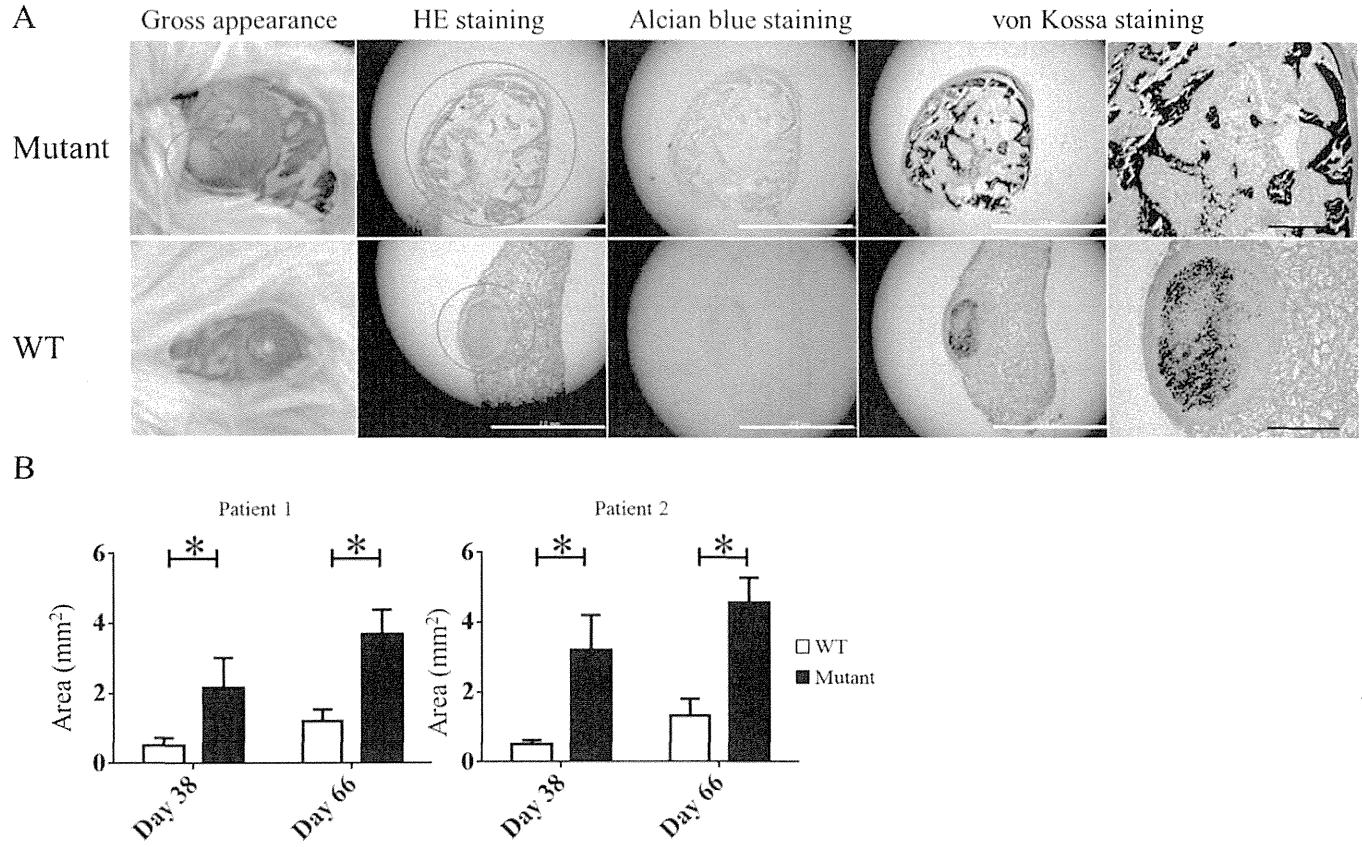

Figure 3. In vivo maturation of 3-D cell pellets derived from mutant or wild-type iPSCs from patients with neonatal-onset multisystem inflammatory disease. **A,** Images of 3-D cell pellets derived from mutant or wild-type iPSCs following transplantation into immunodeficient mice. Gross appearance, hematoxylin and eosin (H&E) staining, Alcian blue staining, von Kossa staining, and higher-magnification images of von Kossa staining are shown. Red circles indicate bone or cartilage pellets in gel form. White bars = 2.0 mm; black bars = 0.2 mm. Results shown were obtained using iPSCs from patient 1; similar results were obtained using iPSCs from patient 2. **B,** Quantitative analysis of the size of pellets when they were transplanted (day 38) and harvested (day 66). Bars show the mean ± SEM of 3 independent clones, from which duplicate measurements were obtained. Data are representative of 3 independent experiments. * = $P < 0.05$. See Figure 1 for other definitions.

with abnormal ossification. The in vitro differentiation system did not induce chondrocyte calcification, probably due to the lack of cell components or factors necessary for the final differentiation step. Therefore, we used an in vivo differentiation system as a model for endochondral ossification, in which immature 3-D pellet samples were transplanted into NOG mice. The transplanted cartilage mass was vascularized in vivo (Figure 3A). Mutant pellets were larger than wild-type pellets, both at transplantation and harvesting, and this size difference increased during in vivo differentiation (Figure 3B). Following von Kossa staining, which detects calcium deposits, calcification was detected in both wild-type and mutant pellets (Figure 3A). Interestingly, Alcian blue staining revealed that mutant pellets contained more residual cartilage components than wild-type pellets. In addition, calcified areas were scattered throughout mutant pellets, whereas they were localized in specific regions and were clearly separated from Alcian blue–positive areas in wild-type pellets. Taken together, these data indicate that in our in vivo model, chondrocyte tissue differentiated from mutant iPSCs grows larger and exhibits disorganized ossification compared to chondrocyte tissue differentiated from wild-type iPSCs.

