Anticentromere antibody induced by immunization with centromere protein and Freund’s complete adjuvant may interfere with mouse early-stage embryo

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Abstract

Background: Anticentromere antibody (ACA) is a member of the antinuclear antibody spectrum (ANAs) which has been speculated to be associated with subfertility. Thus, the present study aimed to investigate the induction of ACA production and its potential interference with early-stage embryos.

Methods: Recombinant centromere protein-A (CENP-A) or centromere protein-B (CENP-B) and complete Freund’s adjuvant (CFA) were used to immunize mice. Serum ACA level was then evaluated by using an indirect immunofluorescence test. Immunofluorescence assay was performed to detect IgG in follicles in ovarian tissues and early-stage embryos.

Results: Following treatment, serum positive ACA was observed in mice treated with CENP and CFA. Furthermore, IgG were detected in follicular fluid and early-stage embryos from mice treated with CENP and CFA.

Conclusions: This study preliminarily indicated that ACA induced by CENP and CFA may penetrate into the living embryos of early-stage in mice.

Keywords: Anticentromere antibody, Early-stage embryo

Background

Anticentromere antibody (ACA), a member of the antinuclear antibody spectrum (ANAs), is regarded as an important autoimmune serological marker for systemic sclerosis (SSc), particularly the form of SSc known as CREST (calcinosis cutis, Raynaud’s phenomenon, esophageal dysfunction, sclerodactyly and telangiectasia) syndrome [1, 2]. The association between ACA and infertility has been discussed in recent papers [3–5].

Centromere protein-A (CENP-A) and -B (CENP-B) are constitutive proteins in the complex centromere protein system, and their crucial role in centromere assembly and function has been studied intensively [6]. CENP-A and CENP-B are the major antigens for ACA in patients with SSc [7, 8]. Human ACA is able to identify and bind to the centromere/kinetochore complex in vertebrates, invertebrates and plants, indicating that the antigenicity of the centromere among different species is highly conserved across evolution [9–12].

Thus, in the present study, recombinant human CENP and complete Freund’s adjuvant (CFA) were used to induce ACA production, and the impact of induced ACA on early-stage embryos was evaluated. Therefore, the purpose of this study was to preliminarily investigate...
whether ACA could enter the living early-stage embryos in mice.

**Methods**

**Mice**

Six-week-old wild-type female C57BL/6J mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All the experiments and procedures were approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University (Guangzhou, China).

**CENP and CFA treatment**

Recombinant human CENP-A or CENP-B (catalog nos. orb81023 and orb81024, respectively; Biorby Ltd., Cambridge, UK) were solubilized in saline (forming HA and HB solution, respectively). HA or HB solution was mixed 1:1 (volume/volume) with complete Freund’s adjuvant (CFA, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). These solutions (200 µl, containing 100 µg HA or HB) were injected subcutaneously at the same site on the shaved back of the mice with a 26-gauge needle three times at an interval of 2 weeks. Mice subcutaneously injected with CFA or saline were used as controls. The mice were categorized into four groups (n=6/group) according to treatments: HA/CFA group, HB/CFA group, CFA group and saline group.

**Mouse early-stage embryos collection**

Supervolution was performed 2 weeks after the last drug injection using pregnant mare serum gonadotrophin (PMSG, 10 IU, i.p) and human chorionic gonadotrophin (HCG, 10 IU i.p after 48h), mice from each group were mated 1:1 with male mice. Subsequently, after 24h, the female mice with the plugs were separated and sacrificed by cervical dislocation, and the fertilized oocytes were collected by sharp dissection of the fallopian tube and transferred to the cleavage stage culture medium for in vitro culture.

**Determination of mouse anti-CENP-A and mouse anti-CENP-B antibodies in the serum**

Mouse serum anti-CENP-A and anti-CENP-B antibodies were assessed using an indirect immunofluorescence test (IIFT) kit for antinuclear IgG antibodies (IIFT Mosaic: HEp-2/Liver [Monkey]; cat. no. FA 1510-1003-1, Euroimmun AG, Luebeck, Germany). IIFT is the standard assay for the determination of antibodies against nuclear antigens. Since the sample to be tested was mouse serum, we substituted the anti-human secondary antibody in the original kit with the Alexa Fluor 488-conjugated goat anti-mouse IgG (Cell Signaling Technology, USA).

**Immunofluorescence assay for the determination of IgG in follicle of ovarian tissue and in early-stage embryos**

Ovarian tissue sections were incubated for 1 h with red fluorescein labeled anti-mouse IgG (H + L) antibody (1:1,000 dilution; cat. no. A21202; Invitrogen; Thermo Fisher Scientific, Inc.). Following washing to remove the excess conjugate, the sections were stained with DAPI and visualized under confocal fluorescence microscope (LSM780; Zeiss GmbH, Jena, Germany).

