patients with hypertrophic cardiomyopathy. *Jpn Circ J* 1998; 62: 499–504.
27 Pravenec M, Churchill PC, Churchill MC, *et al.* Identification of renal Cd36 as a determinant of blood pressure and risk for hypertension. *Nat Genet* 2008; 40: 952–954.
28 Masuda D, Hirano K, Oku H, *et al.* Chylomicron remnants are increased in the postprandial state in CD36 deficiency. *J Lipid Res* 2009; 50: 999–1011.
29 Ghosh A, Li W, Febbraio M, *et al.* Platelet CD36 mediates interactions with endothelial cell-derived microparticles and contributes to thrombosis in mice. *J Clin Invest* 2008; 118: 1934–1943.
30 Cho S, Park EM, Febbraio M, *et al.* The class B scavenger receptor CD36 mediates free radical production and tissue injury in cerebral ischemia. *J Neurosci* 2005; 25: 2504–2512.
31 Hoebe K, Georgel P, Rutschmann S, *et al.* CD36 is a sensor of diacylglycerides. *Nature* 2005; 433: 523–527.
32 Miyakis S, Lockshin MD, Atsumi T, *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; 4: 295–306.
33 Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40: 1725.
34 Brandt JT, Triplett DA, Alving B, Scharrer I. Criteria for the diagnosis of lupus anticoagulants: An update. On behalf of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the ISTH. *Thromb Haemost* 1995; 74: 1185–1190.
35 Harris EN, Gharavi AE, Patel SP, Hughes GR. Evaluation of the anti-cardiolipin antibody test: Report of an international workshop held 4 April 1986. *Clin Exp Immunol* 1987; 68: 215–222.
36 Amengual O, Atsumi T, Khamashta MA, Koike T, Hughes GR. Specificity of ELISA for antibody to beta 2-glycoprotein I in patients with antiphospholipid syndrome. *Br J Rheumatol* 1996; 35: 1239–1243.
37 Atsumi T, Ieko M, Bertolaccini ML, *et al.* Association of autoantibodies against the phosphatidylserine-prothrombin complex with manifestations of the antiphospholipid syndrome and with the presence of lupus anticoagulant. *Arthritis Rheum* 2000; 43: 1982–1993.
38 Moore KJ, El Khoury J, Medeiros LA, *et al.* A CD36-initiated signaling cascade mediates inflammatory effects of beta-amyloid. *J Biol Chem* 2002; 277: 47373–47379.
39 Kopprasch S, Pietzsch J, Westendorf T, Kruse HJ, Grassler J. The pivotal role of scavenger receptor CD36 and phagocyte-derived oxidants in oxidized low density lipoprotein-induced adhesion to endothelial cells. *Int J Biochem Cell Biol* 2004; 36: 460–471.
40 Triantafilou M, Gamper FG, Haston RM, *et al.* Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. *J Biol Chem* 2006; 281: 31002–31011.
41 Bamberger ME, Harris ME, McDonald DR, Husemann J, Landreth GE. A cell surface receptor complex for fibrillar beta-amyloid mediates microglial activation. *J Neurosci* 2003; 23: 2665–2674.
42 Miao WM, Vasile E, Lane WS, Lawler J. CD36 associates with CD9 and integrins on human blood platelets. *Blood* 2001; 97: 1689–1696.
43 Atsumi T, Khamashta MA, Amengual O, Hughes GR. Up-regulated tissue factor expression in antiphospholipid syndrome. *Thromb Haemost* 1997; 77: 222–223.
44 Sorice M, Longo A, Capozzi A, *et al.* Anti-beta2-glycoprotein I antibodies induce monocyte release of tumor necrosis factor alpha and tissue factor by signal transduction pathways involving lipid rafts. *Arthritis Rheum* 2007; 56: 2687–2697.
45 Cervera R, Khamashta MA, Shoenfeld Y, *et al.* Morbidity and mortality in the antiphospholipid syndrome during a 5-year period: A multicentre prospective study of 1000 patients. *Ann Rheum Dis* 2009; 68: 1428–1432.
46 Fischetti F, Durigutto P, Pellis V, *et al.* Thrombus formation induced by antibodies to beta2-glycoprotein I is complement dependent and requires a priming factor. *Blood* 2005; 106: 2340–2346.
47 Silverstein RL, Febbraio M. CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. *Sci Signal* 2009; 2: re3.
48 Han J, Zhou X, Yokoyama T, Hajjar DP, Gotto Jr AM, Nicholson AC. Pitavastatin downregulates expression of the macrophage type B scavenger receptor, CD36. *Circulation* 2004; 109: 790–796.
49 Yun MR, Park HM, Seo KW, Kim CE, Yoon JW, Kim CD. Cilostazol attenuates 4-hydroxynonenal-enhanced CD36 expression on murine macrophages via inhibition of NADPH oxidase-derived reactive oxygen species production. *Korean J Physiol Pharmacol* 2009; 13: 99–106.

Downloaded from lup.sagepub.com by TATSUYA ATSUMI on February 4, 2014

Journal of Perinatology (2013) 33, 569–571
© 2013 Nature America, Inc. All rights reserved 0743-8346/13
www.nature.com/jp

PERINATAL/NEONATAL CASE PRESENTATION

Hemophagocytic lymphohistiocytosis in a newborn infant born to a mother with Sjögren syndrome antibodies

Y Suzuki[1], N Takahashi[1,2], Y Yada[1], Y Koike[1], M Matano[1], H Nishimura[1] and Y Kono[1]

We encountered a neonatal patient with hemophagocytic lymphohistiocytosis (HLH) whose mother was positive for anti-Ro/SSA and anti-La/SSB antibodies. Complete atrioventricular block was found in a male patient at 29 weeks of gestation. The patient was born at 40 weeks of gestation. He showed severe circulatory disturbance at 22 h after the birth, and he also had elevated serum levels of aspartate aminotransferase (1027 IU l^{-1}), alanine aminotransferase (121 IU l^{-1}), lactic dehydrogenase (3490 IU l^{-1}), ferritin (9769.7 ng ml^{-1}) and soluble interleukin-2 (IL-2) receptor (3230 U ml^{-1}). We could not find any known HLH genetic abnormality in the patient, but he fulfilled seven of the eight criteria for HLH. Serum levels of IL-6 and IL-8 had been already elevated in his cord blood, and serum levels of granulocyte-macrophage colony-stimulating factor and IL-8 were significantly increased on the second day of life. His symptoms regressed with the administration of hydrocortisone. We presumed that transplacental transfer of maternal antibodies could be related to the occurrence of HLH.

Journal of Perinatology (2013) **33,** 569–571; doi:10.1038/jp.2012.147

Keywords: autoimmunity; cytokines; maternal antibodies; neonatal lupus; hemophagocytic lymphohistiocytosis (HLH)

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is caused by a prolonged and excessive activation of antigen-presenting cells and CD8+ T cells, and encompasses several entities, including a primary familial HLH and a secondary form associated with infection, malignancies and rheumatologic disorders.[1] Suzuki *et al.*[2] reported only 20 neonatal cases during a 10-year period in Japan, and they did not find any neonatal patients with autoimmune-related secondary HLH.

We encountered a neonatal patient with HLH that may be caused by the transplacental transfer of maternal anti-Ro/SSA and anti-La/SSB antibodies. We determined the cytokine profile at several times in his clinical course.

CASE

Complete atrioventricular block was identified with fetal ultrasonography in a male patient at 29 weeks of gestation. The patient's heart rate was around 70 b.p.m. His mother did not show any signs or symptoms, but her serum was positive for anti-Ro/SSA (64 U ml^{-1}) and anti-La/SSB (32 U ml^{-1}) antibodies. She did not have any other auto-antibodies. The patient was born at 40 weeks of gestation by normal vaginal delivery, and his birth weight was 3292 g. His Apgar scores were 8 at both 1 and 5 min. His heart rate was around 70 b.p.m., but his general condition was fine. His platelet count was low (93.0×10^3 μl^{-1}) and his serum level of C-reactive protein was negative at 0.02 mg dl^{-1}. Serum levels of aspartate aminotransferase and alanine aminotransferase were 62 and 12 IU l^{-1}, respectively, but the serum level of lactic dehydrogenase was high (884 IU l^{-1}). Serum titers of anti-Ro/SSA and anti-La/SSB antibodies in the cord blood were positive (64 and 8 U ml^{-1}, respectively). Immunoglobulin M was <5 mg dl^{-1} and anti-cytomegalovirus immunoglobulin M antibody was negative in the cord blood. At 19 h after birth, his

Table 1. Revised diagnostic criteria for HLH and the patient's results

	Patient's data	*Fulfillment (+/−)*
Family history or known genetic defect	Not identified	−
PRF1, Munc13-4, Munc 18-2, Syntaxin 11		
Clinical and laboratory criteria[a]		
Fever (>38.5 °C)	38.9	+
Splenomegaly	+	+
Cytopenia		+
Hemoglobin <9 g dl^{-1} (below 4 weeks, <12 g dl^{-1})	10.2 g dl^{-1}	+
Platelets <100 000 μl^{-1}	30 000 μl^{-1}	+
Absolute neutrophil count <1000 μl^{-1}	1500 μl^{-1}	−
Hypofibrinogenemia ($\leqslant$1.5 g l^{-1}) or	0.8 g l^{-1}	+
Hypertriglyceridemia ($\geqslant$2.65 g l^{-1})	4.98 g l^{-1}	
Ferritin $\geqslant$500 μg l^{-1}	9769 μg l^{-1}	+
sCD25 $\geqslant$2400 U ml^{-1}	3230 U ml^{-1}	+
Decreased or absent natural killer cell activity[b]	7%	+ or −
Hemophagocytosis images in bone marrow, cerebrospinal fluid or lymph nodes	ND	ND

Abbreviations: HLH, hemophagocytic lymphohistiocytosis; ND, not determined.
[a]Five of the eight clinical and laboratory criteria must be fulfilled, but patients with a molecular diagnosis consistent with HLH do not necessarily need to fulfill the diagnostic criteria.
[b]Natural killer cell activity was measured by ^{51}Cr release assay (15–40% is a standard value in healthy adults according to the laboratory company, SRL, Tokyo, Japan).

[1]Department of Pediatrics, Neonatal Intensive Care Unit, Jichi Medical University School of Medicine, Shimotsuke, Tochigi, Japan and [2]Department of Maternal, Fetal and Neonatal Medicine, The University of Tokyo Hospital, Tokyo, Japan. Correspondence: Dr N Takahashi, Department of Maternal, Fetal and Neonatal Medicine, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: tnaoto-tky@umin.ac.jp
Received 18 July 2012; revised 30 October 2012; accepted 7 November 2012

platelet count had rapidly decreased to $51.0 \times 10^3\ \mu l^{-1}$, and his C-reactive protein level had rapidly increased to $12.0\ mg\ dl^{-1}$. His serum level of lactic dehydrogenase also increased to $974\ IU\ l^{-1}$. At 22 h after the birth, he showed severe pulmonary hypertension and general hypotension. He received cardiopulmonary resuscitation, and then the continuous administration of isoprenaline and nitric oxide inhalation was started. His circulatory condition gradually improved, but he had elevated serum levels of aspartate aminotransferase ($1027\ IU\ l^{-1}$), alanine aminotransferase ($121\ IU\ l^{-1}$) and lactic dehydrogenase ($3490\ IU\ l^{-1}$). At 32 h of life, he demonstrated elevated levels of ferritin ($9769.7\ ng\ ml^{-1}$) and soluble interleukin-2 (IL-2) receptor (sCD25; $3230\ U\ ml^{-1}$). The patient's clinical and laboratory data and the criteria for HLH[3] are shown in Table 1. No genetic abnormalities for *PRF1* and normal expression levels of Munc 13-4/18-2 and Syntaxin 11 were found. We gave him a diagnosis of HLH and presumed that the HLH was related to the acute circulatory disturbance.