The enhanced chondrogenesis of mutant iPSCs is independent of the NLRP3 inflammasome. The inflammatory phenotype of NOMID is caused by gain-of-function NLRP3 mutations, leading to activation of the NLRP3 inflammasome (27). Therefore, we examined the involvement of the NLRP3 inflammasome in the formation of cartilaginous masses. First, we analyzed the

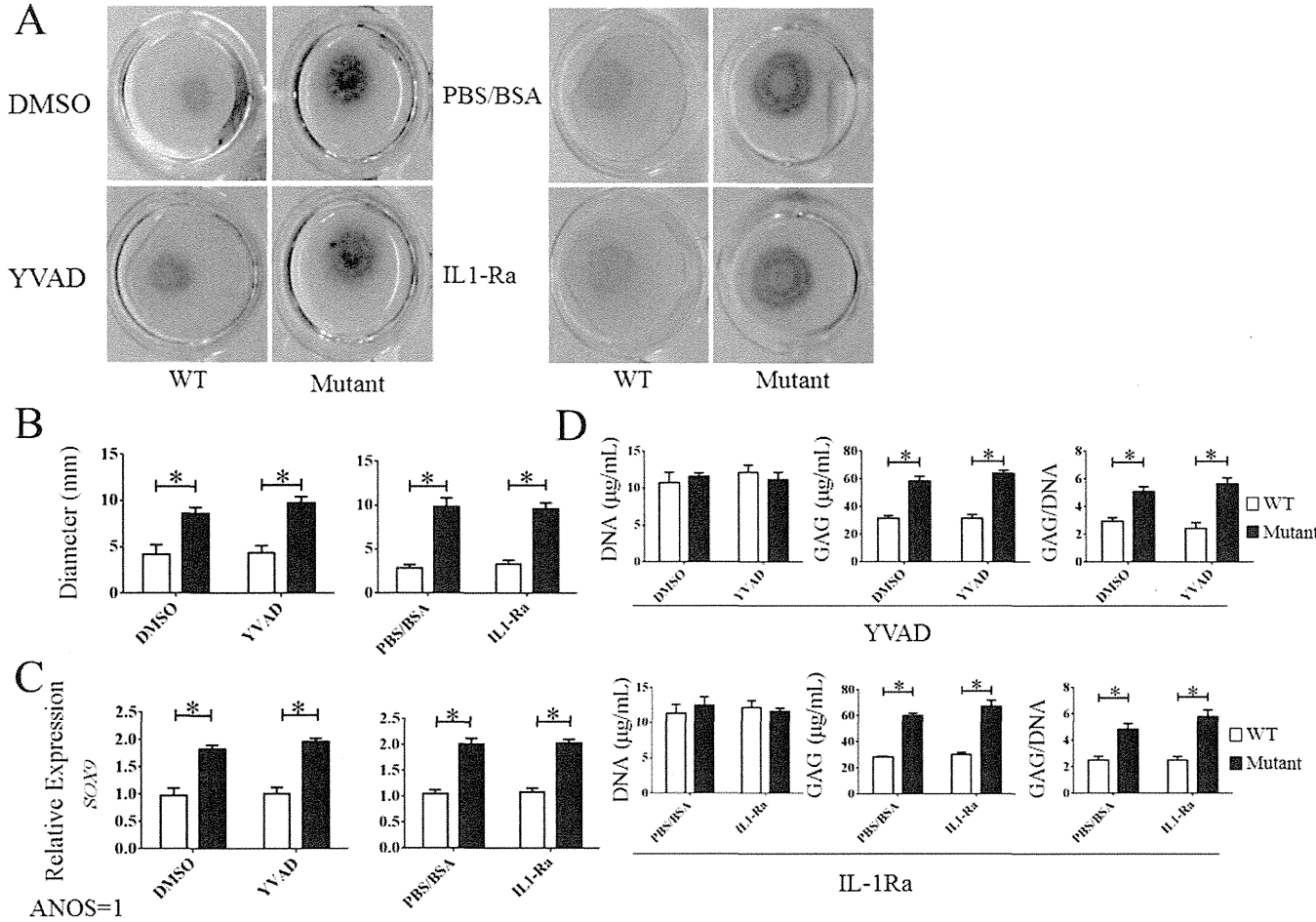

Figure 4. NLRP3 inflammasome–independent enhanced chondrogenesis of mutant iPSCs from patients with neonatal-onset multisystem inflammatory disease. Wild-type and mutant iPSCs were differentiated into chondrocytes in 2-D micromass cultures, and the caspase 1 inhibitor Ac-YVAD-CHO (YVAD; 10 μM) or human recombinant interleukin-1 receptor antagonist (IL-1Ra; 1 μg/ml) was added to the cultures. Control cultures were incubated with DMSO or phosphate buffered saline (PBS) containing bovine serum albumin (BSA). **A**, Representative 2-D micromass cultures treated with YVAD or DMSO as control (left) or with IL-1Ra or PBS/BSA as control (right). **B**, Diameter of the micromass. **C**, *SOX9* expression in chondrocytes derived from wild-type and mutant iPSCs and treated with YVAD or IL-1Ra. Expression levels are shown relative to those in ANOS cells (set at 1). **D**, DNA concentration, glycosaminoglycan (GAG) concentration, and the ratio of GAG concentration to DNA concentration in chondrocytes derived from wild-type or mutant iPSCs and treated with YVAD or IL-1Ra. Bars show the mean ± SEM of 3 independent clones from which triplicate measurements were obtained. Data are representative of 3 independent experiments. Data shown were obtained using iPSCs from patient 1; similar results were obtained using iPSCs from patient 2. $* = P < 0.05$. See Figure 1 for other definitions.

