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A novel bispecific antibody targeting tumor necrosis factor α and ED-B fibronectin effectively inhibits the progression of established collagen-induce arthritis

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ABSTRACT

Specific delivery of TNF-α antagonist to the inflamed site can increase its efficacy and reduce the side effects. In this study, we constructed a bispecific diabody (BsDb) that targets TNF-α and ED-B-containing fibronectin (B-FN), a fibronectin isoform specifically expressed in the pannus of the inflamed joint in rheumatoid arthritis. BsDb was produced in *Escherichia coli* as inclusion bodies, purified to homogeneity, and refolded to the functional form. Our data demonstrate that BsDb could simultaneously bind to human TNF-α and B-FN and neutralize TNF-α action. In the collagen-induced arthritis mouse model, we compared the biodistrubtion and therapeutic efficacy of BsDb with those of the anti-TNF-α scFv (TNF-scFv). Similar to TNF-scFv, BsDb penetrated into the synovial tissue quickly, showing a rapid clearance from blood and normal organs. In contrast, BsDb could selectively accumulate and retain in arthritic joints of mice for a long period time, resulting in a much stronger inhibition of arthritis progression in mice than TNF-scFv. The findings described herein indicate that BsDb has a good specificity to the inflamed joint, with low toxicity to normal organs and seems to be an ideal biological agent for the treatment of RA and other chronic inflammatory disease.

1. Introduction

Tumor necrosis factor (TNF-α) antagonists is considered to be one of the most efficient agents available for rheumatoid arthritis (RA) (Campbell et al., 2003). TNF-α blockade is also highly therapeutic for several other chronic inflammatory diseases, such as inflammatory bowel disease, psoriasis and spondyloarthritis (Stokkers and Hommes, 2004; Tobin and Kirby, 2005; Van den Bosch et al., 2000). Currently, three anti-TNF-α monoclonal antibodies are approved for the treatment of moderate-severe RA and have been shown to dramatically improve the outcomes of the disease (Olsen and Stein, 2004). The major therapeutic goal of RA when administering TNF-α antagonist is to inhibit the action of an excess of TNF-α in blood circulation and inflamed site. TNF-α in the blood circulation can be neutralized without particular problems, whereas neutralization of TNF-α action in the inflamed site requires the effective penetration and an optimal concentration of the antibody in the inflamed synovium. Unfortunately, the synovial tissue has often proven to be relatively resistant to IgG or intact antibody-based therapies. It has been shown that only a small amount of intravenously administered mAbs accumulate in the inflamed synovium (Choy et al., 2000; Tabrizi et al., 2010). Furthermore, due to the FcRn-mediated recycling pathway (Rath et al., 2013), the intact antibody has a long serum half-life. This leads to high level of uptake for the intact antibody by the normal tissues and exposes patients to complication of serious bacterial infection and high risk of tumorigenesis (Curtis et al., 2014; Askling et al.,

Abbreviations: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; HSA, human serum albumin; HRP, horseradish peroxidase; TNF, tumor necrosis factor; TNF-scFv, anti-TNF-α scFv; CD3-scFv, anti-CD3 scFv; IMAC, immobilized-metal affinity chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; Ni-NTA, Ni^{2+}-nitrilotriacetic acid; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; B-FN, ED-B-containing fobronectin; BsDb, bispecific diabody; mAb, monoclonal antibody; ED-B, extra domain B; FcRn, neonatal Fc receptor.

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2009; Rosenblum and Amital, 2011). In an attempt to improve the pharmacokinetic properties, recombinant antibody formats, such as single chain Fv antibody fragment (scFv) and antibody Fab fragment, have been generated. These antibody fragments, without the Fc region, have small size and exhibit efficient tissue penetration, rapid blood clearance and low uptake in normal tissue compared to the intact antibodies (Sanz et al., 2004; Cuesta et al., 2010). However, rapid blood clearance simultaneously results in low uptake of the antibody fragments in the target site (Cuesta et al., 2010; Colcher et al., 1998). One compromising solution to this problem is to extend the half-life of the antibody fragments. The PEGylated anti-TNF-α Fab fragment, Certolizumab, with a suitable serum half-life and excellent tissue penetration, shows promising activity in RA patients (Bykerk et al., 2013). Another attractive possibility is targeted delivery of the anti-TNF-α antibody fragment to the inflamed joint by fusing the agents with a ligand that can bind to a molecule specifically expressed in the inflamed site. Depending on the specific ligand-binding, the bispecific agent may be able to selectively accumulate in the inflamed site. A long retention time of the drug in the inflamed site will overcome the problems caused by the rapid blood clearance.

