INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by T lymphocyte activation that leads to inflammatory synovium and joint destruction. The pathogenesis of RA is still unclear, but the abnormal proliferation and apoptosis of immune cells, especially the effector T cells, is thought to participate in the pathological process of RA. Studies have demonstrated a crucial role of pro-inflammatory and anti-inflammatory cytokines secreted by T lymphocytes in the pathogenic process of RA and also in the maintenance of homeostasis of T lymphocytes. The activated T cells in RA appear to be dysfunctional with continuous proliferation and suppressed cell death.

Kirenol is a major diterpenoid component extracted from the herbal medicine Herba Siegesbeckiae, which has been used for RA treatment since ancient time. Prior study has shown that kirenol can ameliorate inflammation of the ankle joint in rats with adjuvant arthritis (AA) and collagen-induced arthritis (CIA). Kirenol induces synovial cell apoptosis by down-regulating Bcl-2 and up-regulating Fas-L expression. By increasing nuclear annexin levels, kirenol also inhibits nuclear factor-κB (NF-κB) activity in the synovium and reduces interleukin-1β (IL-1β) production in the synovial fluid of CIA rats. In this study, we aimed to further investigate kirenol-induced immunomodulation in bovine type II collagen (CII)-specific lymphocytes in both in vivo and in vitro conditions.

MATERIALS AND METHODS

Animals

Female Wistar rats (6-8 weeks old, 120-160 g) were obtained from the Department of Laboratory Animal Science of Peking University (Beijing, China). All the rats were fed on a standard diet with free access to water and housed under standard laboratory conditions. All the experiments were approved by Animal Welfare and Ethics Branch, Biomedical Ethics Committee of Peking University.
Drugs

Kirenol (purity over 99%) was supplied by the State Key Laboratory of Natural and Biomimetic Drugs of Peking University (Beijing, China). Prednisolone was purchased from Sigma–Aldrich (St. Louis, MO). Both kirenol and prednisolone were suspended in distilled water before use.

Animal grouping and treatment

The rats were randomized into 4 groups, namely the control group, CIA group, kirenol group and prednisolone group. After bovine CII injection, the rats in kirenol and prednisolone groups were treated with daily oral gavage of kirenol (2 mg/kg) or prednisolone (2 mg/kg) for 30 consecutive days. The rats in the control and CIA groups were given an equal volume of distilled water instead.

Induction of CIA

Bovine type II collagen (CII) (Chondrex, WA, USA) was dissolved in 0.05 mol/L acetic acid at the final concentration of 2.0 mg/ml, and vortexed overnight at 4 °C. CII was then emulsified with complete Freund’s adjuvant (CFA) (Chondrex, WA, USA) at the ratio of 1:1. The rats were immunized with 200 µl CII emulsion by subcutaneous injection at the tail root. On day 7, the rats received a subcutaneous booster injection (200 µl) at the tail, and the primary injection site was avoided.

Preparation of splenocytes and lymph node cells (LNCs)

The spleens and draining lymph nodes of the rats were harvested on day 30 after the first immunization. The spleens were pushed through a mesh to prepare the single cell suspension. The cells were washed, transferred to red blood cell lysis buffer (Dukewei, Shenzhen, China), centrifuged at 1200 min/min for 5 min, and then counted; a single cell suspension of the LNCs was prepared according to the protocol described previously.

Assay of cytokines in cell culture supernatant

The splenocytes and LNCs were plated at the density of 4×10^5/well in RPMI supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), and incubated for 48 h in the presence or absence of 20 µg/ml CII. The culture supernatant was then collected for measurement of the levels of interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-10, and tumor necrosis factor-α (TNF-α) using specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (eBioscience, San Diego, CA).

Proliferation assay of LNCs

The LNCs were plated at 4×10^5/well in RPMI supplemented with 10% FBS and incubated for 40 h in the presence of 0-80 µg/ml kirenol (dissolved in RPMI supplemented with 10% FBS) with or without 20 µg/ml CII. The cells were then exposed to pulsed treatment with 1 µCi/well of [3H]-thymidine (MP Biomedicals, Solon, OH), cultured for additional 8 h, and assessed for radioactivity incorporation.

Apoptosis assay of splenocytes and LNCs

The splenocytes and LNCs were plated at 4×10^5/well in RPMI supplemented with 10% FBS containing 0-80 µg/ml kirenol with or without 20 µg/ml CII. After incubation for 24 h, the cells were stained with FITC-annexin V (AV) and propidium iodide (PI) and analyzed using a FACS Calibur flow cytometer and CellQuest software (BD Biosciences, San Jose, California, USA).