expression of the NLRP3 inflammasome components in 2-D cartilage samples. Mutant and wild-type cartilage samples both expressed NLRP3, but did not express ASC, pro–caspase 1, or pro–IL-1β by Western blotting (data not shown). This suggests that the formation of large cartilaginous masses by mutant chondrocytes occurs independently of the NLRP3 inflammasome.

To confirm that chondrogenesis of mutant iPSCs is enhanced independently of the NLRP3 inflammasome, we used inhibitors of components of the NLRP3 inflammasome, namely, Ac-YVAD-CHO, which inhibits caspase 1, and recombinant human IL-1 receptor antagonist (IL-1Ra), which antagonizes IL-1. Neither Ac-YVAD-CHO (10 μM) treatment nor recombinant human IL-1Ra (1 μg/ml) treatment during 2-D micromass culture prevented the formation of large cartilaginous masses (Figures 4A and B), *SOX9* upregulation (Figure 4C), or overproduction of GAG (Figure 4D) by chondrocytes derived from mutant iPSCs. The same was true when samples were treated

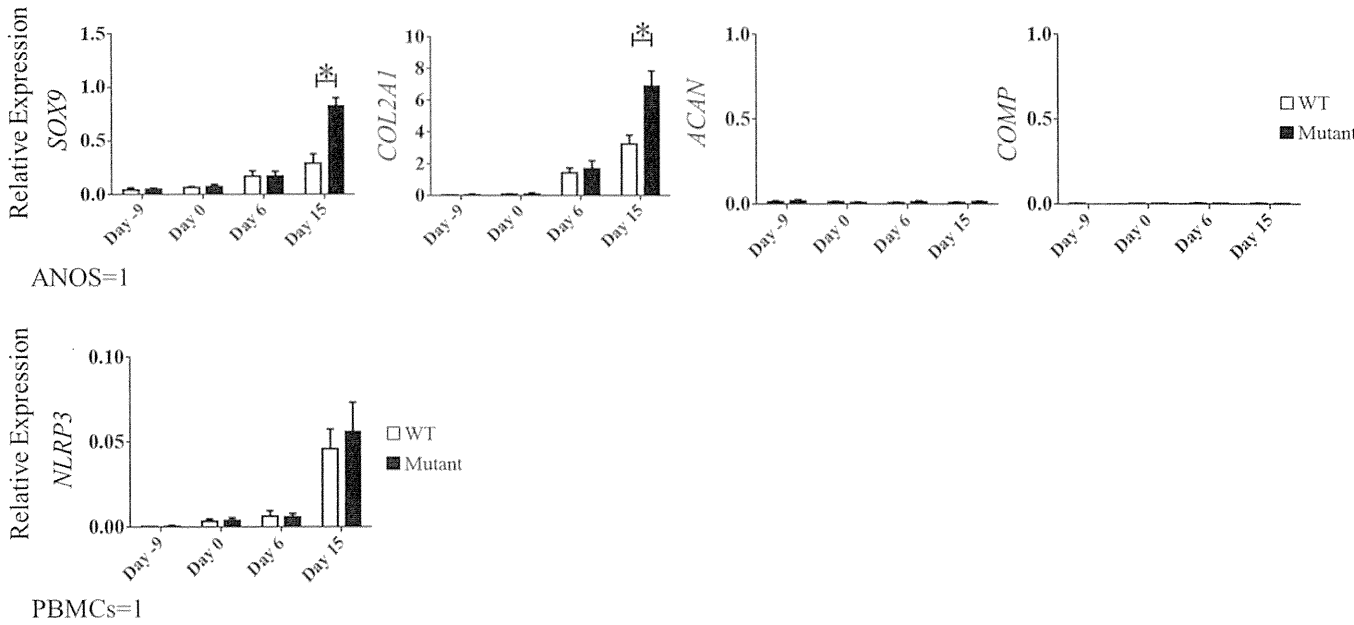

Figure 5. Up-regulation of the expression of *SOX9* and *COL2A1* in chondrocytes with mutated *NLRP3* during the chondroprogenitor cell stage. Expression of *SOX9*, *COL2A1*, *ACAN*, *COMP*, and *NLRP3* in each clone was measured in triplicate from day −9 to day 15 of chondrocyte differentiation of iPSCs from patients with neonatal-onset multisystem inflammatory disease with wild-type or mutant NLRP3. Expression levels of *SOX9*, *COL2A1*, *ACAN*, and *COMP* are shown relative to those in ANOS cells (set at 1), and the expression level of *NLRP3* is shown relative to that in peripheral blood mononuclear cells (PBMCs; set at 1). Bars show the mean ± SEM of 3 independent clones. Data are representative of 3 independent experiments with consistent results and were obtained using iPSCs from patient 1; similar results were obtained using iPSCs from patient 2. * = $P < 0.05$. See Figure 1 for other definitions.

with higher concentrations of these inhibitors (up to 1,000-fold higher) (data not shown). Taken together, these data strongly indicate that the enhanced chondrogenesis of mutant iPSCs is independent of caspase 1 and IL-1, and thus the NLRP3 inflammasome.

