Gonadal macrophage infiltration in congenital lipoid adrenal hyperplasia

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Abstract

Objective: Congenital lipoid adrenal hyperplasia (lipoid CAH) results in impairment of adrenal and gonadal steroidogenesis caused by *STAR* mutations. Our previous study revealed upregulation of genes associated with inflammatory or immune response and macrophage infiltration in the adrenal cortex of *Star*-knockout mice.

This study aimed at investigating macrophage infiltration in the gonads from human patients with lipoid CAH.

Design: This study includes seven patients with lipoid CAH who underwent gonadectomy: two XX women (age, 22 and 40 years) and five XY boys (1 year). Two women with ovarian cysts (32 and 40 years) and six boys with autopsy or tumor (1 year) were examined as controls. Immunohistochemical analysis of their gonads was performed to determine steroidogenic cells by NR5A1 or CYP17A1 and macrophages by IBA1 or CD68.

Results: An increased number of macrophages infiltrated into the ovaries of lipoid CAH and consisted of two subpopulations: one scattered within and around a layer of theca cells of maturing follicles and the other massively aggregated in the stroma. Abundant cytoplasmic lipid droplets were observed not only in the theca cells but also in the stromal macrophages. There was no significant difference in the number of macrophages in the testicular interstitium between lipoid CAH (95% confidence interval (95% CI: 19.3–47.7 per 0.2 mm²) and controls (95% CI: 13.3–25.8 per 0.2 mm²) ($P=0.10$).

Conclusions: These results demonstrate that macrophages infiltrate the ovaries of lipoid CAH, where the theca cells and the stromal macrophages have abundant cytoplasmic lipid droplets.

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Introduction

Steroidogenic acute regulatory protein (STAR) regulates the translocation of cholesterol from the outer to the inner mitochondrial membrane, where it serves as a common precursor of adrenal or gonadal steroid hormones under the acute stimulation of trophic hormones (1, 2). The physiological significance of STAR in steroidogenesis was confirmed by the fact that patients with *STAR* mutations (congenital lipoid adrenal hyperplasia, lipoid CAH) (3, 4, 5)

and knockout mice lacking *Star* (*Star*^{−/−} mice) (6, 7, 8) both had significant defects in the biosynthesis of steroid hormone and diffuse accumulation of lipid droplets in the steroidogenic cells of the adrenal glands and gonads.

By conducting a transcriptome analysis of *Star*^{−/−} mice, we previously demonstrated that a STAR deficiency altered the gene expression profile of adrenocortical cells (9). The significant alteration included the upregulation

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of genes associated with cholesterol efflux as well as a previously undescribed inflammatory or immune response. We also identified increased macrophage infiltration in the adrenal cortex of *Star*^{-/-} mice and proposed a paracrine interaction between adrenocortical cells and macrophages in cases of STAR deficiency (9), although studies on other species or tissues are required to confirm whether these conditions are ubiquitous consequences of STAR deficiency.

We therefore examined macrophage infiltration in the gonads of patients with lipid CAH by immunohistochemical analyses. The present study expands our understanding of the pathophysiology of steroidogenic organs of STAR deficiency.

Subjects and methods

Patients

The present study involved seven patients diagnosed with lipid CAH who underwent gonadectomy (Table 1). All patients or their parents provided informed consent to histological analysis of the extracted gonads and genetic analysis of the *STAR* gene. The protocol of the genetic analysis was approved by the Ethics Committee of Keio University School of Medicine. The direct sequencing of the *STAR* gene was performed for six patients, confirming homozygous or compound heterozygous mutations. The parents of patient 7 did not consent to genetic analysis. This patient was diagnosed with primary adrenal insufficiency with fatty deposition in the hyperplastic adrenal glands

and complete female external genitalia with a 46,XY karyotype. All patients exhibited symptoms of adrenal insufficiency with elevation of plasma ACTH and renin concentrations, and hydrocortisone and fludrocortisone replacement therapy was initiated before 1 year of age. These data indicate that all patients involved in this study showed the classic phenotype of lipid CAH.