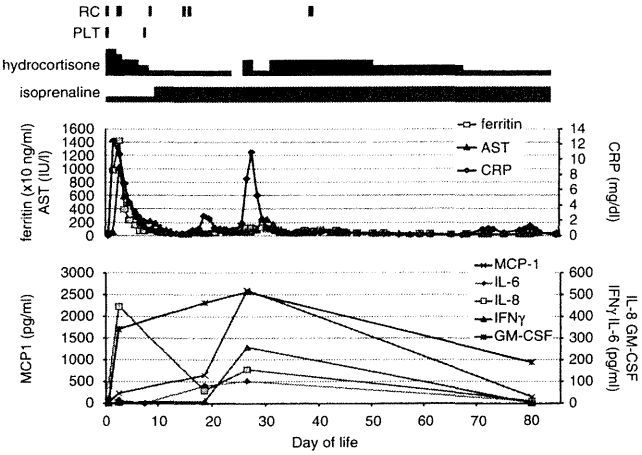


Figure 1. The clinical course of the patient. AST, aspartate aminotransferase; CRP, C-reactive protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; MCP-1, monocyte chemotactic protein-1; PLT, platelet transfusion; RC, red blood cell transfusion.

Figure 1 shows the patient's clinical course. His circulatory condition and serum chemistry were improved by intravenous administration of hydrocortisone ($2\ mg\ kg^{-1}$ every 6 h). We gradually tapered the dose of hydrocortisone and stopped the administration of hydrocortisone on day 24 of life, as shown in Figure 1. The next day, however, his serum C-reactive protein was increased ($7.46\ mg\ dl^{-1}$), and his platelet count was decreased ($90.0 \times 10^3\ \mu l^{-1}$). His ferritin level was $1187.7\ ng\ ml^{-1}$. We resumed the administration of hydrocortisone ($1\ mg\ kg^{-1}$ every 6 h). At this point, the level of anti-Ro/SSA antibody was $8\ U\ ml^{-1}$, and the level of anti-La/SSB antibody was $1\ U\ ml^{-1}$. We more gradually tapered the dose of hydrocortisone and terminated the administration of hydrocortisone on day 85 after birth as shown in Figure 1.

We measured serum cytokine levels, as described previously.[4] As shown in Table 2, serum levels of IL-6 and IL-8 were elevated in the cord blood. On the second day of life, the most striking findings were the significantly elevated levels of

Table 2. Concentrations of serum cytokines ($pg\ ml^{-1}$) in the patient and neonatal controls

	Present patient					*Controls*[a]	
						CB (n = 149)	*PB (n = 8)*
GA (weeks)[b]			40			34.1 ± 3.12	33.4 ± 2.59
BW (g)[b]			3292			1983 ± 701	2047 ± 540
Age (days)	CB	2	18	26	80	CB	5.6 ± 1.2
Pro-inflammatory cytokines							
TNF-α	1.40	4.27	2.52	4.48	1.40	1.80 ± 3.13	6.6 ± 5.8
IL-1β	1.13	0.48	2.9	5.4	0.09	1.28 ± 8.93	2.1 ± 1.9
IL-6	22.1	14.1	82.6	99.6	10.2	4.43 ± 14.3	4.3 ± 3.1
IL-17	<0.02	6.15	22.3	8.51	6.22	1.23 ± 4.33	7.0 ± 7.4
Th1 cytokines							
IFN-γ	0.56	6.79	10.9	260.6	3.12	1.80 ± 3.13	5.3 ± 5.2
IL-2	<0.02	0.5	1.23	2.59	<0.02	1.01 ± 9.74	0.54 ± 1.2
IL-12	1.9	2.32	4.5	4.07	1.46	0.60 ± 4.23	2.6 ± 4.1
Th2 cytokines							
IL-4	0.28	0.37	0.27	0.53	0.21	0.36 ± 1.54	0.14 ± 0.20
IL-5	0.06	0.56	0.39	0.68	<0.04	0.79 ± 1.52	2.0 ± 0.5
IL-10	0.7	7.5	6.55	25.8	1.61	0.89 ± 2.03	1.8 ± 2.1
IL-13	<0.02	2.73	0.82	4.85	0.06	3.77 ± 21.2	1.2 ± 1.1
Growth factors							
IL-7	0.64	4.55	0.06	0.33	<0.02	2.31 ± 2.31	0.22 ± 0.33
GM-CSF	<0.02	342.7	465.8	515.3	187.3	4.72 ± 28.7	17.0 ± 13.7
G-CSF	30.7	152.9	22.65	34.1	17.1	9.12 ± 37.0	11.2 ± 7.0
Chemokines							
IL-8	84.4	447.3	59.2	155.2	11.9	18.8 ± 33.0	20.0 ± 10.4
MCP-1	21.3	245.4	647.4	2611.9	160.5	82.1 ± 88.1	487.0 ± 221.4
MIP-1β	152.5	84.2	147.2	150.8	72.8	224.2 ± 131.7	185.5 ± 73.1

Abbreviations: BW, birth weight; CB, cord blood; GA, gestational age; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MIP-1β, macrophage inhibitory protein-1β; PB, peripheral blood; TNF-α, tumor necrosis factor-α.
[a]Controls contained two groups. One group included 149 CB samples, and the other group included PB samples from eight newborn patients. The samples were collected from patients with various risk factors who were admitted to the Neonatal Intensive Care Unit of Jichi Medical University School of Medicine. The 149 CB samples were extracted from a group of 224 CB samples whose cytokine profiles were investigated in our previous paper.[4] The 75 samples of cases with premature rupture of membrane, chorioamnionitis and placental abruption were excluded. The other control group consisted of eight newborn patients with several minor risks consisting of slightly low birth weight ($n = 5$), diabetic mothers ($n = 2$) and hyperbilirubinemia caused by breast feeding ($n = 1$). PB of controls was taken between the fourth and eighth days of life. Written informed consent was obtained from the controls' parents.
[b]Values are shown as mean ± s.d. of controls.

© 2013 Nature America, Inc.

serum granulocyte-macrophage colony-stimulating factor (342.7 pg ml^{-1}) and IL-8 (447.3 pg ml^{-1}). On the 18th day of life, when the patient's circulatory condition was stable, the serum levels of several cytokines were still high. On the 26th day of life, when the patient's serum C-reactive protein had increased after the initial attempt at hydrocortisone cessation, several cytokine levels were increased further, particularly interferon-γ. On the 80th day of life, just before the successful termination of hydrocortisone administration, the serum levels of almost all cytokines were low (Table 2 and Figure 1).

DISCUSSION

Macrophage activation syndrome is a special form of HLH that occurs in children and adults with autoimmune diseases.[5–10] Imashuku *et al.*[7] studied 96 Japanese patients under 1 year of age with HLH, and they found that four patients (age range, 7 to 12 months) developed rheumatoid arthritis-associated macrophage activation syndrome. To the best of our knowledge, this report may be the first case of neonatal HLH caused by maternal transfer of antibodies.

Fukaya *et al.*[10] identified two adult patients with Sjögren syndrome who had developed HLH in a group of 30 patients with autoimmune diseases. Neonatal lupus erythematosus is caused by the transplacental passage of maternal autoantibodies, most commonly anti-Ro/SSA and anti-La/SSB antibodies. Wisuthsarewong *et al.*[11] reported 17 patients with neonatal lupus erythematosus who were diagnosed at 28 to 84 days of life in Thailand. There has been no report of a neonatal patient with neonatal lupus erythematosus who developed HLH. Autoimmune-related HLH is characterized by very high ferritin levels, severe cardiac impairment and responsiveness to corticosteroid.[9,12] From these facts, it is plausible that anti-Ro/SSA and anti-La/SSB antibodies had a role in the development of HLH in this patient.

Patients with familial or secondary HLH have high serum levels of various pro-inflammatory cytokines such as interferon-γ, IL-6, IL-8, IL-10, IL-12, IL-18 and tumor necrosis factor-α.[9,13] We previously described a patient with familial HLH who developed hydrops fetalis.[14] This patient also had high levels of IL-6 and IL-8 in cord blood. Fetuses must avoid harmful inflammatory immune responses that could lead to preterm delivery; therefore, Th1 immune responses are usually depressed *in utero*. This fact may be related to the low levels of the other cytokines in cord blood. Several reports have identified interferon-γ as the most important cytokine in the development of HLH.[9,13] When corticosteroid therapy was reinitiated, our patient showed high levels of several cytokines including interferon-γ. The immunological reaction at this time point might be different than the immunological reaction during the first hours of life.

This report may be the first report of a neonatal patient with autoimmune-related HLH. We think that it is important to accumulate information on neonatal patients with similar conditions to our patient in future studies to further elucidate the underlying mechanism.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The authors thank Professor E Ishii (Department of Pediatrics, Ehime University, Matsuyama, Japan), Dr I Ueda and Dr T Imamura (Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto, Japan), and Dr H Horiuchi (Department of Cardiovascular Medicine, Kyoto University, Kyoto, Japan) for performing gene analysis for HLH. This work was supported by a grant from the Ministry of Health, Labor and Welfare of Japan (22390215).

REFERENCES

1 Filipovich AH. Hemophagocytic lymphohistiocytosis (HLH) and related disorders. *Hematology Am Soc Hematol Educ Program* 2009; 127–131.
2 Suzuki N, Morimoto A, Ohga S, Kudo K, Ishida Y, Ishii E *et al.* Characteristics of hemophagocytic lymphohistiocytosis in neonates: a nationwide survey in Japan. *J Pediatr* 2009; **155**: 235–238.
3 Janka GE, Schnider EM. Modern management of children with haemophagocytic lymphohistiocytosis. *Br J Haematol* 2004; **124**(1): 4–14.
4 Takahashi N, Uehara R, Kobayashi M, Yada Y, Koike Y, Kawamata R *et al.* Cytokine profiles of seventeen cytokines, growth factors and chemokines in cord blood and its relation to perinatal clinical findings. *Cytokine* 2010; **49**: 331–337.
5 Stephan JL, Kone-Paut I, Galambrun C, Mouy R, Bader-Meunier B, Prieur A-M. Reactive haemophagocytic syndrome in children with inflammatory disorders. A retrospective study of 24 patients. *Rheumatology* 2001; **40**: 1285–1292.
6 Larroche C, Mouthon L. Pathogenesis of hemophagocytic syndrome (HPS). *Autoimmun Rev* 2004; **3**: 69–75.
7 Imashuku S, Ueda I, Teramura T, Mori K, Morimoto A, Sako M *et al.* Occurrence of haemophagocytic lymphohistiocytosis at less than 1 year of age: analysis of 96 patients. *Eur J Pediatr* 2005; **164**: 315–319.
8 Kumakura S, Ishikura H, Kondo M, Murakawa Y, Masuda J, Kobayashi S. Autoimmune-associated hemophagocytic syndrome. *Mod Rheumatol* 2004; **14**: 205–215.
9 Janka GE. Hemophagocytic syndromes. *Blood Rev* 2007; **21**: 245–253.
10 Fukaya S, Yasuda S, Hashimoto T, Oku K, Kataoka H, Horita T *et al.* Clinical features of haemophagocytic syndrome in patients with systemic autoimmune diseases: analysis of 30 cases. *Rheumatology* 2008; **47**: 1686–1691.
11 Wisuthsarewong W, Soongswang J, Chantorn R. Neonatal lupus erythematosus: clinical character, investigation, and outcome. *Pediatr Dermatol* 2011; **28**: 115–121.
12 Stephan JL, Kone-Paut I, Galambrun C, Mouy R, Bader-Meunier B, Prieur AM. Reactive haemophagocytic syndrome in children with inflammatory disorders. A retrospective study of 24 patients. *Rheumatology (Oxford)* 2001; **40**: 1285–1292.
13 Jordan MB, Hildemann D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. *Blood* 2004; **104**: 735–743.
14 Maruyama A, Takahashi N, Gunji Y, Morimoto A, Yada Y, Koike Y *et al.* Congenital hemophagocytic lymphohistiocytosis in a preterm infant: ytokine profile and a review of the disease. *J Pediatr Hematol Oncol* 2011; **33**(5): e209–e212.

© 2013 Nature America, Inc.