Correlation of the up-regulation of NLRP3 with the up-regulation of SOX9 in chondroprogenitor cells. To dissect the mechanism underlying the enhanced chondrogenesis of mutant iPSCs, we analyzed the time course of chondrocyte-specific gene expression in chondroprogenitor cells (Figure 5). Expression of *SOX9*, *COL2A1*, and *NLRP3* started to be up-regulated in chondroprogenitor cells on day 6. Importantly, on day 15, *SOX9* and *COL2A1* were up-regulated more in mutant chondroprogenitor cells than in wild-type chondroprogenitor cells, whereas *NLRP3* was up-regulated similarly in both types of cells (Figure 5). In contrast, at this time point, the other chondrocyte-specific markers *ACAN* and *COMP* were not expressed in either type of cell (Figure 5). Thus, differential up-regulation of *SOX9* in chondroprogenitor cells correlated with the up-regulation of *NLRP3* and preceded the up-regulation of *COMP* and *ACAN*.

Critical role of the CREB/ATF-binding site of the SOX9 promoter in mutated NLRP3–dependent enhancement of SOX9 expression. Next, we focused on *SOX9* because it was up-regulated together with *NLRP3*, and this preceded the up-regulation of other chondrocyte-specific markers. We analyzed the activity of the human *SOX9* promoter in chondroprogenitor cells in which the level of *SOX9* mRNA was increased. We created a luciferase reporter construct containing the 5′-UTR of human *SOX9*, which encompasses −927 to +84 bp of the transcription start site. This fragment has basal promoter activity and putative binding sites for 5 transcription factors, namely, NF-AT, activator protein 1 (AP-1), NF-κB, Sp1, and CREB/ATF (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38912/abstract). This fragment showed no promoter activity in the monocytic cell line THP-1 or the erythroleukemic cell line K562, which do not express endogenous *SOX9* (data not shown). Importantly, human *SOX9* promoter activity was higher in mutant chondroprogenitor cells than in wild-type chondroprogenitor cells (Figure 6A). To identify the element of the human *SOX9*

