Inhibitory effect of CD147/HAb18 monoclonal antibody on cartilage erosion and synovitis in the SCID mouse model for rheumatoid arthritis

Junfeng Jia1, Conghua Wang1, Zhanguo Shi1, Jingkang Zhao1, Yun Jia1, Zheng Zhao-Hui1, Xiaoyan Li1, Zhinan Chen2,* and Ping Zhu1,*

Objective. To explore the therapeutic potential of CD147/HAb18 mAb in the treatment of RA in severe combined immunodeficiency (SCID) mice engrafted with human cartilage and rheumatoid synovium tissue (SCID-HuRAg).

Methods. SCID-HuRAg mice were treated separately with CD147/HAb18 mAb, anti-TNF-α mAb or a combination of both. The mice in control group were treated with anti-Japanese encephalitis virus mAb. The volume of engrafts was measured and the number of inflammatory cells and cartilage erosion score were examined. Expression of MMP-2, -3 and -9 was determined by immunohistochemistry. Human inflammatory cytokine levels in mouse sera were assessed using cytometric bead array kit.

Results. The volume of engrafts decreased significantly in SCID-HuRAg mice treated separately with anti-CD147 mAb or anti-TNF-α mAb, and in the mice treated with anti-CD147 mAb plus anti-TNF-α mAb (P < 0.05). Significant reduction was observed in cartilage erosion score in anti-CD147 treatment group and combined treatment group (P < 0.05). Immunohistochemical analysis showed that expression of MMP-2, -3 and -9 was lower in the anti-CD147 treatment group and combined treatment group than in the control mAb group (P < 0.05). Moreover, the level of TNF-α, IL-6 and -8 in CD147 mAb group showed a significant decrease compared with that of the control mAb group (P < 0.05).

Conclusions. CD147/HAb18 mAb can reduce cartilage erosion and synovitis by inhibition of the MMPs and reduction of inflammatory cytokines in SCID-HuRAg mice, which suggests that CD147/HAb18 mAb is a promising treatment option for RA patients.

Key words: Rheumatoid arthritis, CD147, HAb18, Inflammation, SCID-HuRAg mouse, Pathology.

Introduction
RA is an autoimmune disease characterized by inflammatory synovitis and cartilage erosion [1]. The inflammatory cells and cytokines correlate with the extent of synovitis [2, 3] and a great amount of MMPs at the cartilage-pannus junction of RA patients contribute to the cartilage erosion [4, 5]. Extracellular MMP inducer (EMMPRIN), namely CD147, has been found to be over-expressed on the fibroblast in RA [6, 7]. Our previous studies also demonstrated that the overexpression of CD147 on monocytes/macrophages and synoviocytes in RA patients and the enhanced MMP-2, -9 secretion as well as the invasiveness of synoviocytes may result from the overexpression of CD147 [8, 9]. We have also reported that CD147/HAb18 mAb and CD147/HAb18 antagonist peptide inhibit the MMP production and the invasive potential in vitro [9, 10]. Damsker et al. [11] conducted an in vivo study and reported that rat anti-mouse CD147 mAbs could reduce the incidence rate of CIA in mice. All these findings suggest that CD147 may play an important role in the pathogenesis of RA.

However, most reports of CD147 antagonist on RA are from in vitro studies [6–10] and the anti-mouse mAb used in the in vivo study was a heterogeneous protein to humans [11]. To assess a suppressive effect of CD147/HAb18 mAb (a mouse anti-human CD147 mAb) on the cartilage erosion and synovitis of RA patients in vivo, we conducted a treatment study using severe combined immunodeficiency (SCID) mice, into which rheumatoid synovium and normal cartilage were co-grafted (SCID-HuRAg) [12–14]. Systemic and local parameters of RA, including volume of engrafts, inflammatory cell number and cartilage erosion score, as well as expression of MMPs and inflammatory cytokines levels in the serum, were evaluated.

Materials and methods

Therapeutic mAb
HAb18 mAb (mouse anti-human CD147/HAb18G mAb, subclass IgG1) was provided by the Cell Engineering Research Centre of the Fourth Military Medical University (Xi’an, China). Anti-TNF-α mAb (infliximab) was kindly provided by Xian-Janssen Pharmaceutical Ltd (Xi’an, China). Anti Japanese encephalitis virus mAb (anti-JEV mAb) IgG (negative control mAb) was provided by the Department of Microbiology, the Fourth Military Medical University.

