$.

2 Results

2.1 Expression of lipoxin A_4 receptor in HL-60 and K562 cells

Mainly, LXA_4 play its roles through agonist lipoxin A_4 receptor (ALX) [11]. So the expressions of ALX were measured by qPCR and Western blot. As shown in melting curves, the curves were only a single melting peak, indicating the specialty was good and quantitation was precise (Fig.1A). The quantitation of qPCR and Western blot showed the expression of ALX could be detected in K562 and HL-60 of control and the steady mRNA level of ALX was increased 24 h after treated with LXA_4 (Fig.1B–Fig.1D). It was interesting that treatment with LXA_4 itself could induce the increase of ALX expression. The possible mechanism might be that LXA_4 could also act on other minor receptors, such as $CysLT_1$ and AhR, and up-regulate ALX. This feature might be one of the reasons why small dose of LXA_4 could have potent biological effects.

2.2 LXA_4 inhibited the proliferation of HL-60 and K562 cells

To test the effects of LXA_4 on the proliferation of HL-60 and K562 cells, cells were treated with different dose of LXA_4 for indicated time. The growth inhibition of HL-60 and K562 cells was determined *via* CCK-8 assay. The results showed the proliferation of HL-60 and K562 cells was inhibited by LXA_4 in a dose- and time-dependent manner (Fig.2).

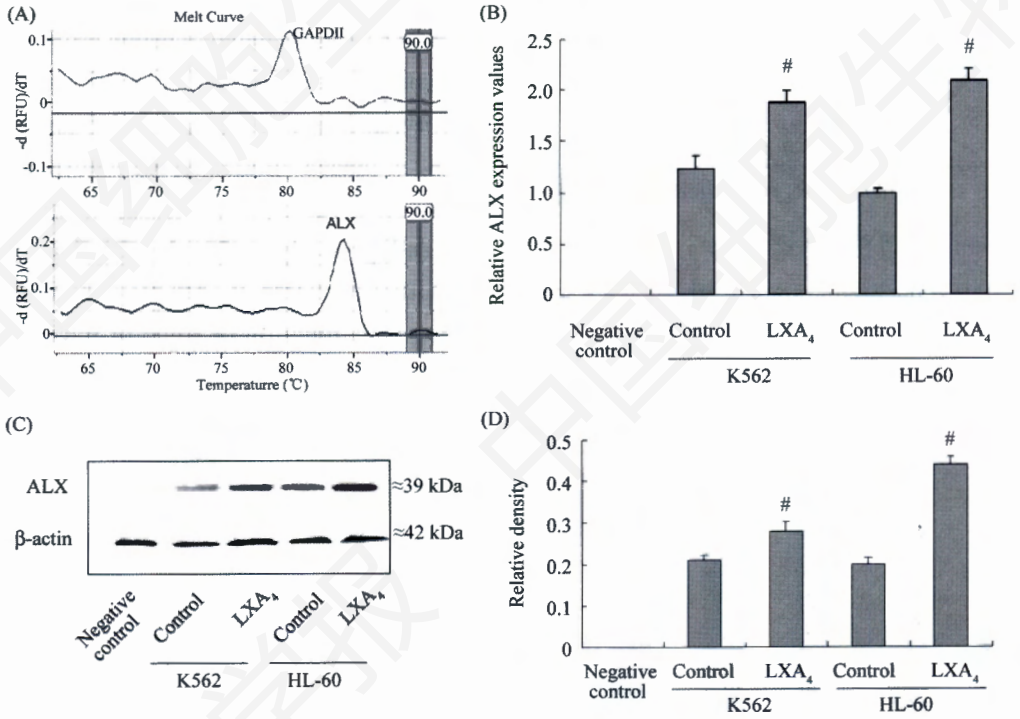

Fig.1 Expression of lipoxin A_4 receptor (ALX) in K562 and HL-60 cells

A: the melting curves; B: data are expressed as folds compared with the control. Total RNA were analyzed by quantitative real time PCR (A and B); C: cells lysates were analyzed by Western blot; D: densitometry was done on all blots using NIH software. HEK293 cell line was used as the negative control. #$P<0.05$, compared with control.