All the patients underwent gonadectomy. In two patients with a 46,XX karyotype, the ovaries were removed for different reasons. Patient 1 exhibited recurrent abdominal pain due to bilateral ovarian torsion and underwent right and left ovariectomies at 19 and 22 years of age, respectively (10). She had menarche at 11 years of age and started estrogen and progestin replacement therapy at 19 years of age. Patient 2 suffered from endometrioid adenocarcinoma and underwent total hysterectomy and bilateral adnexectomy at 40 years of age. She had menarche at 13 years of age and started estrogen and progestin replacement therapy at 25 years of age. In five patients with a 46,XY karyotype (47,XY,+21 for Patient 6), the testes were removed before 2 years of age, because they were reared as girls with complete female external genitalia.

Controls

The testicular tissues of six boys at 1 year of age (with autopsy in two or with gonadectomy because of testicular tumor in four) and the ovarian tissues of 32- and 40-year-old women with ovarian cysts were examined. All controls or their parents provided informed consent to analyze these tissues.

Table 1 Clinical and pathological findings of seven patients with congenital lipid adrenal hyperplasia.

Case	1	2	3	4	5	6	7
Karyotype	46,XX	46,XX	46,XY	46,XY	46,XY	47,XY,+21	46,XY
<i>STAR</i> genotype	Q258*/Q258*	Q258*/E64fs	Q258*/Q258*	Q258*/Q258*	Q258*/R272P	Q258*/Q258*	NA
Age at gonadectomy (years)	22	40	1	1	1	1	1
Lipid droplets in the theca cells	+	+	NA	NA	NA	NA	NA
Lipid droplets in the Leydig cells	NA	NA	–	+	+	+	+
Number of macrophage in gonads	Increased ^a	Increased ^a	38.0±4.4 ^{b,c}	6.0±1.3 ^{b,d}	47.2±4.9 ^{b,c}	33.8±4.4 ^b	42.6±4.2 ^{b,c}
Reason for gonadectomy	Torsion of ovarian cyst	Endometrioid adenocarcinoma	Reared female	Reared female	Reared female	Reared female	Reared female

NA, not applicable; Case 1 was reported previously (10).

^aAssessed qualitatively

^bPresented as mean±S.E.M. per high power field under microscope (95% confidence interval in control testes, 13.3–25.8)

^cSignificantly greater than controls

^dSignificantly lesser than controls.

Histological analyses

Histological characteristics were examined with sections from formalin-fixed or frozen gonadal tissue specimens. The general structure and lipid deposition of the gonadal tissues were assessed by hematoxylin-eosin (HE) and oil red O stainings, respectively. Immunohistochemistry for steroidogenic factor 1 (NR5A1) was used as a marker of steroidogenic cells, and that for ionized calcium-binding adaptor molecule 1 (IBA1) or CD68 was employed as a marker of macrophages. To distinguish between different types of steroidogenic cell in the ovary, 17 α -hydroxylase (CYP17A1) was used as a marker of theca cells. The following primary antibodies were used: mouse monoclonal antibody against NR5A1 (Perseus Proteomics, Tokyo, Japan) at 1 in 100 dilution, rabbit anti-IBA1 polyclonal antibodies (Wako Pure Chemical Industries) at 1 in 4000 dilution, goat anti-CYP17A1 polyclonal antibodies (LifeSpan BioSciences, Inc, Seattle, WA, USA) at 1 in 100 dilution, and mouse anti-CD68 monoclonal antibody (Nichirei, Tokyo, Japan; diluted). The anti-IBA1 polyclonal antibodies, which worked only in formalin-fixed tissues, detected macrophages more specifically than anti-CD68 monoclonal antibody (11). For immunoenzyme detection in double staining, NR5A1 and IBA1 were visualized by horseradish peroxidase using diaminobenzidine and alkaline phosphatase using Permanent Red as chromogen, respectively (EnVision G2 Doublestain System Rabbit/Mouse, Dako). For immunofluorescence detection in double staining, the following secondary antibodies were used: anti-mouse IgG (H+), F(ab')₂ fragment (AlexaFluor 488 Conjugate) (Cell Signaling), anti-rabbit IgG (H+), F(ab')₂ fragment (AlexaFluor 555 Conjugate) (Cell Signaling), and anti-goat IgG (H+), F(ab')₂ fragment (AlexaFluor 350 Conjugate) (Cell Signaling). For immunoenzyme detection in single staining, CYP17A1 or CD68 was visualized by horseradish peroxidase using diaminobenzidine.