Specimen
The diseased human synovium tissues were obtained from four patients with RA, as defined by the ACR [15], at the time of arthroplasty or synovectomy at Xijing Hospital. Written informed consent was obtained from all the patients. The mean age of the patients was 57 years (ranging 38–74 years), with a mean disease duration of 10 years. No patient included in the study was being treated with corticosteroids. Synovium tissues were washed and cut into 5-mm3 pieces. The synovium tissues from each patient were engrafted into 5 or 10 SCID mice. The characteristics of the RA patients are listed in Table 1.

The normal human cartilage specimens were obtained from four non-arthritis patients with femoral head fractured (provided by Department of Orthopaedics, Tangdu Hospital, Fourth Military Medical University). Under sterile conditions, all the cartilage specimens were cut into 2-mm3 pieces. Trimmed synovium and cartilage specimens were kept in 4°C serum-free DMEM (Gibco BRL, Grand Island, NY, USA) conditions after being washed with phosphate buffered saline (PBS) containing penicillin (100 U/ml) and streptomycin (100 μg/ml), until being engrafted.
Preparation of the SCID-HuRAg model

SCID-HuRAg mice were prepared according to previous reports [12–14]. Briefly, 6- to 8-week-old male NOD/SCID mice (SLAC, Shanghai Laboratory Animal Co. Ltd, Shanghai, China), which had been bred under specific pathogen-free conditions, were used for the experiments. A 1-cm incision was made in the left flank. The oblique paraspinal muscle was incised. Normal human cartilage and rheumatoid synovial tissue were placed in the chamber in the muscle using fine forceps. The muscular fasciae and the skin wound were closed using 5-0 Prolene suture material. The entire procedure was performed under sterile conditions. Animal studies were approved by the local regulatory agency (Laboratory Animal Research Centre of the Fourth Military Medical University). Successful implantation of human RA tissue was observed by visual assessment 4 weeks after implantation.

Protocol of CD147 mAb administration

Four weeks after implantation, 30 SCID-HuRAg mice were randomly divided into five groups and used for the treatment study. The CD147/HAb18 mAbs (10 mg/kg, n = 6 mice), anti-TNF-α mAb (10 mg/kg, n = 6 mice) [13], or a combination of both (10 mg/kg, n = 6 mice), anti-human IgG1 mAb (10 mg/kg, n = 6 mice) in 50 µl 0.9% sodium chloride, were administered twice a week within the implanted tissue using a microsyringe. The other six mice were not given any treatment. The injections were repeated over 4 weeks. The mice were anaesthetized under ether, according to the guidelines established by our university’s animal ethics committee, and were euthanized 7 days after the final injection in order to allow removal of the implanted tissue. After removing, the implanted tissues were fixed in 4% paraformaldehyde and decalcified with ethylenediaminetetraacetic acid (EDTA). The implanted tissues were measured with a dialcaliper and the volumes were determined using the formula (length x width²)/2, in accordance with our previous report [10].

Histology evaluation of specimens

After decalcification with EDTA, each tissue sample was embedded with paraffin, cut into 3-µm thick sections and then stained with haematoxylin and eosin (HE) for morphological evaluation. Histological assessments were made under double-blind conditions. Animal researchers and pathologists recorded the data on separate case record forms without exchanging any information until the conclusion of the study. Finally, the data were collected by all the researchers were compiled for the analysis of results. According to previous reports, the number of inflammatory cells per unit was counted using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). Three measurements were made for each unit area, and the mean values were then calculated.

Invasion into the cartilage was quantified according to a semiquantitative score ranging from 0 to 4, referring to the number of invading cell layers and the number of affected cartilage sites: 0 = no invasion; 0.5 = invasion of one to two cell layers; 1 = invasion of three to five cell layers; 1.5 = invasion of three to five cell layers at three independent sites of the cartilage; 2 = invasion of 6-10 cell layers; 2.5 = invasion of 6-10 cell layers at three independent sites; 3 = invasion of more than 10 cell layers; 3.5 = invasion of more than 10 cell layers at two independent sites; and 4 = invasion of more than 10 cell layers at three or more sites of the cartilage. Cellular density was assessed on sections involving invasion and adjacent to the cartilage in three high-power fields at ×400 magnification by counting [16].