ARTHRITIS & RHEUMATISM
Vol. 65, No. 6, June 2013, pp 1477–1486
DOI 10.1002/art.37902
© 2013, American College of Rheumatology

Inhibition of Connective Tissue Growth Factor Ameliorates Disease in a Murine Model of Rheumatoid Arthritis

Kazuhisa Nozawa,[1] Maki Fujishiro,[2] Mikiko Kawasaki,[2] Ayako Yamaguchi,[1] Keigo Ikeda,[3] Shinji Morimoto,[3] Kazuhisa Iwabuchi,[2] Mitsuaki Yanagida,[2] Shouzo Ichinose,[2] Megumi Morioka,[4] Hideoki Ogawa,[2] Kenji Takamori,[2] Yoshinari Takasaki,[5] and Iwao Sekigawa[3]

Objective. **We have shown that connective tissue growth factor (CTGF) plays an important role in the pathogenesis of rheumatoid arthritis (RA). This study was undertaken to evaluate the effects of blockade of the CTGF pathway on the development of collagen-induced arthritis (CIA) in mice.**

Methods. **Arthritis was induced in DBA/1J mice by immunization with a combination of type II collagen (CII) and Freund's complete adjuvant. We evaluated the development of arthritis in mice with CIA left untreated versus treated with neutralizing anti-CTGF monoclonal antibody (mAb).**

Results. **Inhibition of CTGF in mice treated with neutralizing anti-CTGF mAb significantly ameliorated arthritis compared to the untreated mice with CIA. Serum levels of matrix metalloproteinase 3 were reduced by anti-CTGF mAb treatment. Moreover, blockade of CTGF decreased interleukin-17 expression on purified CD4+ T lymphocytes. Although the expression of the retinoic acid receptor–related orphan receptor γt gene was not suppressed by anti-CTGF mAb treatment, that of interferon regulatory factor 4 (IRF-4) and IκBζ (Nfkbiz), which are other important molecules for the differentiation of Th17 cells, was suppressed. In addition, blockade of CTGF inhibited pathologic proliferation of T lymphocytes in response to CII restimulation in vitro. Moreover, aberrant osteoclastogenesis in mice with CIA was restored by anti-CTGF mAb treatment.**

Conclusion. **Our findings indicate that blockade of CTGF prevents the progression of arthritis in mice with CIA. Anti-CTGF mAb treatment suppresses pathologic T cell function and restores aberrant osteoclastogenesis in mice with CIA. CTGF may become a new target for the treatment of RA.**

We previously examined changes in the serum levels of protein biomarkers in infliximab-treated patients with rheumatoid arthritis (RA), via a novel approach to proteomic research using a specially developed serum/plasma protein separation device (hollow-fiber membrane-based device; Toray) and a linked 2-dimensional liquid chromatography system (2-D liquid chromatography mass spectrometry/mass spectrometry) (1). Among the proteins examined in the previous study, we identified connective tissue growth factor (CTGF) as a novel effector molecule in the pathogenesis of RA (1).

CTGF was discovered as a result of cross-reactivity of a platelet-derived growth factor (PDGF) antiserum with a single 38-kd polypeptide secreted by cultured human umbilical vein endothelial cells (HUVECs), and complementary DNA (cDNA) for CTGF isolated from a HUVEC cDNA expression library using an anti-PDGF antibody was shown to en-

Supported by grants from the Institute for Environmental and Gender-Specific Medicine, Juntendo University Graduate School of Medicine.

[1]Kazuhisa Nozawa, MD, PhD, Ayako Yamaguchi, MD, PhD: Juntendo University School of Medicine, Tokyo, Japan, and Juntendo University Graduate School of Medicine, Chiba, Japan; [2]Maki Fujishiro, MS, Mikiko Kawasaki, PhD, Kazuhisa Iwabuchi, PhD, Mitsuaki Yanagida, PhD, Shouzo Ichinose, BS, Hideoki Ogawa, MD, PhD, Kenji Takamori, MD, PhD: Juntendo University Graduate School of Medicine, Chiba, Japan; [3]Keigo Ikeda, MD, PhD, Shinji Morimoto, MD, PhD, Iwao Sekigawa, MD, PhD: Juntendo University Graduate School of Medicine and Juntendo University Urayasu Hospital, Chiba, Japan; [4]Megumi Morioka, MS: Nosan Corporation, Kanagawa, Japan; [5]Yoshinari Takasaki, MD, PhD: Juntendo University School of Medicine, Tokyo, Japan.

Drs. Nozawa and Fujishiro contributed equally to this work.

Address correspondence to Kazuhisa Nozawa, MD, PhD, Department of Rheumatology, School of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail: k-nozawa@juntendo.ac.jp.

Submitted for publication July 9, 2012; accepted in revised form February 7, 2013.

code a 349–amino acid protein (2). CTGF is a bioactive cytokine and is a member of the CCN protein family (3). CCN is believed to be a downstream mediator of transforming growth factor β (TGFβ) (3). Although currently a number of cell surface molecules, such as various kinds of integrins, have been proposed as candidates for specific receptors against CTGF, they have not been completely defined to date. Furthermore, CTGF is produced by various types of cells and is associated with several biologic functions, such as fibrosis, tumorigenesis, angiogenesis, and endochondral ossification (4,5). In addition, although CTGF is known to have multiple biologic effects depending on target cells, few studies have examined its effects on the immune response against immunocompetent cells such as T lymphocytes.

In our previous study, we investigated the contribution of CTGF to the pathogenesis of RA and found that the serum levels and tissue expression of CTGF were up-regulated in patients with RA (6). In addition, we identified integrin $\alpha V\beta 3$ as a cell membrane receptor against CTGF on human osteoclasts. Ligation of CTGF to integrin $\alpha V\beta 3$ recruits ERK-1/2 and is subsequently associated with focal adhesion kinase (FAK) activation (6). To extend our research on the role of CTGF in the pathogenesis of RA, we performed this study to clarify pathologic roles of CTGF in the development of arthritis, using the murine collagen-induced arthritis (CIA) model.

In the present study, we confirmed that CTGF was aberrantly expressed in the synovial tissue of mice with CIA. In addition, we found that blockade of the CTGF pathway by administration of anti-CTGF monoclonal antibody (mAb) significantly prevented the progression of arthritis in mice with CIA. Furthermore, we investigated the effects of CTGF on T cell response because T cells reacting with type II collagen are known to contribute to the development of CIA in mice. We found that the blockade of CTGF inhibited not only the proliferation of autoreactive T cells but also the differentiation of Th17 cells. Moreover, CTGF blockade reduced aberrant osteoclastogenesis in mice with CIA, which results in amelioration of the disease.

We propose that CTGF is an important effector molecule for RA pathogenesis through inhibition of autoreactive T cell proliferation, aberrant Th17 differentiation, and increased osteoclastogenesis. Blockade of the CTGF pathway might become a new therapeutic strategy for RA.

MATERIALS AND METHODS

Mice. Female DBA/1J mice were purchased from Charles River. All mice were 7–8 weeks old at the beginning of the experiments and were maintained under specific pathogen–free conditions. All animal experiments were approved by the Juntendo University Animal Experimental Ethics Committee.

Induction of CIA, mAb treatment, and clinical assessment of arthritis. DBA/1J mice were immunized intradermally at the base of the tail with 100 μg of bovine type II collagen (CII; Chondrex) in 0.05*M* acetic acid, emulsified in Freund's complete adjuvant (CFA). Each group consisted of 12 mice. Neutralizing anti-CTGF mAb was kindly provided by Nosan Corporation; Nosan Corporation developed mAb against the native structure of human CTGF by genetic immunization of mice (7). Mice were immunized by injection of plasmids containing full-length CTGF. The antibody that was produced specifically recognized the insulin-like growth factor binding protein domain of CTGF. This antibody did not recognize a denatured CTGF even if concentration of the antibody was very high. Moreover, the antibody has also been shown to cross-react with mouse CTGF as previously described (8,9). The immunized mice were randomly selected and were intraperitoneally administered 200 μg of anti-CTGF mAb or control purified mouse Ig (Sigma) weekly from 1 week before immunization to 7 weeks after immunization (administration before immunization protocol). In another experiment, mice with CIA were treated with anti-CTGF mAb from 3 weeks to 5 weeks after the onset of arthritis (administration after immunization protocol). The mice with CIA were monitored for arthritis every week and scored in a blinded manner. The degree of swelling in all 4 paws was evaluated by measuring paw thickness, and the severity of arthritis was graded on a scale of 0–4, where 0 = no swelling, 1 = 1 inflamed digit, 2 = 2 inflamed digits, 3 = more than 1 digit and footpad inflamed, and 4 = all digits and footpad inflamed. Each paw was graded, and the 4 scores were totaled so that the maximum possible arthritis score per mouse was 16. Serum samples were collected 7 weeks after immunization. All mice were killed 8 weeks after immunization and used for subsequent experiments.

Immunohistochemical analysis. Briefly, serial paraffin sections derived from articular tissue samples were deparaffinized, rehydrated, and washed with water as previously reported (6). The samples were subjected to heat treatment using citric acid buffer (pH 6.0) with 0.1% Tween 20, and these samples were incubated with peroxidase quenching solution for 10 minutes. The samples were incubated with 1% bovine serum albumin (Sigma) for 60 minutes to eliminate nonspecific binding and then incubated for 60 minutes with goat anti-CTGF antibody (L-20; Santa Cruz Biotechnology) diluted 1:50 in phosphate buffered saline (PBS). After washing with PBS, the bound antibody was visualized using a Super Picture Polymer Detection Kit according to the recommendations of the manufacturer (Zymed). In addition, the sections were counterstained using hematoxylin and eosin (H&E). Histologic examinations were performed as previously described (10).

Enzyme-linked immunosorbent assay (ELISA). The serum levels of matrix metalloproteinase 3 (MMP-3) in mice were measured using an ELISA system (Quantikine MMP-3

Mouse ELISA Kit) according to the recommendations of the manufacturer (R&D Systems). Each sample was analyzed in triplicate, the average optical density was measured at 450 nm, and an appropriate development time was used for the data analysis.

T cell proliferation in vitro. Splenocytes were isolated from each group of mice at 8 weeks after immunization with CII and were cultured in 96-well flat-bottomed microculture plates at a density of 6×10^5 cells/well in the presence or absence of the indicated doses of denatured CII (60°C, 30 minutes) for 72 hours. To evaluate the proliferation of T lymphocytes, interleukin-2 (IL-2) concentration was measured in the supernatants using a Mouse IL-2 ELISA kit according to the recommendations of the manufacturer (Cell Sciences).

Immunoblotting. Immunoblotting analysis was performed as previously described (6). Briefly, the cell extracts of splenic lymphocytes were further subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred to the membranes. After blocking, the primary antibodies, rabbit anti–ERK-1/2 antibody (Abcam) at 1:2,000 dilution, rabbit anti–phosphorylated ERK-1/2 antibody (Abcam) at 1:10,000 dilution, rabbit anti–β-actin antibody (Sigma) at 1:1,000 dilution, rabbit anti-FAK antibody (Santa Cruz Biotechnology) at 1:200 dilution, and rabbit anti–phosphorylated FAK antibody at 1:200 dilution, were incubated with the blots overnight at room temperature. Bound antibodies were detected using horseradish peroxidase–conjugated anti-rabbit IgG antibody (Dako) at 1:2,500 in combination with enhanced chemiluminescence (SuperSignal West Pico; Pierce).

Microarray analysis. CD4+ T cells from splenocytes in each group were purified by passing them through a Pre-Separation filter (30 μm; Miltenyi Biotec) and by using an auto-MACS column with CD4+ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Total RNA was extracted from the purified CD4+ T cells using the RNeasy Mini kit according to the recommendations of the manufacturer (Qiagen). The extracted RNAs were further used for a DNA microarray analysis. Fluorescence-labeled RNA targets were synthesized using an Amino Allyl Messageamp II mRNA Amplification Kit (Ambion). The RNA targets synthesized from untreated mice with CIA were coupled with Cy3 (channel 1) and those from mice treated with anti-CTGF mAb were coupled with Cy5 (channel 2). These labeled targets were competitively hybridized to a 24k 3-dimensional gene mouse oligo chip (Toray) which had been incubated overnight at 37°C, and the hybridized images were scanned and detected using a Gene Pix 440A system (Intermedical).