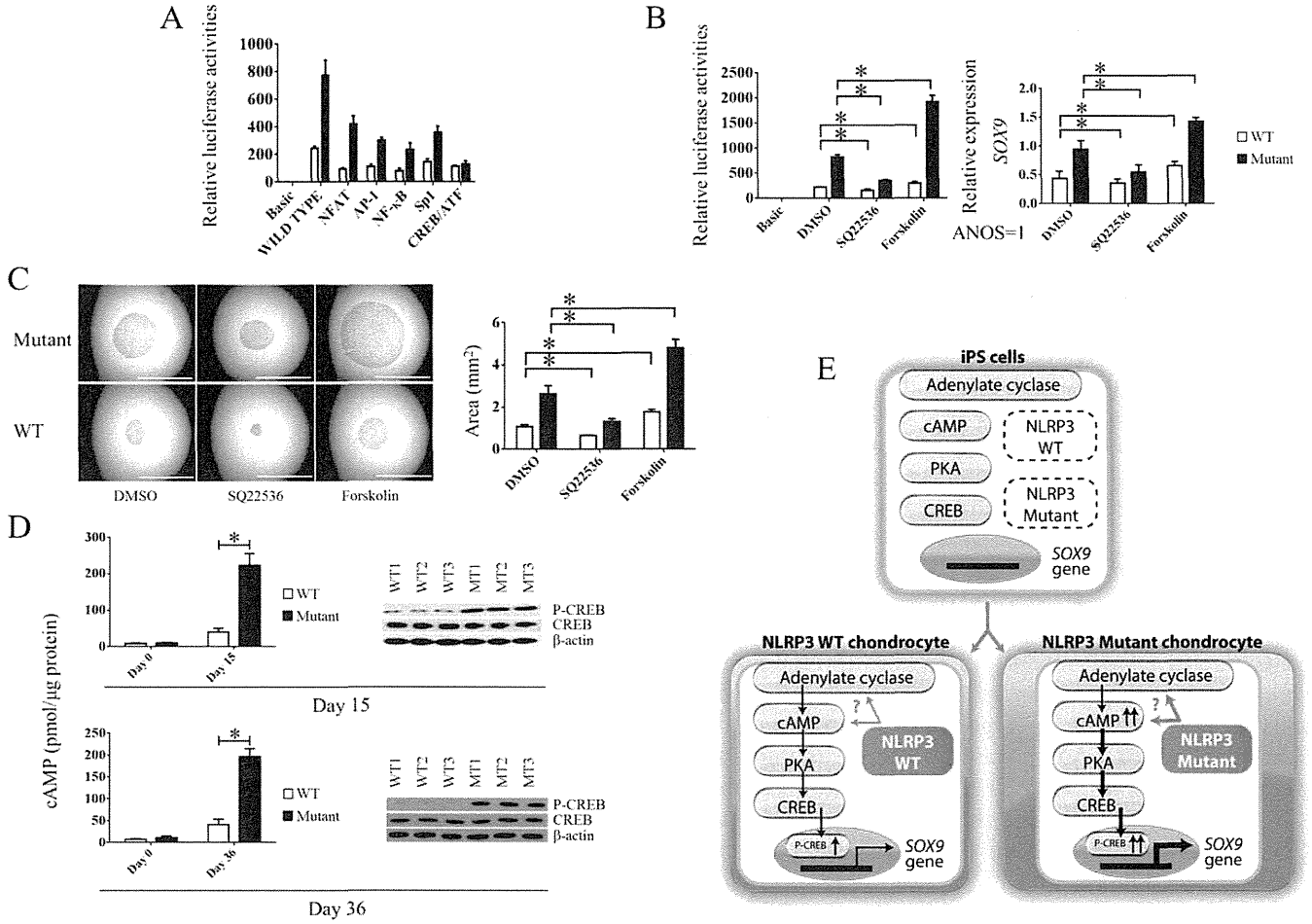

Figure 6. *SOX9* up-regulation in chondrocytes derived from iPSCs from patients with neonatal-onset multisystem inflammatory disease with mutant NLRP3 is dependent on the cAMP/protein kinase A (PKA)/CREB pathway. **A,** *SOX9* promoter activity in wild-type and mutant chondroprogenitor cells after the introduction of mutations into its transcription factor binding sites. **B,** *SOX9* promoter activity and expression in wild-type and mutant chondroprogenitor cells treated with SQ22536 or forskolin. **C,** Effects of SQ22536 and forskolin on 3-D pellets of mutant and wild-type cells. Both reagents were used at a concentration of 10 μM. Bars = 2.0 mm. **D,** Increased activity of the cAMP/PKA/CREB pathway in mutant chondroprogenitor cells compared to wild-type chondroprogenitor cells, as demonstrated by cAMP concentration in wild-type and mutant iPSCs (day 0) and chondroprogenitor cells (day 15 and day 36), and Western blot analysis of phosphorylated CREB in wild-type (WT1–3) and mutant (MT1–3) chondroprogenitor cells. **E,** Schematic diagram summarizing the molecular mechanism elucidated in this study. Bars in **A–D** show the mean ± SEM of 3 independent clones from which triplicate (**A, B,** and **D**) or duplicate (**C**) measurements were obtained. Data are representative of 2 independent experiments with consistent results and were obtained using iPSCs from patient 1; similar results were obtained using iPSCs from patient 2. * = $P < 0.05$. See Figure 1 for other definitions.

promoter region that responds in a mutated NLRP3–dependent manner, we performed site-directed mutagenesis of the sites of this promoter that bind the transcription factors NF-AT, AP-1, NF-κB, Sp1, and CREB/ATF (Supplementary Figure 1). Among the reporters with these mutations, the reporter that harbored a mutation in the CREB/ATF-binding site showed the least up-regulation of *SOX9* promoter activity in mutant cells (Figure 6A and Supplementary Figure 1). Thus, we speculate that the CREB/ATF-binding site is critical for activation of the human *SOX9* promoter in a mutated NLRP3–dependent manner.

Critical role of the cAMP/PKA/CREB pathway in SOX9 up-regulation caused by mutated NLRP3. To further explore the association between mutated NLRP3 and the cAMP/PKA/CREB pathway, we examined the effect of an adenylate cyclase activator and inhibitor (forskolin and SQ22536, respectively) on the activity of the human *SOX9* promoter and *SOX9* mRNA expression (Figure 6B). Among mutant chondroprogenitor

cells, *SOX9* promoter activity was 2.3-fold higher in forskolin-treated cells than in vehicle-treated cells, whereas *SOX9* promoter activity was 2-fold lower in SQ22536-treated cells than in vehicle-treated cells (Figure 6B). Similar effects were observed in wild-type chondroprogenitor cells, although they were less pronounced. These data correlated well with the effects of forskolin and SQ22536 on *SOX9* mRNA expression. We also examined the effects of forskolin and SQ22536 on 3-D chondrocyte pellet formation (Figure 6C). Compared to pellets of vehicle-treated mutant cells, pellets of mutant cells treated with forskolin and SQ22536 were 2.0-fold larger and 2.1-fold smaller, respectively. Similar effects were observed in wild-type cells, although they were less pronounced. These data clearly indicate that up-regulation of *SOX9* following activation of adenylate cyclase is involved in the enhanced chondrogenesis of mutant iPSCs.

We next measured the cAMP concentration to demonstrate that the activity of adenylate cyclase is increased in mutant chondroprogenitor cells. The concentration of cAMP was 4-fold higher in mutant chondroprogenitor cells than in wild-type chondroprogenitor cells on days 15 and 36 (Figure 6D). By contrast, the concentration of cAMP was similar in mutant and wild-type iPSCs, in which NLRP3 expression was low.

Finally, we examined the level of phosphorylated CREB in chondroprogenitor cells. CREB is phosphorylated by cAMP-activated PKA. According to Western blot analysis, the level of phosphorylated CREB was higher in mutant chondroprogenitor cells than in wild-type chondroprogenitor cells on days 15 and 36 (Figure 6D). Taken together, these data indicate that the cAMP/PKA/CREB pathway plays an important role in the up-regulation of *SOX9*, and therefore enhanced chondrogenesis, in chondroprogenitor cells with mutant NLRP3 (Figure 6E).

DISCUSSION

Disease-specific iPSCs have been used extensively to investigate the pathogenesis of diseases and to discover novel drugs. This approach is particularly useful to study rare diseases because tissues are often difficult to obtain from patients with such diseases. In this study, we used disease-specific iPSCs to study NOMID. Using this approach, we produced chondrocyte tissues with mutant and wild-type NLRP3, and revealed a previously unidentified connection between the inflammasome-associated molecule NLRP3 and the master regulator of chondrocyte differentiation SOX9.