Immunohistochemistry on paraffin-embedded SCID mouse graft sections

Tissue specimens obtained from the SCID mouse experiments were sectioned, mounted on polylysine-coated slides and dried at 50°C for at least 2h. Sections were dewaxed in xylol and rehydrated in decreasing concentrations of ethanol, and some slides were pre-treated with microwave heating in citrate buffer (0.01 mol/l, pH 6.0) and kept at 70°C for 30 min in a heat incubator for antigen retrieval when needed. Immunohistochemical staining was performed using a streptavidin/peroxides (SP) kit (Zymed, San Francisco, CA, USA) according to the manufacturer’s instructions. The mAbs used were mouse anti-MMP-2 mAb (NeoMarkers, Fremont, CA, USA), anti-MMP-3 mAb (NeoMarkers) and anti-MMP-9 (NeoMarkers). Sections were reacted in turn with biotin-labelled goat-anti-mouse IgG, horseradish peroxidase (HRP)-labelled streptavidin and diaminobenzidine (DAB) (Zymed) before they were counterstained with haematoxylin for visualization of nuclei. For negative controls, primary antibodies were substituted with normal mouse sera. In the positive sections, the cell membrane and/or cytoplasm were clear brownish-yellow in colour. The percentage of positive cells was determined by counting all cells and the number of the positive cells at ×400 magnification in at least 10 high-power fields, using the Image-Pro Plus 6.0 photogram analysis system.

Serum cytokine detection by cyometric bead array flow cytometry

Mouse blood was obtained by heart puncture at Week 9 following implantation, and serum levels of human cytokine (TNF-α, IL-1β, -6, -8, -10 and -12p70) were measured simultaneously by cyometric bead array (CBA) (BD Biosciences Corp., San Jose, CA, USA). The experimental procedure was described elsewhere [17, 18]. Briefly, 50 μl of samples or known concentrations of standard samples (0-5000 pg/ml) were added to a mixture of 50 μl each of capture Ab-bead reagent and detector Ab-phycocyrthrin (PE) reagent. The mixture was subsequently incubated for 3 h at room temperature away from light, and then was washed to remove unbound detector Ab-PE reagent. Data were acquired by flow cytometry (FACS Calibur; BD Biosciences Corp., San Jose, CA, USA) and analysed on computer (CBA software 1.1; BD Biosciences Corp.).

Statistical analysis

Results were expressed as mean±S.D. Differences between experimental conditions were analysed by t-test or Mann-Whitney U-test where appropriate using SPSS software (V.12.0). P-values < 0.05 were considered statistically significant.

---

*Table 1. Characteristics of the RA patients*

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Gender</th>
<th>Age, years</th>
<th>Duration of disease, years</th>
<th>RF, IU/ml</th>
<th>ESR, mm/h</th>
<th>CRP, mg/dl</th>
<th>Type of surgery</th>
<th>Number of recipient mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA1</td>
<td>F</td>
<td>38</td>
<td>3</td>
<td>52</td>
<td>35</td>
<td>3.45</td>
<td>Synovectomy</td>
<td>5</td>
</tr>
<tr>
<td>RA2</td>
<td>F</td>
<td>63</td>
<td>11</td>
<td>76</td>
<td>27</td>
<td>0.97</td>
<td>Arthroplasty</td>
<td>10</td>
</tr>
<tr>
<td>RA3</td>
<td>F</td>
<td>52</td>
<td>5</td>
<td>178</td>
<td>50</td>
<td>2.98</td>
<td>Synovectomy</td>
<td>70</td>
</tr>
<tr>
<td>RA4</td>
<td>F</td>
<td>74</td>
<td>21</td>
<td>29</td>
<td>78</td>
<td>1.66</td>
<td>Arthroplasty</td>
<td>5</td>
</tr>
</tbody>
</table>

---
Results

Effects of anti-CD147 mAb on the volume of the grafted tissue

Successful implantations of normal human cartilage with rheumatoid synovium were observed by visual assessment after the 4 weeks of implantation. Nine weeks after implantation, the engrafted tissue grew 1.2-fold in size in the control mAb group and non-treated group. In the anti-CD147 mAb-treated group, the anti-TNF-α mAb treatment group and the combined treatment group, the sizes of the grafted tissue were reduced to 85, 72 and 70% of the original size ($P = 0.21, 0.05$ and $0.0086$, respectively). The grafted tissue volume of SCID-HuAg mice was potently reduced by anti-TNF-α mAb, as some previous studies reported [13]. As a result of anti-CD147 mAb treatment, the grafted tissue size significantly decreased at a dose of $10 \text{mg/kg}$ compared with that in the non-treated mice or the control mice treated with anti-human IgG1 mAb ($P < 0.05$). No significant difference in the grafted tissue size was observed between anti-CD147 mAb treatment group and combined treatment group (Fig. 1).