Real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Real-time qRT-PCR was performed as previously reported (6). The primers used were as follows: for mouse IL-17A (GenBank accession no. NM_010552), 5′-CCTTCACTTTCAGGGTCGAG-3′ (forward) and 5′-AAACGTGGGGGTTTCTTAGG-3′ (reverse); for IL-21 (GenBank accession no. NM_021782), 5′-GGAGGGGAGGAAAGAAACAG-3′ (forward) and 5′-GGGAATCTTCTCGGATCCTC-3′ (reverse); for nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor ζ (Nfkbiz; GenBank accession no. NM_030612), 5′-TATCGGGTGACACAGTTGGA-3′ (forward) and 5′-TGAATGGACTTCCCCTTCAG-3′ (reverse); for interferon regulatory factor 4 (IRF-4; GenBank accession no. NM_013674), 5′-CTGAGTGGCTGTATGCCAGA-3′ (forward) and 5′-ATCAGCAATGGGAAAGTTCG-3′ (reverse); and for β-actin controls, 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ (forward) and 5′-ATGGAGCCACCGATCCACA-3′ (reverse). Quantitative real-time RT-PCR was performed using a 10-μl sample volume with 500 ng of cDNA in a SYBR Premix Ex Taq kit (Takara). The amplification cycles consisted of 95°C for 5 seconds as the first step (1 cycle), 95°C for 5 seconds and 60°C for 30 seconds for IL-17A, IRF-4, and Nfkbiz and 65°C for 30 seconds for IL-21 as the second step (40 cycles), and 95°C for 5 seconds, 60°C for 30 seconds, and 95°C for 15 seconds as the third step (1 cycle), according to the protocol described by the manufacturer (Takara). To determine the quantitative expression levels of the transcripts, sample loading was monitored and normalized by the expression of β-actin transcripts.

Osteoclast differentiation. The mice were killed 8 weeks after CII immunization. Spleen cells were derived from untreated mice and mice treated with anti-CTGF mAb weekly from 1 week before immunization to 7 weeks after immunization. The splenocytes were purified into a CD14+ population using biotin anti-mouse CD14 (BioLegend) and anti-biotin microbeads (Miltenyi Biotec) according to the protocol supplied by the manufacturers. The purified CD14+ monocytes (7.5×10^4 cells/well) were cultured and incubated with macrophage colony-stimulating factor (M-CSF; 50 ng/ml) (R&D Systems) and soluble RANKL (sRANKL; 100 ng/ml) (Sigma-Aldrich). After 3 days, the cells were stained for tartrate-resistant acid phosphatase (TRAP) expression using a TRAP staining kit (Primary Cell) for detection of osteoclasts after incubation for 7 days. TRAP-positive multinucleated cells in 3 randomly selected fields examined at 100× magnification were counted as osteoclasts under light microscopy.

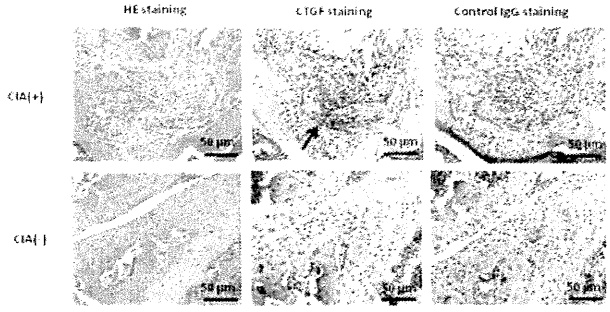


Figure 1. Connective tissue growth factor (CTGF) expression is increased in articular cartilage from mice with collagen-induced arthritis (CIA). Eight weeks after immunization, the mice with CIA were killed and articular tissue was subjected to immunohistochemical analysis. The tissue samples were stained with anti-CTGF antibody or control goat IgG antibody, and the bound antibody was visualized as described in Materials and Methods. Serial sections of the articular tissue samples were also counterstained with hematoxylin and eosin (H&E) to confirm the occurrence of arthritis. The expression of CTGF (**arrow**) was higher in the articular tissue of mice with CIA than in control mice.

Statistical analysis. The experimental data were compared using Student's unpaired *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Increased CTGF expression in the articular tissue of mice with CIA. To investigate the role of CTGF in the development of CIA in mice, we examined CTGF expression in the synovial tissue of mice with CIA by immunohistochemical analysis. H&E staining of the synovial tissue sample revealed massive accumulation of inflammatory cells in mice with CIA compared to control mice without CIA (Figure 1). Although no or very weak CTGF expression was observed in samples from control mice, strong CTGF expression was observed in the synovial tissue of mice with CIA.

Prevention of the development and progression of arthritis by blockade of the CTGF pathway in mice with CIA. To confirm the mechanism by which CTGF contributes to the development of arthritis in mice treated with collagen, we administered neutralizing anti-CTGF mAb to inhibit the biologic function of CTGF in vivo. In this experimental model of CIA, arthritis began to develop 3–4 weeks after immunization with CII and peaked at 7–8 weeks after immunization. The mice with CIA treated with anti-CTGF mAb before the onset of arthritis showed a significant reduction in arthritis score compared to untreated mice with CIA, which indicated that the development of arthritis was prevented (Figure 2A). In addition, swelling of both of the fore paws was prevented by anti-CTGF mAb treatment (Figure 2B). Similar results were observed in the hind

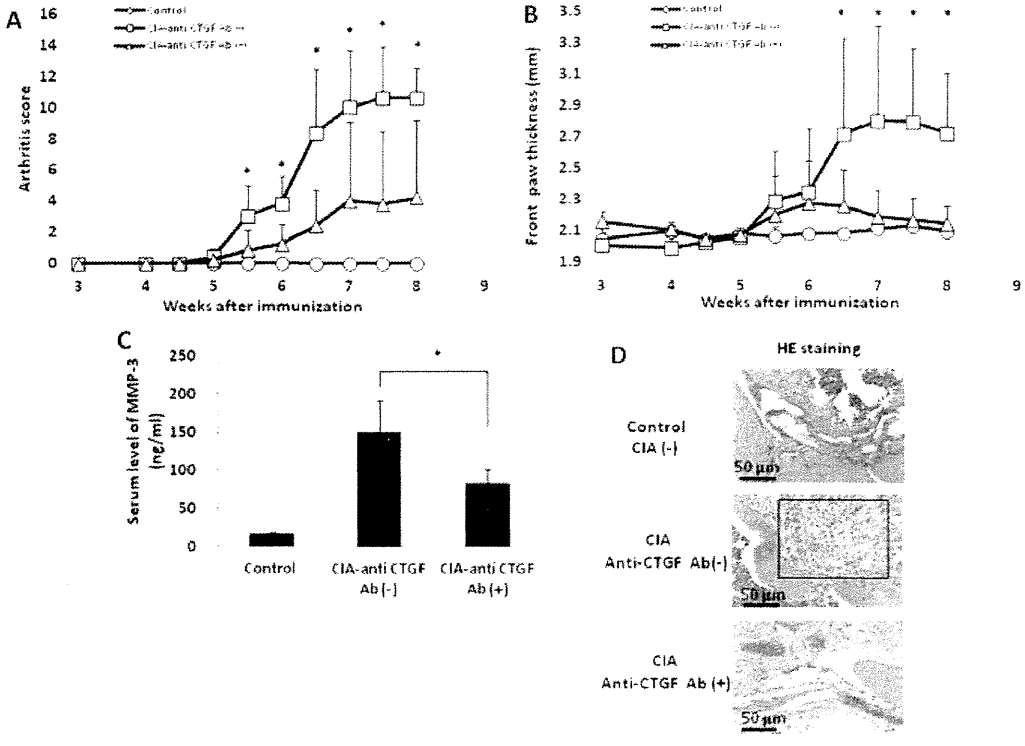


Figure 2. Connective tissue growth factor (CTGF) pathway blockade prevents the development of collagen-induced arthritis (CIA). The mice with CIA were randomly selected and were intraperitoneally administered anti-CTGF monoclonal antibody (Ab) or purified control Ig weekly from 1 week before immunization to 6 weeks after immunization (administration before immunization protocol). **A,** Arthritis score. Mice were monitored for arthritis weekly and scored in a blinded manner. **B,** Paw swelling. The degree of swelling of the front paws was evaluated by measurement of paw thickness. **C,** Serum levels of matrix metalloproteinase 3 (MMP-3). Serum samples were collected from each group of mice 8 weeks after immunization. Values in **A–C** are the mean ± SD ($n = 12$ mice per group). * = $P < 0.05$ versus anti-CTGF–treated mice with CIA. **D,** Staining of articular tissue samples with hematoxylin and eosin (H&E). The boxed area indicates inflamed synovial tissue in an untreated mouse with CIA. Blockade of the CTGF pathway efficiently prevented the development of arthritis in mice with CIA.

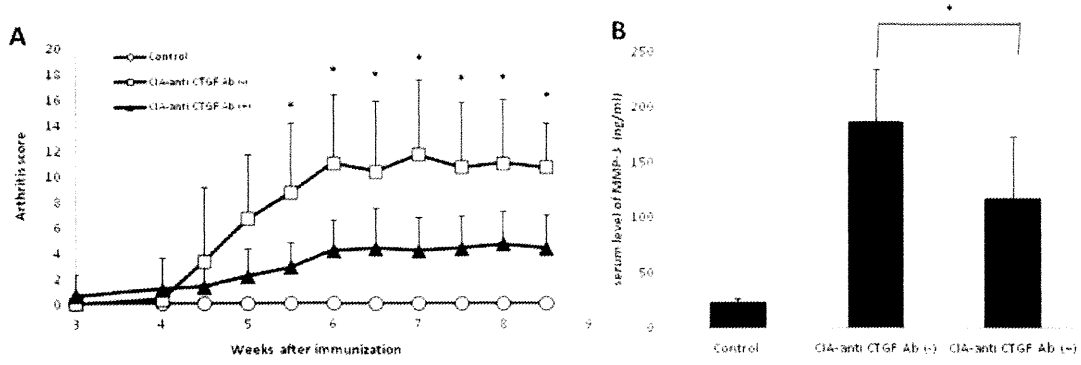


Figure 3. Blockade of the connective tissue growth factor (CTGF) pathway in mice after the onset of collagen-induced arthritis (CIA) ameliorates disease. The mice with CIA were administered anti-CTGF monoclonal antibody (Ab) or purified control mouse Ig weekly from 4 weeks after immunization to 6 weeks after immunization (administration after immunization protocol). **A,** Arthritis score. Mice were monitored for arthritis weekly and scored in a blinded manner. **B,** Serum levels of matrix metalloproteinase 3 (MMP-3). Serum samples were collected from each group of mice 8 weeks after immunization. Blockade of the CTGF pathway efficiently prevented the progression of arthritis despite administration of the antibody after the development of arthritis. Values are the mean ± SD (n = 12 mice per group). * = $P < 0.05$ versus anti-CTGF–treated mice with CIA.

paws (data not shown). We also evaluated serum levels of MMP-3 as a biomarker for synovial tissue inflammation. Administration of anti-CTGF mAb significantly reduced serum levels of MMP-3 (Figure 2C). Histologic analysis with H&E staining revealed a specific area with accumulation of inflammatory cells in samples obtained from untreated mice with CIA compared to control mice (Figure 2D). Mice with CIA treated with anti-CTGF mAb showed a reduction in the accumulation of inflammatory cells (Figure 2D).

Next, we investigated whether anti-CTGF mAb treatment still suppressed arthritis development even after the onset of arthritis. In our experimental model, arthritis began to develop 3–4 weeks after immunization. Therefore, anti-CTGF mAb was administered 3 times, at 4, 5, and 6 weeks after immunization with CII with CFA, to evaluate suppressive effects against the progression of established arthritis. Similar to the effects seen in mice administered anti-CTGF mAb before the onset of arthritis, therapeutic effects were observed in mice treated with anti-CTGF mAb after arthritis onset (Figure 3A). In addition to the arthritis score, serum levels of C-reactive protein and MMP-3 were also reduced in mice with established CIA treated with anti-CTGF mAb (Figure 3B).