SOX9 was up-regulated during the differentiation of iPSCs into chondrocytes, and this was particularly pronounced in mutant iPSCs. During cartilage development, SOX9 is highly expressed in immature chondrocytes and is required for the condensation and differentiation of mesenchymal cells. During the early stages of chondrogenesis, SOX9 activates the transcription of many cartilage-specific ECM genes, including *COL2A1*, *ACAN*, and *COMP*, by directly interacting with SOX5 and SOX6 (28,29). Overexpression of *SOX9* in chondrocytes using a recombinant adeno-associated virus significantly increases the synthesis of major ECM components in chondrocytes, without affecting their proliferation, in vivo and in vitro (30,31). In addition, retroviral transduction of SOX9 increases ECM production in human chondrocytes in vitro (32). These data correlate well with our observation that SOX9 overexpression driven by mutated NLRP3 caused overproduction of ECM, but did not increase chondrocyte proliferation.

It remains to be determined how enhanced expression of SOX9 in chondrocytes leads to epiphyseal overgrowth in NOMID patients. Conditional transgenic mice have been used to show that overexpression of *SOX9* in *COL2A1*-positive cells inhibits terminal differentiation of hypertrophic chondrocytes and endochondral bone formation (29). Although we have not directly confirmed the expression level of SOX9 in samples derived from NOMID patients, this previous study might help to link the findings of the present study with the clinical phenotype of NOMID patients.

We identified the cAMP/PKA/CREB pathway as being critical for the up-regulation of *SOX9* mRNA in a mutant NLRP3–dependent manner. cAMP is an intracellular second messenger that is involved in a variety of cellular processes (33). cAMP/PKA/CREB signaling is crucial in chondrogenesis, and synergism between cAMP and SOX9 is particularly important (34–36). Cotransfection of CREB binding protein (CBP) and p300 increases SOX9 activity (35). PKA phosphorylates SOX9 and thereby increases SOX9 activity, which results in the up-regulation of the *COL2A1* promoter through the interaction between CBP and SOX9 (34). In addition, the PKA inhibitor H89 blocks chondrogenesis in the chick limb bud (36). These data support the idea that cAMP/PKA/CREB signaling up-regulates SOX9 to enhance chondrogenesis.

Using stromal cells established from a tumor-like lesion in a NOMID patient, Almeida et al (37) demonstrated that activation of the cAMP/PKA/CREB pathway leads to caspase 1 activation, release of IL-1β, and

consequently the proliferation of bone stromal cells. This suggests that bone lesions in NOMID are caused in an NLRP3 inflammasome–dependent manner. One explanation for the discrepancy between their data and ours is that no disease-causing NLRP3 mutation was identified in the patient in that previous study; therefore, an unknown genetic alteration may have caused the NOMID phenotype. Another explanation is that different cell types were analyzed in the two studies. The previous study analyzed bone stromal cells established from a tumor-like lesion that might have been a heterogeneous population, while we focused on a single cell type, namely, chondrocytes.

The lack of environmental factors and interactions with other cell populations in our model might have eliminated some contributions of the NLRP3 inflammasome and IL-1β pathway that occur in NOMID patients. Furthermore, our observations relied on an artificial differentiation system in which iPSCs were first differentiated into cells of neural crest character and then into chondrocytes by culture in the presence of various exogenous factors. Abnormal epiphyseal growth is specifically observed around the knee joints of NOMID patients; therefore, additional events might be required to trigger abnormal chondrocyte proliferation in vivo. It is also possible that specific factors produced by surrounding cells in unaffected joints prevent mutant chondrocytes from manifesting their phenotype. Further analyses of patients or patient-derived samples would provide a better understanding of the pathophysiology of arthropathy in NOMID.

The interaction between cAMP and NLRP3 has been studied in monocyte/macrophages, in which the NLRP3 inflammasome is activated following binding of extracellular Ca^{2+} to Ca^{2+}-sensing receptors (CaSRs) (38,39). One study reported that an increase in extracellular Ca^{2+} is detected by CaSRs, which leads to phospholipase C activation and subsequently the release of Ca^{2+} from the endoplasmic reticulum and down-regulation of cAMP. cAMP binds directly to NLRP3 and inhibits assembly of the NLRP3 inflammasome. Therefore, this decrease in the level of intracellular cAMP relieves this inhibition and thereby induces activation of the NLRP3 inflammasome (38). On the other hand, another study reported that an increase in the extracellular Ca^{2+} concentration induces an increase in the intracellular Ca^{2+} concentration, thereby leading to activation of the NLRP3 inflammasome, and this mechanism requires the CaSRs GPRC6A and CaSR, but not the down-regulation of cAMP (39). Thus, the effects of cAMP on the NLRP3 inflammasome in monocyte/macrophages remain a subject of controversy.

In the chondrocyte differentiation system used in the present study, mutated NLRP3 caused SOX9 overexpression via the cAMP/PKA/CREB pathway, which is at odds with the relationship between cAMP and activation of the NLRP3 inflammasome in monocyte/macrophages. This discrepancy might be explained by the absence of other NLRP3 inflammasome components, such as ASC and procaspase 1, in the chondrocytes generated in the present study. Further analysis is needed to determine why cAMP/PKA/CREB signaling elicits different effects on mutated NLRP3 in chondrocytes and monocyte/macrophages, as well as how intracellular cAMP is up-regulated in chondrocytes derived from mutant iPSCs.