2.3 Effects of LXA_4 on the proliferation of PBMCs

We also investigated the effects of LXA_4 on the proliferation of PBMCs. LXA_4 had no inhibitory effect on the proliferation of PBMCs up to the dose of 400 nmol/L (Fig.3). This suggested that LXA_4 has no potential side effect on normal blood cells even though it exhibited significant inhibitory effects on the proliferation of HL-60 and K562 within the dose from 50 to 400 nmol/L.

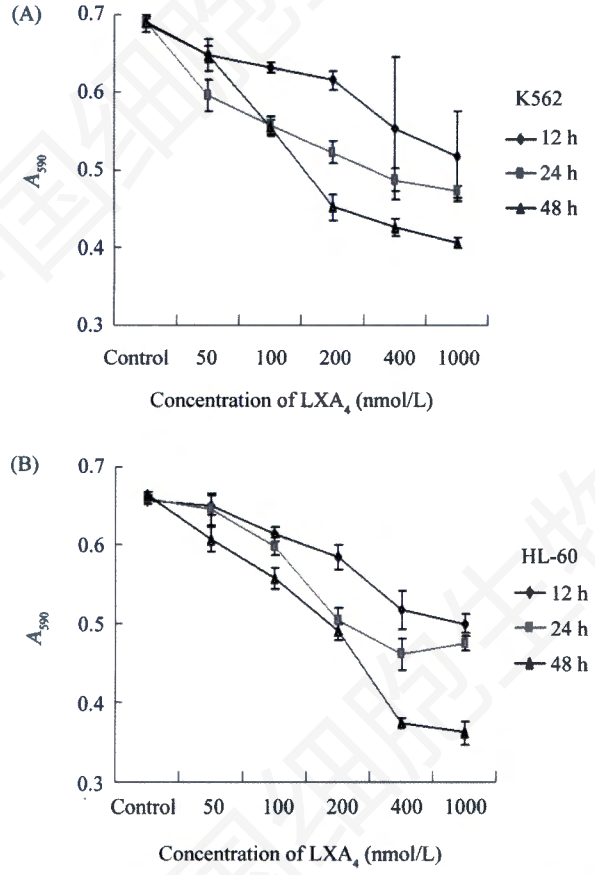

Fig.2 LXA_4 inhibited the proliferation of HL-60 and K562 cells

A: K562; B: HL-60.

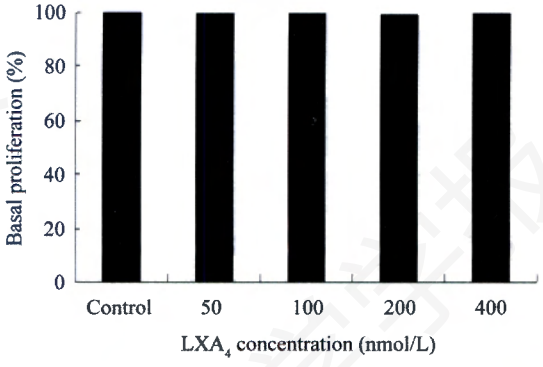

Fig.3 Effects of LXA_4 on the proliferation of PBMCs

2.4 LXA_4 caused a redistribution of cell cycle

After treated with different stimuli for 2 days, cell cycle distribution of exponentially growing cells was analyzed by flow cytometry with propidium iodide staining. HL-60 and K562 cells of control have a normal cell distribution with (28.25±2.24)% and (45.49±2.10)% of the cells in G_0/G_1 respectively. However, LXA_4 redistributed the cell cycle in a dose-dependent manner (Fig.4).

2.5 LXA_4 induction of apoptosis in HL-60 and K562 cells

After treated with different stimuli for 2 days, the apoptosis of cells was analyzed by flow cytometry. Data suggested 100–400 nmol/L of LXA_4 induced of apoptosis in HL-60 and K562 cells (Fig.5).