Effect of anti-CTGF mAb treatment on T cell proliferation in mice with CIA. We hypothesized that amelioration of arthritis by anti-CTGF mAb results from modulation of CII-specific T cell responses, because CII antigen–specific T cells have been considered to play a pathologic role in the mouse model of CIA. To investigate this, splenocytes were isolated 8 weeks after immunization with CII, and proliferative responses against CII were assessed ex vivo. As expected, we confirmed that the CII-specific proliferative response was suppressed in mice treated with anti-CTGF mAb (Figure 4A). To obtain further information about the cell proliferation pathway affected by anti-CTGF mAb treatment, we investigated intracellular signal transduction. We have previously shown that CTGF mediated ERK and FAK activation through ligation with integrin $\alpha V\beta 3$ as a cell membrane receptor on human osteoclasts (6). Therefore, we monitored phosphorylation of ERK and FAK in splenic lymphocytes at 8 weeks after immunization. Both ERK and FAK phosphorylation were up-regulated in mice with CIA compared to control mice. We found that phosphorylation of ERK was suppressed by anti-CTGF mAb treatment in mice with CIA, although phosphorylation of FAK did not change (Figure 4B). These data suggested that CTGF mediated ERK activation through ligation with an unknown receptor, subsequently resulting in T cell proliferation.

Reduction in Th17 differentiation and osteoclastogenesis by blockade of the CTGF pathway in mice with CIA. To further evaluate the effect of anti-CTGF mAb on T cells, we assessed the production of cytokines from CD4+ T cells. Microarray analysis was used to determine whether the gene expression levels of cytokines on CD4+ T cells from mice treated with anti-CTGF mAb were altered compared to those from untreated mice

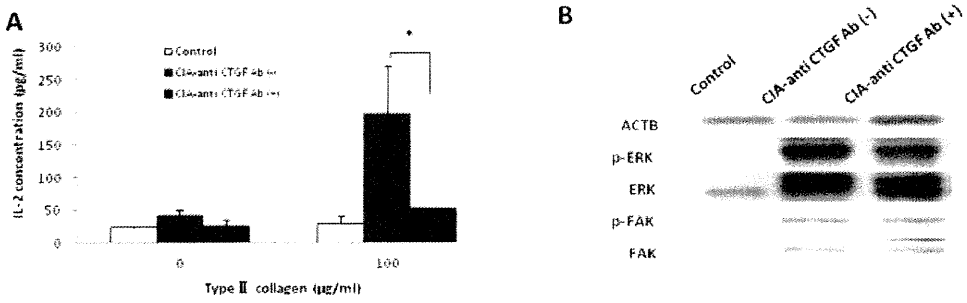


Figure 4. Anti–connective tissue growth factor (anti-CTGF) monoclonal antibody (mAb) treatment suppresses T cell proliferation in mice with collagen-induced arthritis (CIA). Control mice and mice with CIA treated weekly with CTGF mAb or control purified Ig were killed 8 weeks after immunization. Lymphocytes were isolated from the spleen in each group of mice and were cultured in the presence or absence of type II collagen (CII) for restimulation ex vivo. **A,** T cell proliferation restimulated by CII in each group of mice. Anti-CTGF mAb treatment suppressed T cell proliferation. Values are the mean ± SD. * = $P < 0.05$. **B,** Immunoblotting analysis using anti–phosphorylated ERK-1/2, conventional ERK-1/2, anti–phosphorylated focal adhesion kinase (FAK), conventional FAK, and β-actin (ACTB) antibodies in cell extracts of the splenic lymphocytes. Phosphorylation of ERK-1/2 was suppressed in mice with CIA treated with anti-CTGF mAb. IL-2 = interleukin-2.

with CIA. The results of the microarray analysis showed clear up-regulation (gene expression ratio >20) or down-regulation (gene expression ratio <0.5) of several cytokines due to anti-CTGF mAb treatment (Table 1). Interestingly, we found that the expression levels of IL-17 and IL-21 were markedly down-regulated and those of IL-6, IL-10, and IL-23 were up-regulated.

To validate the microarray analysis data, we performed quantitative RT-PCR and confirmed that the expression of IL-17 on CD4+ T cells was significantly suppressed by treatment with anti-CTGF mAb (Figure 5A). Similar results were observed for IL-21 (data not shown). IL-17 is well known to be an important factor in the development of CIA in mice. Furthermore, Th17 cells selectively produce not only IL-17 but also signature cytokines such as IL-21, and these cells play a critical role in the chronic inflammatory response and subsequent tissue damage in RA (11). Therefore, we assumed that anti-CTGF mAb treatment contributes to amelioration of CIA through the suppression of Th17 differentiation.

Next, we analyzed the gene expression of Th17-regulating transcription factors. Although expression of the retinoic acid receptor–related orphan receptor γt (RORγt) gene, which is the major transcription factor for Th17 differentiation, was not significantly altered in the treated mice (data not shown), a nuclear IκB family member, IκBζ (encoded by Nfkbiz), and IRF-4 (encoded by IRF4) on CD4+ T cells were clearly down-regulated in the anti-CTGF mAb–treated mice with CIA (Figures 5B and C). Recently, Nfkbiz has been reported to have a potent effect on the differentiation of Th17 cells by cooperating with RORγt (12). Moreover, IRF-4 has been shown to be essential for IL-21–mediated induction, amplification, and stabilization of Th17 cells (13,14). Taken together, these data suggest that blockade of the CTGF pathway exerts suppressive effects on Th17 differentiation through regulation of Nfkbiz and IRF-4 expression and contributes to the amelioration of CIA in mice.

Our previous study showed that CTGF enhanced osteoclastogenesis and played an important role in bone destruction in patients with RA (6). In the present study we observed that M-CSF/RANKL-mediated osteoclastogenesis from CD14+ progenitor cells was increased in

Table 1. Altered gene expression levels of cytokines on CD4+ T cells in mice treated with anti-CTGF monoclonal antibody*

Gene	RefSeq ID	Description	Gene expression ratio
Up-regulated			
Il23	NM_031252	Interleukin 23, alpha subunit p19	5.220163
Il10	NM_010548	Interleukin 10	2.72609
Il6	NM_031168	Interleukin 6	2.647875
Down-regulated			
Il21	NM_021782	Interleukin 21	0.012748
Il17A	NM_010552	Interleukin 17A	0.020372
Il4	NM_021283	Interleukin 4	0.175501
Il1b	NM_008361	Interleukin 1 beta	0.284016

* Altered gene expression levels were defined as a gene expression ratio of >2.0 (for up-regulation) and <0.5 (for down-regulation) in mice with collagen-induced arthritis (CIA) treated with anti–connective tissue growth factor (anti-CTGF) monoclonal antibody compared to untreated mice with CIA.

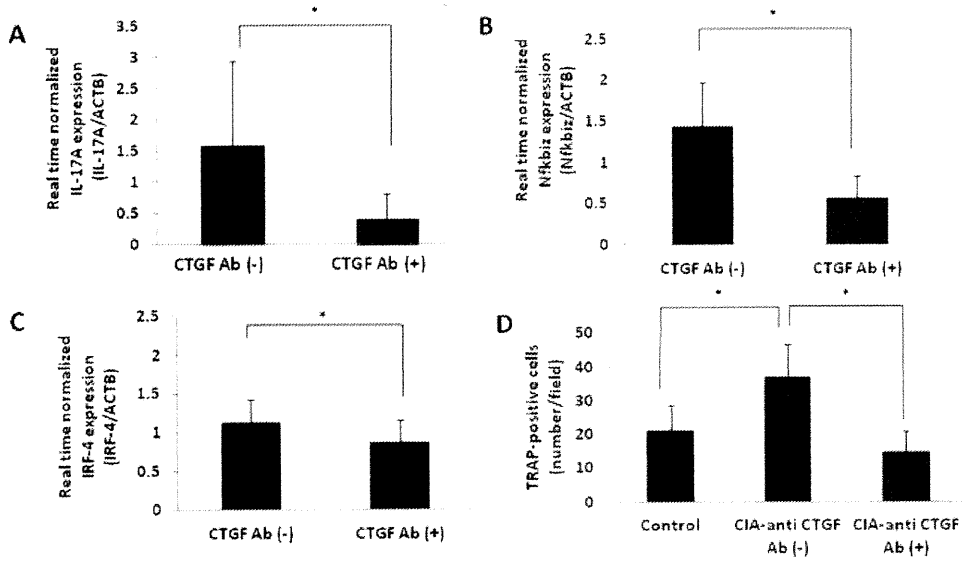


Figure 5. Connective tissue growth factor (CTGF) pathway blockade reduces Th17 cell differentiation and osteoclastogenesis in mice with collagen-induced arthritis (CIA). Mice were killed 8 weeks after immunization in the administration before immunization protocol (9 weeks after the beginning of anti-CTGF monoclonal antibody [mAb] administration). **A–C,** Expression of interleukin-17A (IL-17A) (**A**), nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor ζ (Nfkbiz) (**B**), and interferon regulatory factor 4 (IRF-4) (**C**). CD4+ T cells were isolated from splenic T cells, and total RNA was purified as described in Materials and Methods. The purified RNAs were used for quantitative reverse transcription–polymerase chain reaction, and the expression levels of IL-17A, Nfkbiz, and IRF-4 were evaluated in the RNAs from mice with CIA that were left untreated or treated with anti-CTGF mAb. **D,** Numbers of tartrate-resistant acid phosphatase (TRAP)–positive cells. For evaluation of osteoclastogenesis, CD14+ osteoclastic progenitor cells were purified from splenocytes 8 weeks after immunization and then osteoclasts were induced by macrophage colony-stimulating factor and soluble RANKL, as described in Materials and Methods. Osteoclastogenesis was suppressed in mice with CIA treated with anti-CTGF mAb. Values are the mean ± SD. * = $P < 0.05$. ACTB = β-actin.

mice with CIA compared to that in control mice. As expected, anti-CTGF mAb treatment reduced the number of osteoclasts, which suggested a reduction in aberrant osteoclastogenesis in mice with CIA (Figure 5D).

DISCUSSION

This study was performed to determine the role of CTGF in the development of arthritis in a murine model of RA. There were several novel and interesting findings. Immunohistochemical studies revealed that CTGF appears to be extensively expressed in the articular tissue of mice with CIA (Figure 1). Treatment with anti-CTGF mAb significantly ameliorated arthritis in mice with CIA (Figures 2 and 3), suppressed pathologic proliferation of T cells (Figures 4A and B), and inhibited IL-17 production on CD4+ T cells and differentiation of Th17 cells (Figures 5A–C) in mice with CIA. In addition, anti-CTGF mAb significantly reduced osteoclastogenesis in these mice (Figure 5D).

Although the molecular mechanisms involved in CTGF signaling, such as signal transduction mediated by specific cell receptors, are far from understood, several studies have demonstrated that CTGF plays a significant role in the pathogenesis of RA (15–17). For example, aberrant CTGF expression results in cartilage damage with increased messenger RNA coding for degrading enzymes such as MMP-3 (18). Manns and coworkers also showed that CTGF is up-regulated in an experimental animal model of RA and that treatment with thrombospondin 1–derived peptide is associated with the down-regulation of CTGF occurring simultaneously with the amelioration of the disease (19).

We have previously shown that CTGF enhances osteoclastogenesis in patients with RA and plays an important role in the pathogenesis of RA (6). Osteoclasts are commonly found within the erosive pit at the interface between the synovial inflammatory tissue and subchondral bone (20). RANKL is a membrane protein present on osteoblasts and recognizes its receptor (RANK) expressed on marrow macrophages, which pro-

motes the osteoblasts to differentiate into osteoclasts in the presence of M-CSF (21–23). Therefore, aberrant osteoclastogenesis plays an important role in the development of RA, and this process is further positively regulated by proinflammatory cytokines under pathogenic conditions such as CIA in mice. Our data indicate that aberrant osteoclastogenesis, which occurred in mice with CIA, was reduced by blockade of the anti-CTGF pathway (Figure 5D). These data supported our previous findings. We assumed that one of the important factors for the amelioration of disease in CIA was that CTGF directly affects osteoclastogenesis by interacting with CD14+ osteoclastic precursor cells.