Results

Ovary

Histological analysis revealed similar features in the ovaries of two adult patients with lipid CAH (patients 1 and 2). These features included the maturing follicles with the hyperplastic theca cells (Fig. 1). The granulosa cells, which should be present in the inner layer of the theca cells, were occasionally not clearly identified (data not shown).

Figure 1

Immunohistochemical localization of steroidogenic cells and macrophages in the formalin-fixed adult ovaries. Steroidogenic factor 1 (NR5A1) is a marker for granulosa and theca cells, 17 α -hydroxylase (CYP17A1) for theca cells, and ionized calcium-binding adaptor molecule 1 (IBA1) for resident macrophages. A, C, and E, ovary of a control; B, D, and F, ovary of a patient with congenital lipid adrenal hyperplasia. A and B, magenta and brown colors for immunoreactivities against IBA1 and NR5A1, respectively; C and D, red and green fluorescences for IBA1 and NR5A1, respectively; E and F, red and blue fluorescences for IBA1 and CYP17A1, respectively. An inset shows the magnified view of each white rectangle. Yellow arrows and arrowheads indicate steroidogenic cells and macrophages, respectively. Yellow bars indicate 73 μ m. Original magnification was 200 $\times$ for A and B, and 400 $\times$ for C–F.

The immunohistochemical analyses clarified localization of macrophages in the ovaries (Fig. 1). These macrophages infiltrated sparsely in the control ovaries; compared with the control, the number of macrophages was markedly increased in the ovaries of those with lipid CAH. These macrophages were located in patchy distribution, hardly allowing quantitative assessment between control and patients, and divided into two subpopulations. One group of macrophages was detected in the layer of follicular epithelia, closely to the theca

Figure 2

Immunohistochemical localization of steroidogenic cells and macrophages and distribution of lipid droplets in the fresh-frozen adult ovary of congenital lipoid adrenal hyperplasia (lipoid CAH). Four consecutive sections were stained differently. Oil red O staining is indicative of lipid droplets as fresh red. 17 α -hydroxylase (CYP17A1) is a marker for theca cells, and CD68 for resident macrophages. A–H, ovarian follicle; I–P, ovarian stroma. A, B, I, and H, hematoxylin-eosin staining; C, D, K, and L, oil red O staining; E, F, M, and N, light brown colors for immunoreactivities against CYP17A1; G, H, O, and P, dark brown colors for immunoreactivities against CD68. Yellow bars indicate 500 μ m for A, C, E, G, I, K, M, and O (original magnification was 40 $\times$) and 100 μ m for B, D, F, H, J, L, N, and P (original magnification was 200 $\times$).

cells that expressed NR5A1 and CYP17A1 (follicular macrophages); the other group massively aggregated in stroma and was probably associated with atretic follicles or corpora albicans, apart from other steroidogenic cells that expressed NR5A1 or CYP17A1 (stromal macrophages).

The oil red O staining and CD68 immunostaining using consecutive sections revealed the positional overlap between the lipid droplets and the macrophages (Fig. 2). The stromal macrophages with cytoplasmic lipid droplets were even predominantly observed compared with the theca cells with cytoplasmic lipid droplets. No steroidogenic cells were observed among or around the stromal macrophages. The lipid-laden cells in the follicular layer partly appeared to originate from the theca cells because they were immuno-positive for CYP17A1. We could not determine whether or not the follicular macrophages also had cytoplasmic lipid droplets, because these macrophages and the lipid-laden theca cells resided too closely to distinguish them.

Testis

The histological analysis of HE sections revealed fewer seminiferous tubules and fewer germ cells in patients with lipoid CAH than in controls as reported previously (12). In the XY patients (except for Patient 3), the Leydig cells

in the interstitium had a foamy appearance. Any of the XY patients did not have Leydig cells hyperplasia that was reported in only a few patients with lipoid CAH (12).

The immunohistochemical analyses identified resident macrophages within the testicular interstitium of both controls and patients with lipoid CAH (Fig. 3). These macrophages spread through the interstitium, allowing quantitative assessment in a specific microscope field (0.2 mm²). There was no significant difference in the mean number of macrophages between the controls (95% confidence interval, 13.3–25.8 per 0.2 mm²) and patients (19.3–47.7) ($P=0.10$ by Mann–Whitney test). Three out of five patients, however, had significantly more macrophages than the controls (Table 1).