Statistical analysis

The results are presented as Mean ± SE. The differences between the groups were tested using one-way ANOVA. A P value less than 0.05 were considered to indicate a significant difference.

RESULTS

Effect of kirenol on cytokine production in splenocytes

IFN-γ levels in the culture supernatant of the splenocytes from CIA model rats was significantly decreased as compared with those in the control group (P<0.05). Kirenol and prednisolone treatments of CIA rats significantly decreased IFN-γ levels in the culture supernatant of the splenocytes (Fig.1A; P<0.05, P<0.01). The production of TNF-α showed similar changes (Fig.1D).

Compared with those in the control group, IL-10 and IL-4 levels in the supernatant of the splenocytes were significantly lowered in CIA model group. Kirenol and prednisolone significantly up-regulated IL-10 level in the culture supernatant of the splenocytes compared with those in CIA group (Fig.1B, P<0.05). Similarly, the levels of IL-4 in the culture supernatant of splenocytes from rats treated with kirenol and prednisolone were significantly lower than those in CIA group (Fig.1C, P<0.01 or 0.05).

Effect of kirenol on IFN-γ, TNF-α, IL-10, and IL-4 in LNCs from CIA rats

Compared with those of the control group, the levels of IFN-γ and TNF-α in the culture supernatant of LNCs increased significantly in CIA model group (P<0.05 and P<0.001, respectively). Kirenol treatment obviously lowered IFN-γ level in the culture supernatant of LNCs from CIA rats (Fig.2A, P<0.05). Similar results were observed in TNF-α levels in different groups (Fig. 2D). The CIA group showed significantly lowered levels of IL-10 (Fig. 2B, P<0.01) and IL-4 (Fig.2C, P<0.001),
Effects of kirenol on bovine type II collagen-induced rat lymphocytes *in vivo* and *in vitro*

**Fig. 1** Levels of IFN-γ (A), IL-10 (B), IL-4 (C), and TNF-α (D) in the culture supernatant of splenocytes from rats in different groups. *P*<0.05, **P**<0.01 vs control group; *P*<0.05, ***P**<0.01 vs CIA group (Mean±SE, n=6).

**Fig. 2** Effects of kirenol on the levels of IFN-γ (A), IL-10 (B), IL-4 (C), and TNF-α (D) in the culture supernatant of LNCs. *P*<0.05, **P**<0.01, ***P**<0.001 vs control group; *P*<0.05, ***P**<0.001 vs CIA group (Mean±SE, n=6).
which increased obviously in response to kirenol treatment ($P<0.05$ and $P<0.001$, respectively).

**Kirenol blocked CII-specific LNC proliferation**

LNCs from the control rats were stimulated with 20 μg/ml bovine CII in the presence of kirenol at increasing concentrations (0-80 μg/ml), and kirenol showed a dose-dependent suppression of CII-induced proliferation of the LNCs (Fig.3).

**Kirenol-induced apoptosis of CII-specific splenocytes and LNCs**

Splenocytes and LNCs from the control rats were stimulated with bovine CII and increasing concentrations (0-80 μg/ml) of kirenol. A dose-dependent effect was seen in kirenol-induced CII-specific apoptosis of the cells, and at the concentration of 80 μg/ml, kirenol caused a percentage of early apoptosis cells of 9.84% and 14.32% in the splenocytes and LNCs, respectively (Fig.4).

**DISCUSSION**

Excessive proliferation of effector T cells inhibits the differentiation of normal T cells in RA patients. The T helper (Th) cells have two distinct subpopulations, namely Th1 cells that secrete mainly IFN-γ and Th2 cells secreting IL-4 and IL-10. Th1/Th2 cells and the cytokines secreted by these cells maintain a homeostasis, and the balance between the pro-inflammatory and anti-inflammatory forces is the heart of immune tolerance.