3 Discussion

Leukemia is a disease manifested by inability of hematopoietic cells to differentiate into functional mature cells, cell cycle disorder, and the failure of cell death. Inhibition proliferation, induction of cell apoptosis has been applied for leukemia therapy. However, effective doses of present chemotherapeutic drugs in clinical studies produced objectionable side effects, physiological toxicity, and drug resistance [15,16]. Significant emphasis has been placed on identifying natural cancer preventive or chemotherapeutic agents in recent years. LXA_4 is an endogenously produced anti-inflammatory and pro-resoluting mediator. It only plays its important effects during inflammation or some of the related diseases, but does not participate in maintenance the physiological functions and has no obvious influences on normal physiologies, so LXA_4 has few secondary effects, physiological toxicity, and drug resistance. LXA_4 has been shown to inhibit the proliferation of mesangial cells induced by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), leukotriene D_4 (LTD_4), and tumor necrosis factor-α (TNF-α) [17-20]; the proliferation of of macrophages activated by lipopolysaccharide (LPS) [21]; the proliferation of of fibroblasts induced by connective tissue growth factor [22]; the proliferation of of umbilical vein endothelial cells stimulated with

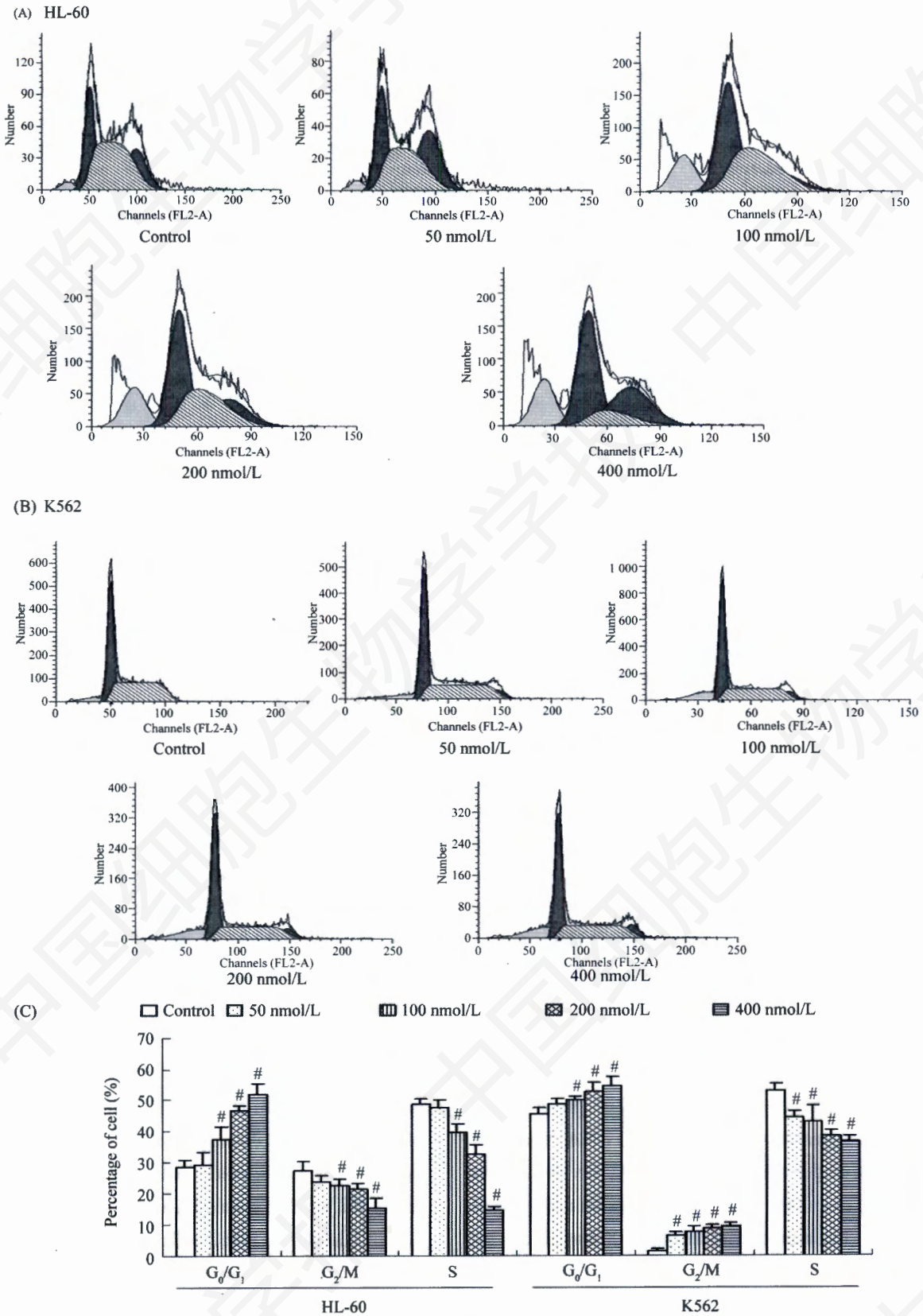

Fig.4 LXA_4 caused a redistribution of cell cycle

A: HL-60; B: K562; C: analysis of DNA content in A and B. $^{\#}P<0.05$, conpared with control.

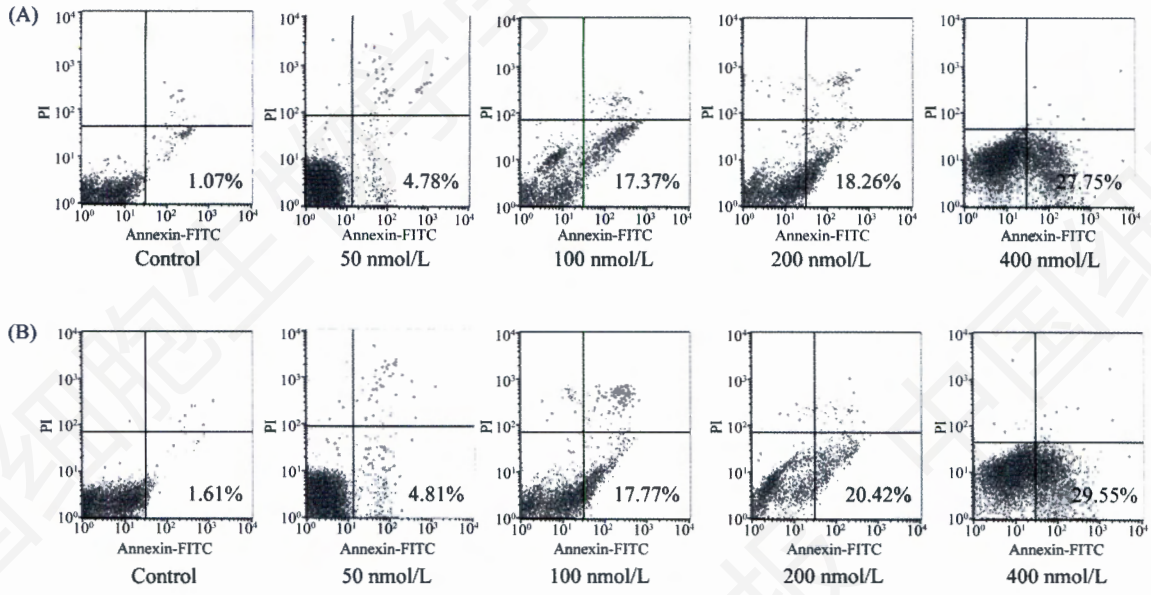

Fig.5 LXA_4 induced of apoptosis in HL-60 and K562 cells

A: HL-60; B: K562.

vascular endothelial growth factor (VEGF) [23]. Also, LXA_4 could promote renal interstitial fibroblasts apoptosis [24]. Moreover, aspirin-triggered lipoxins (15-epi-LXs) were generated by the human lung adenocarcinoma cell line (A549)-neutrophil interactions, and LXA_4 was potent inhibitors for cell proliferation in lung adenocarcinoma cell line [25]. Claria *et al.*[25] considered that LXs, when generated within the microenvironment of tissues, may contribute to aspirin's therapeutic role in decreasing the risk of human cancer. In addition, aspirin, triggered the generation of LXs, has been reported to be a potent antileukemia [4-8]. Basis on all of these data, we researched whether and how LXA_4 influenced the proliferation and apoptosis in HL-60 and K562 cells.