Discussion

The present study revealed that the ovary of adult patients with lipoid CAH is characterized by prominent infiltration of macrophages with cytoplasmic lipid accumulation, although the macrophage infiltration did not differ significantly in the testis of infants between controls and patients. To our knowledge, this observation regarding ovarian macrophage in lipoid CAH has not been studied, partly because the ovarian specimens are difficult to obtain. These results

Figure 3

Immunohistochemical localization of steroidogenic cells and macrophages in the formalin-fixed infantile testes. Steroidogenic factor 1 (NR5A1) is a marker for Sertoli and Leydig cells and ionized calcium-binding adaptor molecule 1 (IBA1) for resident macrophages. A and C, testis of a control; B and D, testis of an infant with congenital lipid adrenal hyperplasia. A and B, magenta and brown colors for immunoreactivities against IBA1 and NR5A1, respectively; C and D, red and green fluorescences for IBA1 and NR5A1, respectively. An inset shows the magnified view of each white rectangle. Yellow arrows and arrowheads indicate steroidogenic cells and macrophages, respectively. Yellow bars indicate 73 μ m. Original magnification was 200 $\times$ for A and B, and 400 $\times$ for C and D.

are consistent with our previous observation that macrophages infiltrated the adrenal cortex of fetal *Star*^{-/-} mice (9), thereby suggesting that this phenomenon is common for STAR deficiency among different species and different steroidogenic tissues.

The macrophages in the ovaries could play a specific role in the pathophysiology of lipid CAH. The excessive follicular atresia results in premature ovarian failure, which is one of the features described for 46,XX adolescents or adults with lipid CAH (3). The underlying mechanism of the atresia is explained by the two-hit model in which accumulated cholesterol esters abrogate the residual capacity of the STAR-independent steroidogenesis initially preserved in STAR-deficient steroidogenic cells (3). The follicular macrophages adjacent to the lipid-laden theca cells may modulate the adaptive response to excessive cholesterol accumulation. The aggregation of the lipid-laden stromal macrophages could be the consequence of

depletion and replacement for atretic follicle or corpus luteum in which cholesterol esters were accumulated abundantly. We described previously that the adrenal steroidogenic cells of *Star*^{-/-} mice had significantly increased the expression of genes involved in inflammatory or immune response (9), some of which have a pivotal role in the chronic inflammation in the adipose tissue of obese individuals (13). Thus, it is plausible to hypothesize the possible link between cholesterol accumulation and macrophage infiltration in the ovaries of lipid CAH.

Several lines of evidence suggest that the paracrine interactions between macrophages and Leydig cells are physiologically relevant (14). In this study, three XY infants with lipid CAH showed significant increase in macrophage infiltration, the degree of which was less obvious in the infant testes compared with that in the adult ovaries. This is partly explained by the notion that hyperplasia of Leydig cells in infants is less manifested than that of the theca cells in adult patients (10, 12). Intriguingly, testicular macrophages have the potential to provide 25-hydroxycholesterol as a direct substrate for P450 side-chain cleavage enzyme in an alternate pathway for steroidogenesis, which bypasses the STAR-dependent delivery of cholesterol into the inner mitochondrial membrane (15, 16). Thus, potential steroidogenic cooperation may also occur between the macrophages and the ovarian theca cells that, similar to the testicular Leydig cells, make androgens as well.

In summary, we have further demonstrated a potentially pivotal role of the inflammatory or immune response in pathological conditions of steroidogenic tissues. The ovaries and adrenal glands in patients with STAR deficiency may share some common features of homeotic inflammation with white adipose tissue in patients with obesity and with the vascular walls in those with atherosclerosis (17). This speculation needs to be proved by different strategies including *in vitro* experiment, and further questions still remain unsolved including where the macrophages come from and what is the role of macrophages in steroidogenic tissues of lipid CAH.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

TI contributed to collection of the samples, analysis and interpretation of the data, and preparation of the manuscript. R F contributed to histological analyses of the samples and interpretation of the data. T S, K M, M A, K I, J I, Y H, S S, and T M contributed to collection of the samples. T H contributed to analysis and interpretation of the data and review of the manuscript.

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