Th1 cells are considered the principal T cell fraction involved in the pathogenesis of RA, and accumulations of Th1 and IFN-γ were observed in the synovial fluid of RA patients. We also found increased IFN-γ production in the splenocytes and LNCs from CIA rats. Kirenol treatment of the CIA rats significantly reduced IFN-γ production by the splenocytes and LNCs, demonstrating the capacity of kirenol in suppressing Th1 response in CIA. The joints of patients with RA showed an increased ratio of Th1/Th2 cells, and earlier studies of childhood arthritis suggested an important immunoregulatory role of the Th2 cytokines, IL-4 and IL-10. In the CIA rat models, we found that IL-10 and IL-4 secretions were decreased in the splenocytes and LNCs, suggesting a disturbance of Th1/Th2 cell balance. Kirenol significantly inhibited IFN-γ production and increased IL-4 and IL-10 secretions in the lymphocytes and thereby modify Th1/Th2 cell balance.
imbalance in CIA rats.

The pro-inflammatory cytokine TNF-α play an important role in RA [18-19]. Blocking TNF-α with monoclonal antibodies can ameliorate disease activity in a murine model of CIA [20], and this strategy also proved effective in RA patients [19]. In our experiment, kirenol significantly reduced TNF-α production in the splenocytes and LNCs of CIA rats, suggesting a strong effect of kirenol in suppressing TNF-α secretion by the lymphocytes.

Excessive proliferation and reduced apoptosis of the effector T cells is another characteristic pathology of RA [21]. The T cell clones from RA patients showed a suppressed cell apoptosis as compared with the T cells from normal individuals [4]. In the in vitro experiment, we found that kirenol added in the cell culture resulted in suppressed CII-specific proliferation of LNCs and induced CII-specific apoptosis of the splenocytes and LNCs from CIA rats in a dose-dependent manner. These results suggest an immunosuppressive role of kirenol on CII-specific lymphocytes. Still, further study is needed to explore the mechanisms underlying the immuno-modulatory effect of kirenol.

Glucocorticoid drugs (GCs) are potent immunosuppressive agents in RA therapy. Long-term treatment with GCs is necessary to control the symptoms of RA. However, long-term steroid therapy is often associated with a variety of side effects including disorders of the hypothalamus-pituitary-adrenal axis and disturbances of the cardiovascular system and bone metabolism [22]. An alternative therapeutic agent with fewer side effects is therefore desirable for RA treatment. Our recent studies showed that kirenol did not produce obvious adverse effect on the hypothalamus-pituitary-adrenal axis in CIA rats [5]. The results of this present study show a similar therapeutic effect of kirenol and prednisolone in regulating the cytokines secretion by the lymphocytes, suggesting the potential of kirenol as an alternative of steroids for treatment of RA.

In conclusion, we found that kirenol could reduce the secretion of IFN-γ and TNF-α and increase the IL-10 and IL-4 production by CII-specific lymphocytes from CIA rats. Kirenol also shows a strong effect in inhibiting CII-induced lymphocyte proliferation and in inducing their apoptosis in vitro to cause immunosuppression of CII-specific lymphocytes.

References

摘要:目的 观察奇壬醇对牛II型胶原特异性淋巴细胞体内和体外的免疫调节作用,探讨奇壬醇对抗原特异性淋巴细胞的免疫抑制作用机制。方法 将Wistar大鼠随机分为对照组、模型组、奇壬醇组和泼尼松龙组。除对照组外,均复制胶原诱导关节炎大鼠模型,造模当天开始给药。造模30 d后取脾脏和引流淋巴结,制备单细胞悬液,经体外培养48 h后,ELISA法检测培养上清中干扰素-γ(IFN-γ)、白介素-10(IL-10)、IL-4、肿瘤坏死因子-α(TNF-α)含量;在II型胶原诱导下,取正常大鼠淋巴结细胞、脾细胞与不同浓度的奇壬醇共同培养,检测胶原诱导的淋巴细胞增殖及凋亡。结果 与模型组相比,奇壬醇组脾细胞培养上清中IFN-γ和TNF-α含量降低(P<0.05, P<0.01), IL-10和IL-4水平升高(P<0.05, P<0.01);淋巴结细胞培养上清中IFN-γ和TNF-α含量降低(P<0.05, P<0.001), IL-10和IL-4水平升高(P<0.05, P<0.001);体外实验中,奇壬醇抑制II型胶原特异性淋巴结细胞增殖,促进II型胶原特异性淋巴结细胞和脾细胞凋亡,均呈剂量依赖关系。结论 奇壬醇可通过抑制II型胶原特异性淋巴细胞分泌促炎因子,促进胶原特异性淋巴细胞分泌抗炎因子,抑制胶原特异性淋巴细胞增殖和促进其凋亡,以多条途径发挥免疫抑制作用。关键词:奇壬醇;牛II型胶原;淋巴细胞;类风湿性关节炎