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The bispecific antibody aimed at the vicious circle of IL-1β and IL-17A, is beneficial for the collagen-induced rheumatoid arthritis of mice through NF-κB signaling pathway

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic, systemic and autoimmune disease with overexpression inflammation cytokines. The biological therapy targeting these inflammatory cytokines has been applied for the clinic. Drugs aimed at a single target are ineffective in some patients with RA. However, double-target and multi-target drugs have huge advantages in therapy. Interleukin-1β (IL-1β) and Interleukin-17A (IL-17A) are the keys to inflammatory factors in RA. The bispecific antibody(BsAb)against both human IL-1β and human IL-17A was formed and expressed in *E. coli*. The binding specificity and efficiency of the BsAb was tested by enzyme-linked immunosorbent assay, Western blotting and several cells experiments including THP-1, 3T3-L1 and L929 in vitro. Different doses of BsAb (5, 2, 0.8 mg/kg) were compared in collagen-induced arthritis (CIA) mice, with Adalimumab and Dexamethasone as the positive control. The effects on mice were determined by the degree of arthritis severity, cytokines levels, the level of IgG against CII and rheumatoid factor level in serum, the transcription levels of relative cytokines in the spleen, and histological damage. Furthermore, the activation of NF-κB was analyzed by Western blotting. In conclusion, BsAb can bind with IL-1β and IL-17A to alleviate the severity of arthritis, to decrease the levels of inflammation cytokines in serum, to down-regulate the expression of IL-1β, IL-2, IL-6, IL-17A, TNF-α, IFN-γ, and MMP-3, to up-regulate the expression of IL-10, to relieve histological damage and to inhibit bone destruction. BsAb inhibited nuclear translocation of the p65 subunit and cytoplasm IκB-α degradation by blocking IL-1β and IL-17A, the upstream of NF-κB pathway. High doses of BsAb had a more beneficial effect on CIA mice than Adalimumab and Dexamethasone. Thus, these results indicate that BsAb can be regarded as an ideal candidate for RA therapy.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, systemic and autoimmune disease associated with chronic inflammation, joint swelling, joint tenderness, synovial hyperplasia leading to the destruction of cartilage and bone, and causes functional limitations, working disability, and poor quality of life [1]. Cartilage degeneration and bone destruction are the main features of RA. Autoimmune diseases are caused by direct or indirect immune response. The immune system has the ability to distinguish itself or non-itself but it also attacks their own tissue in some special circumstances when the autoimmune diseases occur. With the development of our studies in the immune response, it is known that a large number of inflammatory factors produce aggravating inflammatory reaction.

Inflammatory cascade including overproduction and overexpression of interleukin1 (IL-1), interleukin17 (IL-17) and TNF-α can drive disease pathology [2–6]. It is well established that IL-1β and TNF-α could induce RA progress through mediators such as cyclooxygenase-2 (COX-2), which increases prostaglandin E2 (PGE_2) production followed by synovial inflammation, and matrix metalloproteinases (MMPs) [7,8]. At present, most of the drugs which the treatment of rheumatoid arthritis that has been listed for are aimed at IL-1β and TNF-α. However, there is no satisfactory

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effect on some clinical experiments. Other inflammatory targets are taken into consideration. IL-17A, mainly released by CD4+T-lymphocytes, also plays a critical role in inflammation response. IL-17 may play an upstream role in T-cell triggered inflammation by stimulating stromal cells to secrete cytokines and growth factors [9] .The synergistic effect of IL-1β and IL-17A in inflammatory response were proved [10]. At the same time, the mutual promotion between IL-1β and IL-17A formed a vicious circle [11]. The vicious circle of IL-1β and IL-17A is a serious culprit in the pathogenesis of RA.

In this report, the therapeutic efficacy of bispecific antibody(BsAb)against the vicious circle of IL-1β and IL-17A was verified on Chick Type II collagen-induced rheumatoid arthritis (CIA) mice, compared with Dexamethasone and Adalimumab. Furthermore, it is explored that the preliminary related anti-inflammatory mechanism based on NF-κB signaling pathway. BsAb had a more obvious effect on the vicious circle of IL-1β and IL-17A. It is speculated that BsAb can inhibit NF-κB activation by blocking the upstream inflammatory factors like IL-1β and IL-17A to ameliorate the downstream inflammation factors related with the of NF-κB pathway.

2. Material and methods

2.1. Animals

Female C57BL/6 mice, 6–8 weeks old, were purchased from Changchun Yisi Animal Research Institute of China. They were housed at the animal facility of the Laboratory Animals Centre of Northeast Agricultural University. Animals were fed ad libitum. This study was carried out in strict accordance with the recommendations of the Guide to the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Institutional Review Board of the Northeast Agricultural University Institute of Biomedicine.