Effects of anti-CD147 mAb on the inflammatory cells in the implanted synovium

A large number of inflammatory cells (monocytes, lymphocytes and granulocytes) were observed in implanted tissues in the control mAb group ($410 \pm 82 \text{cells/2500}\mu^2$). Though the number of these inflammatory cells decreased in the anti-CD147 mAb-treated group ($330 \pm 48 \text{cells/2500}\mu^2$), no significant decrease was found compared with that in the control mAb group ($P = 0.066$). Anti-TNF-α mAb ($278 \pm 56 \text{cells/2500}\mu^2$) treatment and combined treatment ($259 \pm 47 \text{cells/2500}\mu^2$) significantly reduced the numbers of inflammatory cells compared with the control mAb treatment ($P = 0.086$ and $0.029$, respectively; Fig. 2).

Effects of anti-CD147 mAb on cartilage invasion

Four weeks after the treatment, histological evaluation revealed a significant reduction of the invasiveness into the cartilage in the anti-CD147 mAb-treated group compared with that in the control mAb group ($P = 0.0014$). The combined treatment group showed consistent amelioration compared with the control mAb group. The cartilage erosion in the anti-CD147 mAb-treated group was significantly less severe than that in the anti-TNF-α mAb-treated group ($P = 0.0022$). Deep invasion (an invasion score of $>2.5$) was observed in five of the six cartilage sections in the control mAb group, but in only one of the six mice in the anti-CD147 mAb-treated group (Fig. 3). The mean invasion score in cartilage

![Fig. 1. Volume of implanted tissue in each treatment group (n=6). Each bar represents the mean ± s.d. of different groups. *$P < 0.05$ vs control mAb and non-treated group.](image)

![Fig. 2. Light microscopic features of implanted synovium in SCID-HuAg mice in each treatment group. (A) Control mAb-treated group, (B) anti-CD147 mAb-treated group, (C) group treated with anti-TNF-α mAb-treated group and (D) group that received combination therapy (original magnification ×400), (E) Numbers of inflammatory cells in each treatment group, *$P < 0.05$ vs control antibody group.](image)

![Fig. 3. Light microscopic features of cartilage erosion in SCID-HuAg mice from each treatment group. RA synovium with normal human articular cartilage were co-implanted under the back muscle chamber of the SCID-HuAg mice. After 4 weeks of treatment, the co-implants were taken out. They were then stained by HE, and evaluated histologically. (A) Control antibody group; (B) anti-CD147 mAb group; (C) anti-TNF-α mAb group; and (D) combination group (original magnification ×400). (E) Cartilage erosion score after CD147 inhibition in each treatment group ($*P < 0.05$ vs control mAb group, **$P < 0.05$ vs anti-TNF-α mAb group). Arrows show the invasive front of the synovial tissue.](image)
those in the control mAb group, the levels of TNF-α, IL-1β, -6, -8 and -12p70 decreased significantly in the anti-TNF-α mAb-treated group (P < 0.0001, 0.028, <0.0001, <0.0001 and 0.056, respectively). Similar results were achieved in the combined treatment group (P < 0.0001, 0.030, <0.0001, <0.0001 and 0.023, respectively). It is notable that the concentrations of TNF-α, IL-6 and -8 in the CD147 mAb-treated group decreased significantly compared with those in the control mAb group (P = 0.043, 0.003 and <0.001, respectively, in turn). No significant difference was observed in IL-10 levels between different groups (Fig. 5).

Discussion

Although the pathogenesis of RA is still not entirely clear, disease progression is characterized by inflammatory synovitis and cartilage erosion [1]. It has been well established that pro-inflammatory cytokines and MMPs are involved in these two major pathogenic processes [2, 19]. By using the SCID-HuRAg mouse model, we found a beneficial effect of CD147/HAb18 mAb and of the combined treatment on the number of inflammatory cells, erosion score and expression of MMP-2, -3 and -9. Moreover, we demonstrated a decrease in the plasma levels of TNF-α, IL-6 and -8.