In addition to the direct effect of CTGF on osteoclastogenesis, we observed that blockade of the anti-CTGF pathway suppressed Th17 differentiation (Figure 5A). IL-17 is well known to play a significant role in CIA, and was responsible for the priming of collagen-specific T cells. These observations suggest that IL-17 plays a crucial role in the development of CIA by activating the cellular response (24). IL-17 is mainly produced by CD4+ Th17 cells, and classic Th17 cells are also important contributors to the development of arthritis in mouse models such as CIA and in human RA (25); further, IL-17 has recently been correlated with inflammatory activity in RA (26). Notably, in the mouse, Th17 cells directly contribute to bone damage because they express RANKL and have the ability to activate RANK expression on osteoclasts (23). In the present study, we found that blockade of CTGF inhibited IL-17 expression on CD4+ T cells (Figure 5A). These results suggest that CTGF blockade could reduce aberrant osteoclastogenesis not only through a direct effect but also through indirect effects, such as inhibition of Th-17 responses, in mice with CIA.

The precise mechanism underlying CTGF-mediated suppression of Th17 differentiation is completely unknown. Microarray analysis showed that the expression levels of IL-23, IL-6, and IL-10 were up-regulated, and IL-17 and IL-21 were down-regulated, in anti-CTGF mAb–treated mice compared to untreated mice with CIA (Table 1). Moreover, the expression levels of TGFβ and tumor necrosis factor α were not altered (data not shown). Although IL-23 has long been recognized to be an inducer of IL-17 (27), it has more recently been established that naive T cells do not express IL-23 receptor (IL-23R) (28,29). This has led to the notion that IL-23 cannot be the sole inducer of Th17 differentiation. IL-6 and IL-21 are implicated in the regulation/induction of IL-17 production (30,31). In particular, IL-6 is a potent inducer of Th17 cells but only when combined with other cytokines, including TGFβ1 (31,32). This led to the finding that the combination of TGFβ1 with IL-6 is the initial driver of Th17 specification (32). In addition, IL-21 induces the expression of IL-23R. Thus, it appears that both IL-6 and IL-21 can promote Th17 differentiation. We assume that the reason the anti-CTGF mAb–treated mice did not exhibit increased Th17 differentiation with increased IL-6 levels is that TGFβ and IL-21 expression were not increased concomitantly.

Furthermore, we found that IL-10 expression on CD4+ T cells was up-regulated in the anti-CTGF mAb–treated mice. Chaudhry and coworkers showed that IL-10, and not proinflammatory IL-6 and IL-23, cytokine signaling endowed Treg cells with the ability to suppress pathogenic Th17 cell responses (33). One possible explanation why blockade of the CTGF pathway inhibited IL-17 production in CD4+ T cells is that IL-10 was up-regulated by blockade of the CTGF pathway. Although few studies have examined the correlation between CTGF and IL-10 expression, we assume that blockade of the CTGF pathway positively regulates IL-10 expression of CD4+ T cells and subsequently leads to the amelioration of CIA.

We analyzed the expression of genes related to Th17 differentiation in the anti-CTGF mAb–treated mice (Figures 5B and C). RORγt acts as a transcription factor for Th17 differentiation, and therefore, has been proposed to be a "master regulator" for Th17 differentiation (34). However, the expression of the RORγt gene was not altered by anti-CTGF mAb treatment in the present study. This result suggested that the blockade of CTGF prevents Th17 differentiation by other mechanisms of RORγt gene suppression. We found that the expression of Nfkbiz and IRF-4 genes on CD4+ T cells was significantly suppressed by treatment with anti-CTGF mAb. Although IRF-4 was originally implicated as a key inducer of GATA-3 expression in Th2 lineage differentiation (35), IRF-4–deficient T cells were shown to impair IL-17 production in response to TGFβ and IL-6 (36). Furthermore, IRF-4 has been shown to regulate IL-17 and IL-21 production (14). In addition to IRF-4, Okamoto and coworkers showed that a nuclear IκB family member, IκBζ (encoded by the Nfkbiz gene), was a transcription factor required for Th17 development in mice, and they also showed that Nfkbiz enhanced IL-17 expression by binding directly to the regulatory region of the IL-17 gene in cooperation with RORγt (12). The blockade of CTGF may negatively regulate IRF-4 and Nfkbiz expression independent

of RORγt and inhibit Th17 differentiation, subsequently leading to the amelioration of CIA.

Taken together, our findings provide evidence of an important role of CTGF in the development of arthritis in mice with CIA. We propose that CTGF exerts significant actions in RA pathogenesis. Blockade of the CTGF pathway can ameliorate CIA, especially through the inhibition of pathologic proliferation of T cells and differentiation of Th17 cells and the reduction of aberrant osteoclastogenesis. These results may lead to new therapeutic strategies for RA and the possibility of the development of more specific biologic therapies.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Nozawa and Sekigawa had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Nozawa, Fujishiro, Kawasaki, Yamaguchi, Ikeda, Morimoto, Iwabuchi, Yanagida, Ichinose, Morioka, Ogawa, Takamori, Takasaki, Sekigawa.

Acquisition of data. Nozawa, Fujishiro, Kawasaki, Yamaguchi, Ikeda, Morimoto, Iwabuchi, Yanagida, Ichinose, Morioka, Ogawa, Takamori, Takasaki, Sekigawa.

Analysis and interpretation of data. Nozawa, Fujishiro, Kawasaki, Yamaguchi, Ikeda, Morimoto, Iwabuchi, Yanagida, Ichinose, Morioka, Ogawa, Takamori, Takasaki, Sekigawa.

ADDITIONAL DISCLOSURES

Author Morioka is an employee of Nosan Corporation.

REFERENCES

1. Sekigawa I, Yanagida M, Iwabuchi K, Kaneda K, Kaneko H, Takasaki Y, et al. Protein biomarker analysis by mass spectrometry in patients with rheumatoid arthritis receiving anti-tumor necrosis factor-α antibody therapy. Clin Exp Rheumatol 2008;26:261–7.
2. Bradham DM, Igarashi A, Potter RL, Grotendorst GR. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. J Cell Biol 1991;114: 1285–94.
3. Grotendorst GR. Connective tissue growth factor: a mediator of TGF-β action on fibroblasts. Cytokine Growth Factor Rev 1997; 8:171–9.
4. Perbal B. CCN proteins: multifunctional signalling regulators. Lancet 2004;363:62–4.
5. Takigawa M, Nakanishi T, Kubota S, Nishida T. Role of CTGF/ HCS24/ecogenin in skeletal growth control. J Cell Physiol 2003; 194:256–66.
6. Nozawa K, Fujishiro M, Kawasaki M, Kaneko H, Iwabuchi K, Yanagida M, et al. Connective tissue growth factor promotes articular damage by increased osteoclastogenesis in patients with rheumatoid arthritis. Arthritis Res Ther 2009;11:R174.
7. Grotendorst GR, Duncan MR. Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. FASEB J 2005;19:729–38.
8. Chujo S, Shirasaki F, Kondo-Miyazaki M, Ikawa Y, Takehara K. Role of connective tissue growth factor and its interaction with basic fibroblast growth factor and macrophage chemoattractant protein-1 in skin fibrosis. J Cell Physiol 2009;220:189–95.
9. Ikawa Y, Ng PS, Endo K, Kondo M, Chujo S, Ishida W, et al. Neutralizing monoclonal antibody to human connective tissue growth factor ameliorates transforming growth factor-β-induced mouse fibrosis. J Cell Physiol 2008;216:680–7.
10. Nakasa T, Shibuya H, Nagata Y, Niimoto T, Ochi M. The inhibitory effect of microRNA-146a expression on bone destruction in collagen-induced arthritis. Arthritis Rheum 2011;63:1582–90.
11. Dong W, Zhu P. Functional niche of inflamed synovium for Th17-cell expansion and activation in rheumatoid arthritis: implication to clinical therapeutics. Autoimmun Rev 2012;11:844–51.
12. Okamoto K, Iwai Y, Oh-Hora M, Yamamoto M, Morio T, Aoki K, et al. IκBζ regulates T_H17 development by cooperating with ROR nuclear receptors. Nature 2010;464:1381–5.
13. Huber M, Brustle A, Reinhard K, Guralnik A, Walter G, Mahiny A, et al. IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. Proc Natl Acad Sci U S A 2008;105:20846–51.
14. Biswas PS, Gupta S, Chang E, Song L, Stirzaker RA, Liao JK, et al. Phosphorylation of IRF4 by ROCK2 regulates IL-17 and IL-21 production and the development of autoimmunity in mice. J Clin Invest 2010;120:3280–95.
15. Cicha I, Yilmaz A, Klein M, Raithel D, Brigstock DR, Daniel WG, et al. Connective tissue growth factor is overexpressed in complicated atherosclerotic plaques and induces mononuclear cell chemotaxis in vitro. Arterioscler Thromb Vasc Biol 2005;25:1008–13.
16. Crean JK, Finlay D, Murphy M, Moss C, Godson C, Martin F, et al. The role of p42/44 MAPK and protein kinase B in connective tissue growth factor induced extracellular matrix protein production, cell migration, and actin cytoskeletal rearrangement in human mesangial cells. J Biol Chem 2002;277:44187–94.
17. Wu SH, Wu XH, Lu C, Dong L, Zhou GP, Chen ZQ. Lipoxin A4 inhibits connective tissue growth factor-induced production of chemokines in rat mesangial cells. Kidney Int 2006;69:248–56.
18. Blaney Davidson EN, Vitters EL, Mooren FM, Oliver N, van den Berg WB, van der Kraan PM. Connective tissue growth factor/ CCN2 overexpression in mouse synovial lining results in transient fibrosis and cartilage damage. Arthritis Rheum 2006;54:1653–61.
19. Manns JM, Uknis AB, Rico MC, Agelan A, Castaneda J, Arango I, et al. A peptide from thrombospondin 1 modulates experimental erosive arthritis by regulating connective tissue growth factor. Arthritis Rheum 2006;54:2415–22.
20. Schett G. Rheumatoid arthritis: inflammation and bone loss. Wien Med Wochenschr 2006;156:34–41.
21. Gravallese EM, Manning C, Tsay A, Naito A, Pan C, Amento E, et al. Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. Arthritis Rheum 2000;43:250–8.
22. Shigeyama Y, Pap T, Kunzler P, Simmen BR, Gay RE, Gay S. Expression of osteoclast differentiation factor in rheumatoid arthritis. Arthritis Rheum 2000;43:2523–30.
23. Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. J Exp Med 2006;203:2673–82.
24. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol 2003;171:6173–7.
25. Pollinger B. IL-17 producing T cells in mouse models of multiple sclerosis and rheumatoid arthritis. J Mol Med 2012;90:613–24.
26. Zizzo G, De Santis M, Bosello SL, Fedele AL, Peluso G, Gremese E, et al. Synovial fluid-derived T helper 17 cells correlate with inflammatory activity in arthritis, irrespectively of diagnosis. Clin Immunol 2011;138:107–16.
27. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL.

Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 2003;278:1910–4.
28. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17$^+$ T helper cells. Cell 2006;126:1121–33.
29. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T_H-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol 2007;8:967–74.
30. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature 2007;448:480–3.
31. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006; 24:179–89.
32. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector T_H17 and regulatory T cells. Nature 2006;441:235–8.
33. Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM, et al. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity 2011;34:566–78.
34. Hirahara K, Ghoreschi K, Laurence A, Yang XP, Kanno Y, O'Shea JJ. Signal transduction pathways and transcriptional regulation in Th17 cell differentiation. Cytokine Growth Factor Rev 2010;21:425–34.
35. Lohoff M, Mittrucker HW, Prechtl S, Bischof S, Sommer F, Kock S, et al. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. Proc Natl Acad Sci U S A 2002;99:11808–12.
36. Brustle A, Heink S, Huber M, Rosenplanter C, Stadelmann C, Yu P, et al. The development of inflammatory T_H-17 cells requires interferon-regulatory factor 4. Nat Immunol 2007;8:958–66.