2.2. Construction of BsAb

BsAb was composed of functional domains of anti IL-1β antibody and anti-IL-17A antibody. Two polypeptides chains constituted BsAb by disulfide bond. Each chain was composed of three parts. The structure pattern of BsAb was showed as followed. One chain contained VH of an anti IL-1β antibody, VL of anti-IL-17A antibody and CL of anti-IL-17A antibody. VH was linked with VL by the fragment which contained the first 15 amino acids of the whole CH1 of anti- IL-1β antibody. Another chain contained VL of anti- IL-1β antibody, VH of anti-IL-17A antibody and CH1 of anti-IL-17A antibody. VL was linked with VH by the fragment which contained the first 15 amino acids of the whole CL of anti- IL-1β antibody. The two chains had the similar structure but contained the opposite component. The upper part of BsAb bound IL-1β and the lower part of BsAb bound IL-17A. Each unit in the construction was cloned from plasmids pEAI(containing anti-hIL-1β antibody) and pEAP(containing anti-hIL-17A antibody) and overlapped by PCR. Finally, each chain was subcloned into the pET30a expression vector.

2.3. Preparation of BsAb

The recombinant pET30a vector containing the one gene sequence of the BsAb was transformed into a host bacterium, Rosetta (DE3). Single colonies of *E.coli* Rosetta were grown in LB liquid media containing kanamycin (100 μg/mL). Until the density reached OD_{600} = 0.4, β-*D*-thiongalactopyranoside (IPTG) (Dingguo Bio Inc, Beijing, China) was added at the final concentration of 0.25 mmol/L and the *E.coli* were cultured at 37 °C for 4 h continuously. After that, they were harvested by centrifugation (4000 × *g*, 4 °C, for 30 min) and resuspended in PBS. Subsequently, the bacteria were disrupted by sonication on the ice and centrifuged (12,000 × *g*, 4 °C, for 15 min). The insoluble materials were denatured in lysis buffer (20 mmol/L Tris-HCl, pH 8.0, 0.5 mol/L NaCl, 8 mol/L Urea) and then renatured them(two chains) together by dialysis refold method of refolding buffer (20 mmol/L Tris-HCl pH 8.0 containing 1 mol/L Urea, 1 mmol/L GSH, 0.1 mmol/L GSSG, 300 mmol/L NaCl). Finally, the proteins were purified by ion exchange chromatography and dialyzed in phosphate buffer saline (PBS). The purified proteins were analyzed by 15% SDS-PAGE under reducing conditions and non-reducing conditions, following stained by Coomassie Brillant Blue R250.

2.4. BsAb specificity analysis

The antigens of hIL-1β and hIL-17A were analyzed on 15% SDS-PAGE under reducing conditions and then were transferred to the nitrocellulose membrane (Invitrogen, USA). The BSA was the negative control. Next, the membrane was blocked for 2 h in phosphate-buffered saline (PBS) with 5% nonfat dried milk. Incubate the membrane with BsAb diluted in PBS for 1 h at room temperature. The membrane was washed five times with PBST and was incubated with HRP-coupled rabbit anti-human IgG (R&D, USA) for 1 h at room temperature. The membrane was washed as before and detected by ECL chemiluminescence.

2.5. ELISA binding assay

The specificity and efficiency of the BsAb was verified by enzyme-linked immunosorbent assay. In the assay, ELISA plates were coated into 10 μg antigen of hIL-1β per well (diluted in 0.1 mol/L Na_2CO_3-$NaHCO_3$ buffer, pH 9.6) at 4 °C overnight. The plates were washed three times with PBST(0.05%Tween-20 in PBS) and blocked for 2 h with 300 μL 5% nonfat dried milk-PBS. Different doses(0.1, 0.5, 1, 5, 10, 20, 30 μg) of BsAb as first antibody were added into the well incubated for 1 h at 37 °C. Then the plates were washed three times with PBST and 100 μL rabbit anti-human IgG-HRP antibody (R&D, USA) as the second antibody added and incubated for another hour. Negative controls were included: control 1(uncoated with hIL-1β); control 2 (coated with hTNF-α to replace hIL-1β); controls 3(human anti-hTNF-α antibody; Adalimumab to replace BsAb as the first antibody); control 4 (without hIL-1β and BsAb). control 5 (human anti- hIL-1β McAb to replace BsAb) was a positive control. Another panel was coated into hIL-17A, different doses(0.1, 0.5, 1, 5, 10, 20, 30 μg) of BsAb as the first antibody were added into the well. Negative controls were included: control 1(uncoated with hIL-17A); control 2 (coated with hTNF-α to replace hIL-17A); controls 3(human anti-hTNF-α antibody; Adalimumab to replace BsAb as first antibody); control 4 (without hIL-17A and BsAb). control 5 (human anti- hIL-17A McAb to replace BsAb) was a positive control. After washing the plates five times with PBST, a total of 100 μL TMB solution (1 mg/mL TMB, sodium acetate buffer, pH6.0, 0.006%H_2O_2) was added into the plates. The reaction was stopped with 50 μL of 1 M H_2SO_4. Absorbance was measured at 450 nm in an ELISA reader.

2.6. Biological activity assay of BsAb in vitro

THP-1 cells were stimulated by IL-1β and IL-17A producing MCP-1 [12,13]. THP-1 were grown in RPMI 1640 medium with Glutamax™ I (Gibco, France) supplemented with antibiotics(100U/ml penicillin and 100 μg/mL streptomycin) and 10% (v/v) heat-inactivated fetal calf serum (Gibco, France). THP-1 cells were added into the Tissue Culture Flask (5 mL/flask) and incubated with different doses of BsAb (2,5,10 nmol/L) which has been incubated with 10 nM IL-1β or 10nmol/L IL-17A in advance. No