CD147/HAb18G is a highly glycosylated cell surface transmembrane protein that belongs to the immunoglobulin superfamily [20, 21]. It is a cellular adhesion molecule involved in cell–cell and cell–extracellular matrix interactions. CD147 has been reported to play a role in the pathogenesis of human RA [8, 9], as well as of CIA [11]. For all these findings from in vitro studies, whether CD147 inhibition is helpful for RA patients remains unclear. Determination of the effects of CD147/HAb18 mAb on human inflammatory synovium in vivo is an indispensable step in progressing from mouse therapy to human biologics therapy. The various arthritis animal models, including antigen-induced as well as mutation-, knockin- and knockout-induced arthritides, all have the limitation of a certain experimental and clinical distance from human diseases [22]. To fill the gap, we conducted a treatment study using SCID mice, into which rheumatoid synovium and normal cartilage were co-grafted (SCID-HuRAG).

The SCID-HuRAG mouse is considered to be a useful model for the evaluation of biological agents [13]. A previous study has shown that the implanted tissue in SCID-HuRAG could grow one to two times bigger in size by 8 weeks after implantation [14]. In this study, we measured the size of grafted tissues in different groups and found that the volume of grafts reduced significantly in the group treated with anti-CD147 mAb, anti-TNF-α mAb and the combined treatment compared with that in the control mAb group, showing that CD147 mAb inhibited the proliferation of RA synovium. This result was consistent with the findings reported in previous studies on the efficacy of TNF-α inhibition in SCID-HuRAG mice [13, 23]. Our results also showed that anti-CD147 has stronger anti-erosion effects than TNF-α mAb [12, 16, 24].

Several studies have indicated that fibroblast-like cells and MMPs contribute significantly to the perpetuation of RA [22, 25, 27] and thus targeting such cells and mediators would provide additional benefits to control the disease besides focusing on the immune system [28]. CD147 has been considered to be an important regulator of MMP production and function [20]. Our previous in vitro studies have also shown that CD147/HAb18 mAb and CD147/HAb18G antagonistic peptide decrease the autocrine production of MMPs [8, 10]. In the present study, we also found that the expression of MMP-2, -3 and -9 in the grafted tissue on the SCID-HuRAG decreased after CD147 inhibition, especially that of MMP-3. These results suggest that
CD147 mAb may achieve its anti-erosion function through down-regulating MMP-2, -9 and -10. This may explain why CD147/HAb18 mAb exhibited stronger anti-erosion effects than anti-TNF-α mAb.

Previous studies have found that anti-CD147 mAb can reduce the accumulation of lymphocytes and neutrophils [29, 30]. Cyclophilin A (CypA), as one of the ligands of CD147, may stimulate macrophages to degrade joint cartilage via MMP-9 expression and promote inflammation via pro-inflammatory cytokine secretion [31, 32]. In the present study, secretion of TNF-α, IL-6 and -8 was found to have decreased following anti-CD147 mAb treatment using the CBA method. The CBA is a flow cytometric-based assay for the detection of cytokines. It is accurate and sensitive for measuring cytokine levels with a smaller volume of samples, which is well-suited for our model based on the fact that human cytokine can be detected in the sera from SCID-HuRAg mice [33]. These findings indicate that CD147/HAb18 mAb has an anti-inflammatory effect on RA by pro-inflammatory cytokine inhibition. Further studies using the SCID-HuRAg mouse model may be useful to clarify the mechanism of CypA-CD147 interaction in inflammation.

In conclusion, our study reported here shows that CD147/HAb18 mAb reduces cartilage erosion and synovitis in vivo by the inhibition of MMP-9 and reduction of inflammatory cytokines in SCID-HuRAg mice. CD147/HAb18 mAb is a promising agent for RA therapy.

Rheumatology key messages

- **Significant inhibitory effects of CD147/HAb18 mAb on cartilage erosion and synovitis were reached in SCID-HuRAg mice.**
- **CD147 inhibition could be a new strategy to control joint destruction in RA.**

Acknowledgements

We wish to thank Y. H. Wang and C. M. Fan for their excellent technical assistance; W. J. Dong and Y. Y. Ma for their assistance in image and statistical analysis, respectively; and G. C. Ge for critical reading of the manuscript.

Funding: This work was supported by grant from the Key Program of the National Natural Science Foundation of China (No. 30530720) and the National Basic Research Program (No. 2006CB708504, No. 2009CB521705).

**Disclosure statement:** The authors have declared no conflicts of interest.

References