日本IVF学会雑誌 Vol.16, No.1, 21-25, 2013

—総説—

不育症　Up to date —不妊症と不育症の境界領域も含めて—

齋藤 滋

富山大学産科婦人科　〒930-0194　富山市杉谷 2630

はじめに

2008－2010年度に厚生労働科学研究(成育疾患克服等次世代育成基盤研究事業)「不育症治療に関する再評価と新たなる治療法の開発に関する研究」が行なわれ[1,2]，これまで混沌としていた不育症の定義，不育症のスクリーニング法，不育症の治療成績が明らかになるとともに，抗リン脂質抗体症候群に対するヘパリンカルシウム製剤の保険収載，プロテインS活性の保険収載，血栓性素因(先天性アンチトロンビン欠乏症，プロテインC欠乏症，プロテインS欠乏症，抗リン脂質抗体症候群など)を有する患者へのヘパリン在宅自己注射が保険収載され，不育症の治療の大半が保険診療で行なえるようになった．加えて，全国の都道府県に不育症相談窓口(49ヶ所)が開設され，24ヶ所の市町村(一部県単位)で，不育症に対しての助成金給付が行なわれている．

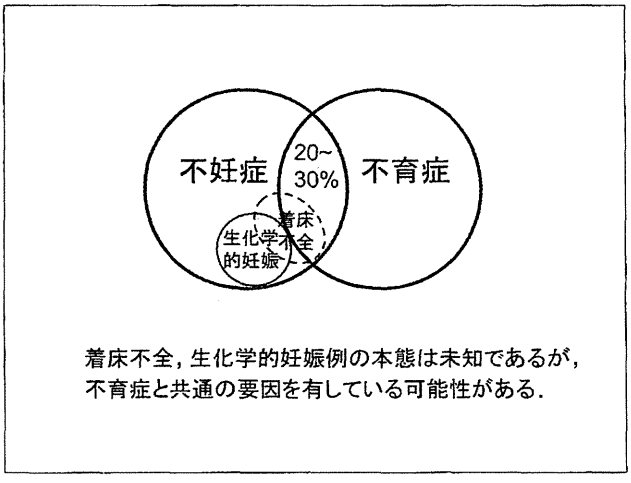
このような状況の変化に加え，不育症と不妊症を併せ持つ症例，不育症と不妊症の境界領域にある症例，対象者高齢化など，不妊症をプロフェッショナルとする方々にも，不育症のことを知っていただき，適切な対処を行なうか，もしくは不妊クリニックと不育症外来とのコラボレーションが必要な時期に来ている．本稿では，不育症の現状と今後の展望につき解説する．

不育症の定義

アメリカ産科婦人科学会(2001)[3]，アメリカ生殖医療学会(2008)[4]ともに，3回以上の流産，死産の既往がある場合をrecurrent pregnancy loss (recurrent miscarriage)と呼ぶと提唱している．しかし，いずれの報告でも，多くのエクスパートは，2回以上の流産・死産の既往でもrecurrent pregnancy lossに含めて良いと考えているという但し書きがある．そのため，厚生労働研究班では，2回以上の流産・死産あるいは，早期新生児死亡の既往があることを提唱した．早期新生児死亡には，抗リン脂質抗体症候群による重度のIUGR/FGR症例や，早発型の妊娠高血圧症候群など，不育症関連の疾患が含まれる一方で，遺伝的代謝疾患や免疫異常など，明らかに流産とは関連性がない疾患も含まれることを留意すべきである．また，班員で意見が分かれたが，現在のところ生化学的妊娠は流産に含めないこととした．これは，生化学的妊娠(化学妊娠，化学流産)の頻度が高いため[5]，臨床的流産のみを不育症の流産に含めることとした．しかし，**図1**に示すように生化学的妊娠，着床不全と不育症は基盤を同じくしており，また不妊症と不育症を併せ持つ症例が20～30%存在している．現在，筆者の不育症外来の約30%が不妊クリニックからの紹介患者であり，今後，益々不妊クリニックと，不育症外来を開設する病院，もしくはクリニックとの連携が必要となってきている．

I. 不育症の頻度

海外の不育症の頻度は約1%とされているが[6]，日本ではこれまでその頻度は不明であった．Sugiura-Ogasawaraらは愛知県岡崎地区における住民健診で，



着床不全，生化学的妊娠例の本態は未知であるが，
不育症と共通の要因を有している可能性がある．

図1　不妊症，着床不全，生化学的妊娠，不育症の関係

受付　2013年1月24日／受理　2013年3月7日
著者連絡先：齋藤 滋　e-mail［s30saito@med.u-toyama.ac.jp］

妊娠歴，流産歴を聴取し，2回以上の流産歴が4.2%，3回以上の流産歴が0.88%であることを明らかにした[8]．また，2回以上の流産歴のある89.5%が生児を得ていた．不育症患者で通院している患者の生児獲得率も85.5%と高かった[8]．

流産を繰り返すと，次回の妊娠自体が恐くなり，避妊をしたり，性交渉を拒むケースもしばしば経験するが，不育症であっても生児を得られる率は高いことや，スクリーニングを勧め，各リスクに応じた対応すれば，85%以上が生児を獲得できていることを，知ってもらうことは重要である．また，不育症例では，離婚のリスクが3.1倍（95%CI:1.474－6.53）と高いことも明らかとなり[7]，流産後の夫を交えたカウンセリング等の心のケアも重要となる．体外受精胚移植(IVF－ET)で，不成功になった場合の心の落ち込みは，流産と同等とも推察されるので，不妊クリニックにおいても，IVF治療が不成功になった後の心のケアが，益々重要となっている．

II. 不育症のスクリーニング

図2に不育症のリスク因子の検査項目を一次スクリーニングと選択的検査に分けて示した．一部の検査が薬事未承認のため自費となるが，検査にかかる負担は以前に比して軽減した．**図3**にこれらリスク因子の頻度を示した．なお，抗リン脂質抗体陽性であれば，12週間後に再検査を行ない，再度陽性であれば抗リン脂質抗体症候群と確定診断できる．陰性化した際は，偶発的抗リン脂質抗体(まだ用語は確定していない)となる．

III. 各リスク別の治療

不育症の治療は多岐に渡るため，専門医師の診断を仰いだ方が良い場合もあるが，ここでは代表的な治療方法を提示する．

1)子宮形態異常

子宮奇形という用語は子供にも遺伝するという誤解を招くため，用いない方が良い．子宮形態異常で手術の対象となるのは，中隔子宮と双角子宮になる．その他の弓状子宮などは，手術療法の有効性に対する十分なエビデンスがない．中隔子宮でも，すべての症例が流産のリスクになるのではなく，中隔の突出度（D）と，内子宮口から中隔頂部への長さ(C)の比(D/C)が0.61以上だと流産のリスクが高くなるという報告があるので[8]，D/C比が0.61未満であれば，経過観察も選択肢の一つになる．最近，3D超音波により子宮内膜が明瞭に描出されるようになったので，スクリーニングとしては子宮卵管造影法（HSG）より簡便で，患者に与える苦痛も少ない．

2)甲状腺機能異常

内科の専門医を紹介し，正常機能となってから妊娠を許可する．妊娠後も引き続き治療が必要である．なお，潜在性甲状腺機能低下症に対して，積極的に治療すべきか否かは，結論が出ていないが，筆者は少量の甲状腺剤の投与を内科医に依頼している．

3)染色体異常

まず検査をする前に十分なカウンセリングを行ない，結果によっては一方の配偶者が不利益を得ないような配慮が必要である．すなわち，夫婦のいずれかに異常があるということを知らせる選択肢があることを，あらかじめ伝えておく．

異常が認められた際，充分な遺伝カウンセリングを行なうとともに，正しい知識を伝えることが大切である．すなわち，流産率は高いが，妊娠を繰り返すことで，80%以上の高い累積生児獲得率が得られること，不均衡型染色体異常を持って生まれる確率は，1%と低い(不均衡型だと，多くは流産するため，流産しないで生まれてくる子供の異常は低い）ことを説明する．均衡型転座の場合は，上記を説明した上で，羊水染色体検査のメリット，デメリットを述べた上で，検査を受けるかどうかを夫婦で選択してもらう．しかし，Robertson型転座では，トリソミーの可能性があるので，羊水検査を勧めた方が良い．

4)抗リン脂質抗体症候群

低用量アスピリン(50～100mg/日)を，できれば妊娠を計画した時点から黄体期に内服してもらい(月経期には中止する)，妊娠した時点から陣痛発来時点まで，ヘパリンカルシウム5,000単位を朝，夕2回(一日10,000単位)皮下注射する．治療開始後に，ヘパリンに対する抗体が産生されると，ヘパリン起因性血小板減少症(HIT)が生じ，血栓症をきたすことがある．HITは投与後2週間以内に生じることが多いので，ヘパリン投与2週間以内に複数回，血小板数を測定し，低下していればヘパリン投与を中止する．自己注射に関するDVDやマニュアルが作製されているので，参考にしていただきたい．またAPTT値は，初期値の1.5倍を越えないように容量を調節する．なお，偶発的抗リン脂質抗体例も，無治療だと流産率が高いことが，経験上知られているため，低用量アスピリン療法を行なうことも一法である．

5)プロテインS 欠乏症

白人には，プロテインS 欠乏症が0.03～0.13%しかいないが，日本人では，1.5%程度がプロテイン

S欠乏症で，しかも不育症では7.2%と高率である．妊娠10週以降の流産・死産の既往歴が1回でもあるプロテインS欠乏症では，ヘパリンと低用量アスピリン群の生児獲得率が78.6%（11/14）であるのに対して，低用量アスピリン群では，7.1%（1/14）の生児獲得率に留まる[9]．したがって，妊娠10週以降の胎児染色体異常のない流産・死産の既往があるプロテインS欠乏症では，ヘパリンと低用量アスピリン療法が勧められる．

これまで，妊娠10週までの流産歴のあるプロテインS欠乏症に対しての治療方針は，欧米での症例数の少なさもあり，確定していなかった．厚生労働研究班の不育症データベースで明らかになったこととして，プロテインS欠乏症に対して，無治療，低用量アスピリン，ヘパリン＋低用量アスピリンでの生児獲得率は，それぞれ10.5%（2/19），71.4%（25/35），76.9%（40/52）であった．以上より，妊娠10週までに流産を繰り返すプロテインS欠乏症には，低用量アスピリン療法を行なうことも一法である[2]．

6）抗PE抗体陽性例

抗PE-IgG，抗PE-IgM高値は，不育症全体で34.3%，リスク因子不明/偶発的流産例の22.6%を占める（**図3**）．抗PE抗体陽性例に対する治療方針は，厚生労働研究班員の間でも意見の一致を見ていない．研究班のデータベースでは，低用量アスピリン療法の有効性が認められたので，低用量アスピリン療法を行なうのも一法である．

7）偶発的流産/リスク因子不明

検査を行なっても，リスク因子が判明しない際，これまでの流産が胎児染色体異常により生じた可能性が高いことを十分に説明し，原則的に無治療で臨んでも良好な治療成績が得られていることを説明すべきである[2]．既往流産回数が2回，もしくは3回の場合，カウンセリングを受け，その他は無治療群での生児獲得率は，それぞれ81.4%（35/43），81.0%（17/21）と高い．これらの率は，胎児染色体異常例の流産を除くと，92.1%（35/38），85.0%（17/20）とさらに高率となる．これらの症例に対するカウンセリングは，極めて有用である．

		検査内容	医療保険の適応	保険点数
一次スクリーニング	子宮形態検査	経腟超音波	○	530点 (800点)
		子宮卵管造影		
		子宮鏡		
	内分泌検査	甲状腺機能	○	T4: 120点 TSH: 115点
		糖尿病検査		
	夫婦染色体検査		○	2600点 分染法加算　400点
	抗リン脂質抗体	抗カルジオリピンβ_2グルコプロテインI複合体抗体	○	230点
		ループスアンチコアグラント	○	290点
		抗CLIgG抗体	○	250点
		抗CLIgM抗体	×（薬事未承認）	自費
選択的検査	抗リン脂質抗体	抗PEIgG抗体（抗フォスファチジルエタノールアミン抗体）	×（薬事未承認）	自費
		抗PEIgM抗体	×（薬事未承認）	自費
	凝固因子検査	第XII因子活性	○	240点
		プロテインS活性もしくは抗原	○	170点
		プロテインC活性もしくは抗原	○	260点
		APTT	○	29点