**Statistical analysis**

Statistical analysis was performed using SPSS 13 (SPSS, Inc., Chicago, IL, USA). All continuous variables were expressed as the mean ± standard deviation. The Mann-Whitney U test was used to evaluate differences among groups, and analysis of variance followed by Bonferroni adjustment was used for multiple comparisons. P < 0.05 was considered statistically significant.

**Results**

**Serum positive ACA in mice treated with CENP and CFA**

Treatment with CENP-A and CFA or CENP-B and CFA induced the production of ACA in mice. The immunofluorescence assay exhibited a positive nuclear staining in the serum samples from the HA/CFA and HB/CFA groups; none of the serum samples from the CFA and saline groups exhibited any fluorescence signal (Fig. 1).

**Accumulation of IgG in follicular fluid induced by CENP and CFA treatment**

Immunofluorescence assay of ovarian tissue section showed strong fluorescence of IgG in follicular fluid in mice from both the HA/CFA and HB/CFA groups; none of the serum samples from the CFA and saline groups exhibited any fluoresence signal (Fig. 2).

**IgG fluorescence in early-stage embryos are observed following treatment with CENP and CFA**

All embryos from mice treated with CENP-A and CFA or CENP-B and CFA exhibited fluorescence of IgG dispersed in the nucleus. This phenomenon was based on the overlap of green fluorescence of antibodies and the blue fluorescence of DAPI staining. However, none of the embryos from mice treated with CFA or saline exhibited any fluorescence of antibodies (Fig. 3).
Discussion

In the current study, mice treated with CENP and CFA not only exhibited higher levels of ACA in the serum, but also exhibited a large quantity of IgG in the follicular fluid. We speculate that the majority of immunoglobulins in the follicular fluid could be ACA, and the potential impact of these IgG on oocytes and early-stage embryos should be considered in further studies.

It was reported that different types of ANAs could enter living cells [13, 14]. Nevertheless, there is little evidence to suggest that ANAs could enter the oocyte or the embryo. In 1999, researchers identified that early-stage mouse embryos cultured with purified IgG from ANA-positive serum exhibited strong fluorescence of antibodies and experienced significant growth impairment, whereas other types of autoantibodies, such as anti-thyroid and control immunoglobulins, were not able to bind to embryos, suggesting a specific binding between ANAs and embryo [15]. We have identified in our recent study the development and maturation of oocytes...
were impaired in peripheral ACA positive mice, which exhibited severe chromosomal misalignments in metaphase meiosis, however, no evidence of ACA entering the oocytes was observed, thus the underlying mechanism needs further exploration [16].

However in this study, the results of immunofluorescence assay showed strong immunofluorescence of antibody against nuclear components (which were speculated to be ACA), indicating that mouse embryos may be a direct target for some ACA in vivo prior to implantation.

In addition, for the majority of tested embryos, always only one or some of the blastomeres showed fluorescence. Perhaps, the density of structures in and around the centromere prevents ACA accessibility, or the blastomere with detectable fluorescence was inclined to apoptosis and displayed relatively loose structures that enabled ACA accessibility. However, the precise mechanism needs further clarification.

In the present study, the mechanism by which antibodies entered the living cells has not been elucidated, and the concept of antibodies entering living cells has not been fully defined. However, previous studies have provided some evidence of this. For instance, it was reported that the entry of antibodies into cells was via glycosaminoglycans [17], Fc receptors [18], DNA-histone complexes [19], and myosin 1 [20].

**Conclusions**

The presence of ACA induced by immunization with CENP and CFA may penetrate into the living early-stage mouse embryo. The mechanism by which antibodies enter the living cells and the underlying interference mechanism on the early-stage embryos require further exploration.

**Abbreviations**

ACA: Anticentromere antibody; ANAs: Antinuclear antibodyspectrum; CENP: Centromere protein; CFA: Complete Freund’s adjuvant; CREST: Calcinosis cutis, Raynaud’sphenomenon, esophageal dysfunction, sclerodactyly and telangiectasia; IIFT: Indirect immunofluorescence test

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Not applicable.

**Authors’ contributions**

Hanyan Liu and Yufen Zhang carried out the experiments and performed data analysis; Haiying Liu and Qing Huang wrote the manuscript; Ying Ying conceived the original idea, designed the study, and revise the manuscript. The author(s) read and approved the final manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.
Declarations

Ethics approval and consent to participate
Medical Ethics Committee of The Third Affiliated Hospital of Guangzhou Medical University approved the study (approval number: 2017−156).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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