There have been many reports on the differentiation of chondrocytes from embryonic stem cells (ESCs) or iPSCs (40–42). However, previously, it was difficult to differentiate a sufficient number of chondrocytes with a relatively mature phenotype from ESCs or iPSCs, especially human ESCs or iPSCs. We have recently established a cartilage differentiation system in which iPSCs first differentiate into cells of neural crest character and then into chondrocytes, which enabled us to obtain a large number of chondrocytes with the phenotype of growth plate cartilage chondrocytes. An important aspect of the present study is that this differentiation system can generate a large number of chondrocytes that could share functional properties causing the arthropathy observed in NOMID. This system could thereby be used to screen for novel therapeutic agents.

In conclusion, we showed that SOX9 is overexpressed via the cAMP/PKA/CREB signaling pathway in chondrocytes with disease-causing mutations in NLRP3, and this causes overproduction of ECM independently of the NLRP3 inflammasome. We used iPSC technology to elucidate the role of chondrocytes in the pathophysiology of the human disease NOMID.

ACKNOWLEDGMENTS

We are grateful to K. Hayakawa, M. Fukuta, S. Nagata, and M. Hiraga of Kyoto University for technical assistance and to N. Kambe of Chiba University for critical reading of the manuscript.

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. Nishikomori and Toguchida 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. Yokoyama, Ikeya, Tanaka, Nishikomori, Nakayama, Nakahata, Heike, Toguchida.
Acquisition of data. Yokoyama, Umeda, Nodomi, Horigome, Kusaka, Ohara.
Analysis and interpretation of data. Yokoyama, Umeda, Oda, Nodomi, Nasu, Matsumoto, Izawa, Kusaka, Saito, Yasumi, Nishikomori, Ohara.

ADDITIONAL DISCLOSURES

Author Horigome is an employee of Dainippon Sumitomo Pharma.

REFERENCES

1. Kufer TA, Sansonetti PJ. NLR functions beyond pathogen recognition. Nat Immunol 2011;12:121–8.
2. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat Genet 2001;29:301–5.
3. Tanaka N, Izawa K, Saito MK, Sakuma M, Oshima K, Ohara O, et al. High incidence of NLRP3 somatic mosaicism in patients with chronic infantile neurologic, cutaneous, articular syndrome: results of an international multicenter collaborative study. Arthritis Rheum 2011;63:3625–32.
4. Feldmann J, Prieur AM, Quartier P, Berquin P, Certain S, Cortis E, et al. Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes. Am J Hum Genet 2002;71:198–203.
5. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. Nat Rev Immunol 2013;13:397–411.
6. Gattorno M, Martini A. Beyond the NLRP3 inflammasome: autoinflammatory diseases reach adolescence [review]. Arthritis Rheum 2013;65:1137–47.
7. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, et al. NF-κB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol 2009;183:787–91.
8. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, et al. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 2006;440:228–32.
9. Hoffman HM, Rosengren S, Boyle DL, Cho JY, Nayar J, Mueller JL, et al. Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. Lancet 2004;364:1779–85.
10. Hawkins PN, Lachmann HJ, McDermott MF. Interleukin-1-receptor antagonist in the Muckle-Wells syndrome. N Engl J Med 2003;348:2583–4.
11. Lachmann HJ, Kone-Paut I, Kuemmerle-Deschner JB, Leslie KS, Hachulla E, Quartier P, et al. Use of canakinumab in the cryopyrin-associated periodic syndrome. N Engl J Med 2009;360:2416–25.
12. Arostegui JI, Lopez Saldana MD, Pascal M, Clemente D, Aymerich M, Balaguer F, et al. A somatic NLRP3 mutation as a cause of a sporadic case of chronic infantile neurologic, cutaneous, articular syndrome/neonatal-onset multisystem inflammatory disease: novel evidence of the role of low-level mosaicism as the pathophysiologic mechanism underlying Mendelian inherited diseases. Arthritis Rheum 2010;62:1158–66.
13. Hill SC, Namde M, Dwyer A, Poznanski A, Canna S, Goldbach-Mansky R. Arthropathy of neonatal onset multisystem inflammatory disease (NOMID/CINCA). Pediatr Radiol 2007;37:145–52.
14. Mackie EJ, Tatarczuch L, Mirams M. The skeleton: a multifunctional complex organ. The growth plate chondrocyte and endochondral ossification. J Endocrinol 2011;211:109–21.
15. Goldring MB, Tsuchimochi K, Ijiri K. The control of chondrogenesis. J Cell Biochem 2006;97:33–44.
16. Bonar SL, Brydges SD, Mueller JL, McGeough MD, Pena C, Chen D, et al. Constitutively activated NLRP3 inflammasome causes inflammation and abnormal skeletal development in mice. PloS One 2012;7:e35979.
17. Tanaka T, Takahashi K, Yamane M, Tomida S, Nakamura S, Oshima K, et al. Induced pluripotent stem cells from CINCA syndrome patients as a model for dissecting somatic mosaicism and drug discovery. Blood 2012;120:1299–308.
18. Umeda K, Zhao J, Simmons P, Stanley E, Elefanty A, Nakayama N. Human chondrogenic paraxial mesoderm, directed specification and prospective isolation from pluripotent stem cells. Sci Rep 2012;2:455.
19. Nasu A, Ikeya M, Yamamoto T, Watanabe A, Jin Y, Matsumoto Y, et al. Genetically matched human iPS cells reveal that propensity for cartilage and bone differentiation differs with clones, not cell type of origin. PloS One 2013;8:e53771.
20. Sakai H, Okafuji I, Nishikomori R, Abe J, Izawa K, Kambe N, et al. The CD40-CD40L axis and IFN-γ play critical roles in Langhans giant cell formation. Int Immunol 2012;24:5–15.
21. Aoyama T, Okamoto T, Nagayama S, Nishijo K, Ishibe T, Yasura K, et al. Methylation in the core-promoter region of the chondromodulin-I gene determines the cell-specific expression by regulating the binding of transcriptional activator Sp3. J Biol Chem 2004;279:28789–97.
22. Ushita M, Saito T, Ikeda T, Yano F, Higashikawa A, Ogata N, et al. Transcriptional induction of SOX9 by NF-κB family member RelA in chondrogenic cells. Osteoarthritis Cartilage 2009;17:1065–75.
23. Kajita Y, Kato T Jr, Tamaki S, Furu M, Takahashi R, Nagayama S, et al. The transcription factor Sp3 regulates the expression of a metastasis-related marker of sarcoma, actin filament-associated protein 1-like 1 (AFAP1L1). PloS One 2013;8:e49709.
24. Sandelin A, Alkema W, Engstrom P, Wasserman WW, Lenhard B. JASPAR: an open-access database for eukaryotic transcription factor binding profiles. Nucleic Acids Res 2004;32:D91–4.
25. Saito M, Fujisawa A, Nishikomori R, Kambe N, Nakata-Hizume M, Yoshimoto M, et al. Somatic mosaicism of CIAS1 in a patient with chronic infantile neurologic, cutaneous, articular syndrome. Arthritis Rheum 2005;52:3579–85.
26. Saito M, Nishikomori R, Kambe N, Fujisawa A, Tanizaki H, Takeichi K, et al. Disease-associated CIAS1 mutations induce monocyte death, revealing low-level mosaicism in mutation-negative cryopyrin-associated periodic syndrome patients. Blood 2008;111:2132–41.
27. Aksentijevich I, Nowak M, Mallah M, Chae JJ, Watford WT, Hofmann SR, et al. De novo CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): a new member of the expanding family of pyrin-associated autoinflammatory diseases. Arthritis Rheum 2002;46:3340–8.
28. Akiyama H, Lefebvre V. Unraveling the transcriptional regulatory machinery in chondrogenesis. J Bone Miner Metab 2011;29:390–5.
29. Kim Y, Murao H, Yamamoto K, Deng JM, Behringer RR, Nakamura T, et al. Generation of transgenic mice for conditional overexpression of Sox9. J Bone Miner Metab 2011;29:123–9.
30. Cucchiarini M, Orth P, Madry H. Direct rAAV SOX9 administration for durable articular cartilage repair with delayed terminal differentiation and hypertrophy in vivo. J Mol Med (Berlin) 2013;91:625–36.
31. Cucchiarini M, Thurn T, Weimer A, Kohn D, Terwilliger EF, Madry H. Restoration of the extracellular matrix in human