計　5234点
3割負担だと15,700円

図2　不育症のリスク因子の検査の医療保険適応

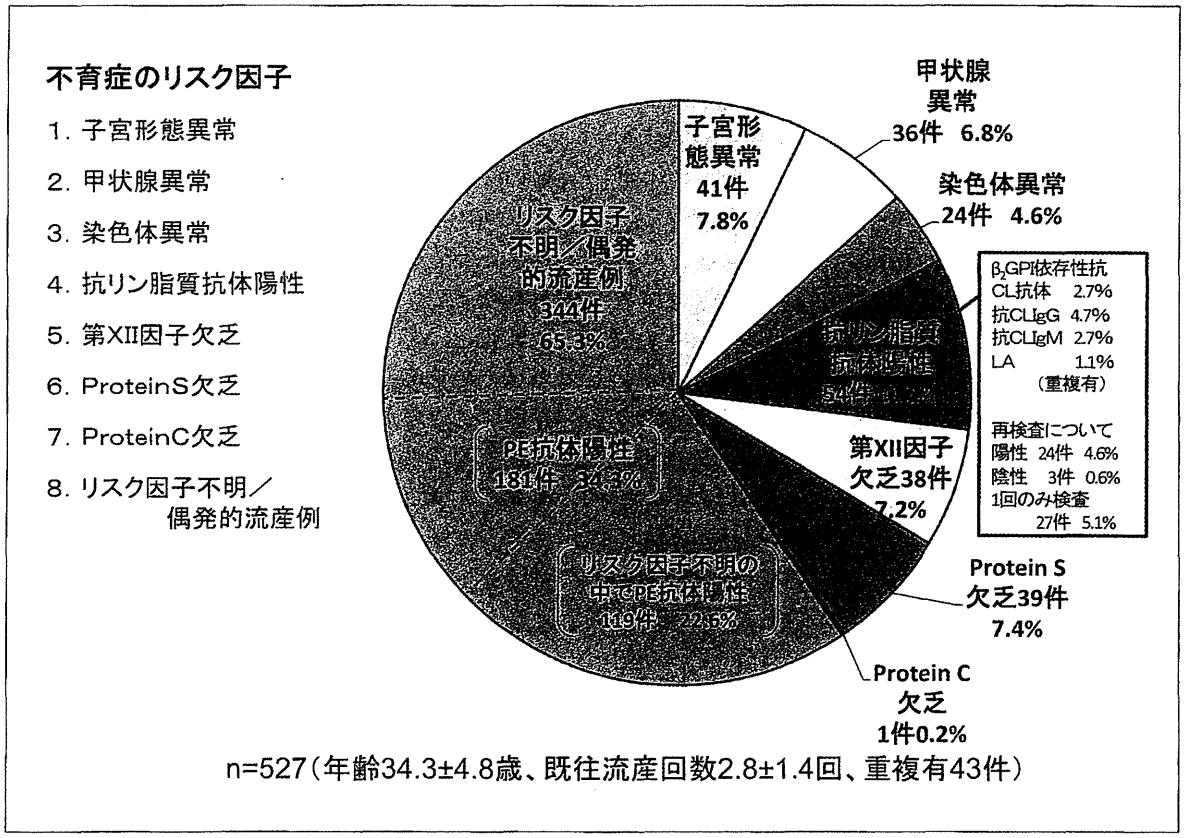


図3 不育症のリスク別頻度

表1 カウンセリングや Tender loving care

表：原因不明不育症例に対する Tender loving care の有用性

対象	成功率		報告者
	Tender loving care	No Treatment	
Unknown etiology（この頃はまだ APS の概念ない．子宮形態異常，染色体異常，内分泌異常は除かれている．）	86% (32/37)	33% (8/24)	Stray-Pedersen *et al.* AJOG 148:140-146, 1984.
Unexplained recurrent pregnancy loss	73.8% (118/160)（妊娠初期から来院）	48.8% (20/41)（妊娠初期に受診せず）	Clifford *et al.* Hum. Reprod. 12:387389, 1997.
厚労研究データ	75.9% (41/54)	39.6% (19/48)	

Tender loving care は有効

TLC: カウンセリングの他，リスクを十分にスクリーニングして説明すること，治療方針を明確にすること，家族や友人が話を聞いてあげること，職場や近所で気を使わなくてよい配慮，妊娠後に超音波で胎児の状態を観察することも含まれます．

IV. カウンセリングや Tender loving care の有用性

表1に示すように，カウンセリングや優しく労りのある態度で接するTender loving careは，生児獲得率を高める[10,11,12]．妊娠する前に十分な時間を取って，カウンセリングをしたり，夫婦でじっくり話を聞いてあげることも重要で，患者同士のピアサポートも有益である．なかなか，外来では時間を取れないため，各地方自治体に開設された不育症相談窓口を紹介しても良い．また，妊娠してからのTender loving careは，極めて重要となる．なお，これらのマニュアルは，インターネットで入手できる[12]．また，総説を参考にしていただきたい[13]．また，カウンセリングは不妊治療における成功率を向上させるので，今後は保険診療で可能なように働きかけていく必要がある．

V. 不妊症に対する低用量アスピリン，ヘパリン療法の有効性

現在のところ，着床不全や生化学的妊娠を繰り返す症例に対して，低用量アスピリン療法やヘパリン療法を行なっても，その有益性は証明されていない．しかし，これらの症例は確実に存在し，臨床家として，何とかしてあげたいと正直思うところはある．今後，不妊症と不育症の境界領域にあるこれらの症例に対して，どのように対応すべきかを，真剣に議論し，解決策を見出していく必要がある．

VI. 不妊治療を行っても臨床的流産を繰り返す症例

不妊治療を行ない，良好胚を移植しても，臨床的流産を繰り返す症例は「不育症」と「不妊症」を併せ持つ症例として認識すべきである．不育症のスクリーニングを行なった上で，不妊クリニックと不育クリニックとのコラボレーションが必要となる．

おわりに

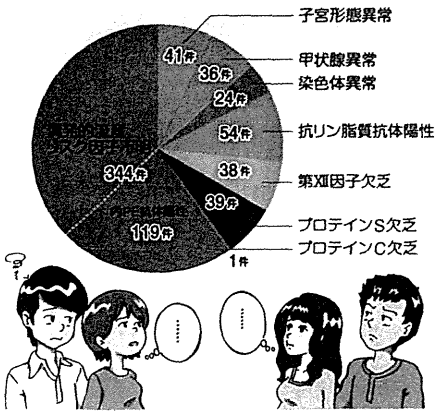
不妊症治療を専門に行なっている医療関係者が集まるIVF学会で，不育症のことをお話しする機会を得たことは，筆者にとり大きな喜びである．不育症のことが，ようやく注目されるようになってきたので，今後はエビデンスに基づいた検査や治療が行なわれ，従来，軽視されていたカウンセリング療法を行なって，不育症で悩んでおられる方々が無事出産されることを心から願っている．

参考文献

1) 厚生労働科学研究費補助金（成育疾患克服等次世代育成基盤研究事業）「不育症治療に関する再評価と新たなる治療法の開発に関する研究」．平成20～22年度総合研究報告書，2011．研究代表者　齋藤　滋．
2) 厚生労働科学研究費補助金（成育疾患克服等次世代育成基盤研究事業）「不育症治療に関する再評価と新たなる治療法の開発に関する研究班を基にした不育症管理に関する提言」，2011．研究代表者　齋藤　滋．
3) ACOG practice bulletin: management of recurrent early pregnancy loss. No. 24. Washinton, DC : American College of Obstetricians and Gynecologists, February 2001.
4) Birmingham, AL.: Patient's fact sheet: recurrent pregnancy loss. American Society for Reproductive Medicine, 2008.
5) Zinaman, MJ., Clegg, ED., Brown, CC., O'Connor, J., Selevan, SG.: Estimates of human fertility and pregnancy loss. Fertil. Steril., 65 : 503-509, 1996.
6) Branch, DW., Gibson, M., Silver, RM.: Recurrent miscarriage. N Engl. J. Med., 363 : 1740-1747, 2010.
7) Sugiura-Ogasawara, M., Suzuki, S., Ozaki, Y., Katano, K., Suzumori, N., Kitaori, T.: Frequency of recurrent spontaneous abortion and its influence on further marital relationship and illness: the Okazaki Cohort Study in Japan. J. Obstet. Gynaecol. Res., 39 : 126-131, 2013.
8) Sugiura-Ogasawara, M., Ozaki, Y., Kitaori, T., Kumagai, K., Suzuki, S.: Midline uterine defect size is correlated with miscarriage of euploid embryos in recurrent cases. Fertil. Steril., 93 : 1983-1988, 2010.
9) Gris, JC., Mercier, E., Quéré, I., Lavigne-Lissalde, G., Cochery-Nouvellon, E., Hoffet, M., Ripart-Neveu, S., Tailland, ML., Dauzat, M., Marès, P.: Low-molecular-weight heparin versus low-dose aspirin in women with one fetal loss and a constitutional thrombophilic disorder. Blood., 103 : 3695-3699, 2004.
10) Stray-Pedersen, B., Stray-Pedersen, S.: Etiologic factors and subsequent reproductive performance in 195 couples with a prior history of habitual abortion. Am. J. Obstet. Gynecol., 148 : 140-146, 1984.
11) Clifford, K., Rai, R., Regan, L.: Future pregnancy outcome in unexplained recurrent first trimester miscarriage. Hum. Reprod., 12 : 387-389, 1997.
12) 厚生労働科学研究費補助金（成育疾患克服等次世代育成基盤研究事業）「地域における周産期医療システムの充実と医療資源の適正配置に関する研究」：反復・習慣流産（いわゆる「不育症」）の相談対応マニュアル．2011．http://fuiku.jp
13) 齋藤　滋：「反復・習慣流産（いわゆる『不育症』）の相談対応マニュアル」について．助産雑誌，66 : 845-848, 2012.

図版作成／藤田としお

■ 不育症のリスク因子別頻度



平成22年度に厚生労働省研究班が調査したデータ(「Fuiku-Labo」より)。リスク因子によって、投薬や手術など対処の方法は異なる

不育症になって大変だったこと

(不育症そだってねっと調べ)

- 周りに経験を共有したり、理解してくれる人がいない
- 「また流産するのでは?」と、妊娠するのが怖くなる
- 専門の治療をしてくれる病院を、自分で探すのが大変
- 人の妊娠や出産を羨んでしまい、自己嫌悪に陥る
- 流産・死産をしたときの病院側の対応で嫌な思いをした

体にも心にも負担の多い不育症。正しい理解のもとに、適切な対応をとりたいものだ

健康

年間3万人が発症していると推定

男性も知っておきたい!「不育症」の基礎知識

妊娠はするものの、流産や死産を繰り返す「不育症」。妊娠自体が叶わない不妊症に比べると認知度は低いが、2011年に厚生労働省研究班が算出した統計では、年間でおよそ3万人もの人が発症していると推測されている。血液凝固の異常や子宮の形状、内分泌に問題があったり、夫婦どちらかの染色体に異常があったりと、原因は様々。夫の理解がなくひとりで悩んだり、知識がないために治療を受ける機会に恵まれず、妊娠のたびに悲しい思いをする女性が多くいるという。一体、不育症とはどんな症状なのか。厚生労働省研究班の代表・齋藤滋先生に伺った。

「はっきりした定義はまだ定められていませんが、2回以上連続した流産や死産があれば、一般的に不育症と診断します。妊娠22週以降の死産や、生後1週間の新生児死亡も含め、広い意味で用いられる呼称です。第一子を問題なく出産した後、2人目以降の妊娠で見つかることもあります」

血液や子宮などの検査でリスク因子を調べれば、次回の妊娠に向けて適切な対策ができる。ただし、特別のリスクがなく、偶発的な流産を繰り返している場合もあるのだとか。また、治療費は病院によって異なり、検査の一部は保険の適用外なので費用がかさむことも…。ただし、保険で認められる薬が増えるなど、経済的な負担はやや軽減されつつある。

認知度アップのために活動する「不育症そだってねっと」の代表・工藤智子さんによると「本人や家族にとっては、精神的なプレッシャーもかなりのもの。周りの理解不足や『妊娠すれば当たり前に出産できる』という雰囲気に傷つく女性もいます」とのこと。

現状で予防策はないが、心当たりがあるなら早めの検査が肝心。正しい知識を持ち、いざというときは病院を頼りながらケアすることが、よい夫の務めですよ!

(菅原さくら／アバンギャルド)