Fibronectin (FN) is an extracellular matrix (ECM) component widely expressed in a variety of normal tissues and body fluids. Different FN isoforms are produced by the alternative splicing of the FN pre-mRNA in three sites including complete type III repeat ED-A, ED-B, and type III repeats connection sequence (IIICS) (Carnemolla et al., 1989; Borsi et al., 2002; Borsi et al., 1990). The complete type III repeat ED-B, highly conserved in different species with 100% homology in all mammalians, may be entirely included or omitted in the FN molecule (Zardi et al., 1987). The FN isoform containing ED-B (B-FN) is rarely expressed in normal adult tissues except for some tissues undergoing physiologic remodeling such as endometrium, ovary and wound tissues during healing process (Carnemolla et al., 1989; Ffrench-Constant et al., 1989). By contrast, it is abundant in fetal and neoplastic tissues (Carnemolla et al., 1989). As maker of angiogenesis (Castellani et al., 1994), its mAb BC1 and antibody fragment L19 have been used to selectively target cancer in vivo, and very promising tumor targeting data were reported (Moosmayer et al., 2006; Pini et al., 1998; Sauer et al., 2009). Recently, a number of data showed that B-FN was also highly expressed in the pannus of RA (Claudepierre et al., 1999; Vollmer et al., 2009; Kriegsmann et al., 2004), a vascularised inflammatory granulation tissue that spreads from the inflamed synovial membrane and invades the articular cartilage and bone. L19 together with the F8, an antibody specific for the ED-A of fibronectin, have been used to target IL-10 to the inflamed joint, inhibiting the progression of collagen-induced arthritis (CIA) more effectively than IL-10 alone (Trachsel et al., 2007; Schwager et al., 2009). Another phage library-deriving scFv A7, specifically recognizing an epitope expressed in the microvasculature of human arthritic synovium, has a good potential to be developed as a joint-specific pharmacodelivery tool (Kamperidis et al., 2011). These data indicate that targeting therapeutic agents to the inflamed sites might be a promising approach for the treatment of chronic inflammatory diseases.

We have generated an anti-TNF-α single chain Fv fragment (TNF-scFv) and its multivalent forms (Liu et al., 2006–2008), and demonstrated that the antibody constructs could inhibit the action of human and mouse TNF-α and suppress the progression of established collagen-induced arthritis (CIA) in mice (Liu et al., 2007). In the present study, a bispecific diabody, named BsDb, was constructed by covalently linking the anti-TNF-α scFv and the L19 scFv via a peptide linker derived from the human serum albumin. BsDb was produced in *Escherichia coli* as inclusion bodies, purified to homogeneity, and refolded to the functional form. Our data demonstrate that BsDb could simultaneously bind to human TNF-α and B-FN and neutralize TNF-α action. In the collagen-induced arthritis mouse model, BsDb could penetrate into the synovial tissue rapidly and selectively accumulate in arthritic joints of mice for a long period time, with a rapid blood clearance and low uptake in normal organs. Additionally, BsDb inhibited the progression of the established arthritis in mice more effectively than did TNF-scFv. The findings described herein indicate that the BsDb seems to be an ideal biological agent for the treatment of RA and other chronic inflammatory disease.

2. Materials and methods

2.1. Plasmid, strains and cells

Plasmid TNF-scFv/pTS, for the production of the anti-TNF-α scFv (TNF-scFv), was constructed previously in our lab and maintained in the *E. coli* strain Top10 (Liu et al., 2006). Plasmid pMD18T/L19 was constructed by cloning of the encoding sequence of the anti-ED-B scFv L19 (EMBL accession no. AJ006113) into plasmid pMD18T and maintained in the *E. coli* strain Top10. *E. coli* strain Top10 (Tiangen Biotech., Beijing, China) was used for cloning and maintaining the plasmids throughout the experiments. *E. coli* strain BL21 star (DE3) (Tiangen Biotech., Beijing, China) was used as a host cell for protein expression. A murine fibrosarcoma cell line L929 (ATCC, Manassas, VA, USA) was stored in our laboratory and, when required, cultured in RPMI-1640 medium containing10% (v/v) FBS (fetal bovine serum; Hyclone) at 37 °C in a 5% CO2 incubator.

2.2. Regents and materials

HRP (horseradish peroxidase)-conjugated mouse anti-His tag mAb and HRP-conjugated mouse anti-*c*-Myc tag mAb were from Santa Cruz Biotechnology (Santa Cruz, CA, USA.). Anti-CD3 scFv (CD3-scFv), anti-ED-B scFv L19, and anti-TNF-α scFv (TNF-scFv) were cloned and prepared in our laboratory with the same expression system described in this study. Recombinant human TNF-α was from PeproTech. Restriction endonuclease, T4 DNA ligase, DNase, and RNase were from TaKaRa Biotechnology (Dalian, China). Pfu (Pyrococcus furiosus) DNA polymerase was a product of BioAsia (Shanghai, China). 2-Mercaptoethanol, Triton X-100, reduced and oxidized glutathione, Bovine type II collagen, and complete Freund's adjuvant were from Sigma Chemical Co. (St Louis, MO, USA). Actinomycin D was from Fluka Chemical Company (Buchs, Switzerland). Ni-NTA (Ni^{2+}-nitrilotriacetate)–agarose was from Pharmacia Biotechnology Company (Piscataway, NJ, USA). The ELISA commercial kits for mouse TNF-α, IL-1β (interleukin-1β) and IL-6 (interleukin-6) were from Biosource International (Camarillo, CA,USA).

2.3. Cloning of bispecific diabody BsDb

For construction of the diabody BsDb, the HSA linker—scFv L19 fusion gene, composed of the sequence encoding residues 490–513 of HSA and scFv L19 was amplified by using the plasmid pMD18T/L19 as template with two primers, P1 (sense, 5′-TTC*GAATTC*GCGCTGGAAGTGGATGAAACCTATGTGCCGAAAGAATTTAACGCGGAAACCTTTACCTTTCATGCGGATATTGAAATTGTGTTGACGCAGTC-3′) and P2 (antisense, 5′-GGC*GGATCC*ACTCGAGACGGTGACCAGGG-3′). An *Eco*R I and a *Bam*H I restriction sites were introduced to the P1 and P2, respectively, for cloning purpose. The conditions for the amplification were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The final extension was 72 °C for 5 min. The recombinant plasmid pTES for the production of BsDb was constructed by insertion of the fragment containing HSA linker and scFv L19 into