As shown in this study, LXA_4 suppressed the proliferation of HL-60 and K562 in a dose- and time-dependent manner (Fig.2A and Fig.2B). To both cell lines, data suggested that 400 nmol/L LXA_4 was most efficacious, and 1 000 nmol/L LXA_4 has not shown stronger but even less inhibitory effects on cell growth. In addition, the concentration of 1 000 nmol/L is too much high relatively. So, we observed how 0–400 nmol/L LXA_4 affected HL-60 and K562 cells. Proliferating cells pass through several cell cycle checkpoints, mainly the G_1 to S and G_2 to M transitions. The former checkpoint is considered to be the most important one in the replication of DNA and mitosis. As revealed from cell cycle analysis (Fig.4), both cell lines treated with 0–400 nmol/L of LXA_4 showed that LXA_4 blocked cell cycle progression at the G_1 transition and prevented cells entering S phase from G_0/G_1 phase, consequently inhibiting DNA replication and the growth of HL-60 and K562 cells in concentration dependent manner at 48 h. Additionally, apoptosis rates were detected in K562 and HL-60 cells after treated with 0–400 nmol/L LXA_4 as evidenced by the detection of positive Annexin V-staining. Data demonstrated that LXA_4 at 100–400 nmol/L could induce apoptosis in K562 and HL-60 cells, and the effectiveness of both cell lines had no obvious difference (Fig.5). Further studies are necessary to clarify the mechanisms of LXA_4 induction of apoptosis in K562 and HL-60 cells.

The data demonstrated that LXA_4 inhibited the proliferation of HL-60 and K562, increased the number of cells in G_0/G_1 phase, and decreased that in S phase. Furthermore, LXA_4 could induce the apoptosis of HL-60 and K562 cells. It seemed LXA_4 inhibited the proliferation of HL-60 and K562 cells and the mechanism might be associated with cell cycle arrest in G_0/G_1 phase and induction of apoptosis. These results suggested LXA_4, with powerful growth inhibition and apoptosis induction on leukemia cells, will be a good candidate for chemoprevention or chemotherapeutic adjuvant for hu-

man leukemia in the future. Recently, some researchers thought cancer was the result of a failure of the resolution of inflammation [26]. On the other hand, leukemia itself is the caner of inflammatory cells. Then, we speculate insufficient LXs, the important pro-resolving mediators, might be the main agent for leukemia, and exogenous addition of LXs will markedly improve pathogenetic condition of leukemia. However, further investigation is needed to demonstrate clinical application and the mechanisms.

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脂氧素对 K562 和 HL-60 白血病细胞增殖和凋亡的影响

周晓燕[1,2] 吴 萍[2] 蔡震宇[1] 徐方云[1] 王红梅[1] 叶笃篪[2]*

([1] 南昌大学医学院病理生理系, 南昌 330006; [2] 华中科技大学同济医学院病理生理系, 武汉 430030)

摘要 炎症在肿瘤的发生发展过程中扮演重要角色, 脂氧素是一类重要的内源性抗炎介质。但是迄今为止, 脂氧素对肿瘤的影响报道极少。为此, 本文研究了脂氧素对 HL-60 和 K562 白血病细胞增殖和凋亡的影响。体外培养白血病细胞株 HL-60 和 K562, Western 印迹和实时荧光定量 PCR 检测脂氧素受体的表达情况; CCK-8 法(cell counting kit-8 assay)检测 HL-60 和 K562 的增殖能力; PI 染色后利用流式细胞仪进行细胞周期分析; 膜联蛋白 V 试剂盒检测脂氧素对细胞凋亡的影响。实验结果表明脂氧素抑制 HL-60 和 K562 白血病细胞增殖($P<0.05$); 脂氧素处理组 S 期细胞比例明显减少而 G_0/G_1 期细胞比例增加; 脂氧素还可以诱导 HL-60 和 K562 白血病细胞凋亡。由此可见, 脂氧素抑制 HL-60 和 K562 白血病细胞增殖, 其机制可能与诱导白血病细胞 G_0/G_1 期阻滞和细胞凋亡有关。

关键词 类花生四烯酸物质; 白血病细胞; 增殖; 凋亡

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* 通讯作者。Tel: 027-83690562, E-mail: yedy@mails.tjmu.edu.cn