osteoarthritic articular cartilage by overexpression of the transcription factor SOX9. Arthritis Rheum 2007;56:158–67.
32. Tew SR, Li Y, Pothacharoen P, Tweats LM, Hawkins RE, Hardingham TE. Retroviral transduction with SOX9 enhances re-expression of the chondrocyte phenotype in passaged osteoarthritic human articular chondrocytes. Osteoarthritis Cartilage 2005;13:80–9.
33. Carroll SH, Ravid K. Differentiation of mesenchymal stem cells to osteoblasts and chondrocytes: a focus on adenosine receptors. Expert Rev Mol Med 2013;15:e1.
34. Huang W, Zhou X, Lefebvre V, de Crombrugghe B. Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer. Mol Cell Biol 2000;20:4149–58.
35. Tsuda M, Takahashi S, Takahashi Y, Asahara H. Transcriptional co-activators CREB-binding protein and p300 regulate chondrocyte-specific gene expression via association with Sox9. J Biol Chem 2003;278:27224–9.
36. Yoon YM, Oh CD, Kang SS, Chun JS. Protein kinase A regulates chondrogenesis of mesenchymal cells at the post-precartilage condensation stage via protein kinase C-α signaling. J Bone Miner Res 2000;15:2197–205.
37. Almeida MQ, Tsang KM, Cheadle C, Watkins T, Grivel JC, Nesterova M, et al. Protein kinase A regulates caspase-1 via Ets-1 in bone stromal cell-derived lesions: a link between cyclic AMP and pro-inflammatory pathways in osteoblast progenitors. Hum Mol Genet 2011;20:165–75.
38. Lee GS, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, Sacks DB, et al. The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca^{2+} and cAMP. Nature 2012;492:123–7.
39. Rossol M, Pierer M, Raulien N, Quandt D, Meusch U, Rothe K, et al. Extracellular Ca^{2+} is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. Nat Commun 2012;3:1329.
40. Barberi T, Willis LM, Socci ND, Studer L. Derivation of multipotent mesenchymal precursors from human embryonic stem cells. PLoS Med 2005;2:e161.
41. Kawaguchi J, Mee PJ, Smith AG. Osteogenic and chondrogenic differentiation of embryonic stem cells in response to specific growth factors. Bone 2005;36:758–69.
42. Zur Nieden NI, Kempka G, Rancourt DE, Ahr HJ. Induction of chondro-, osteo- and adipogenesis in embryonic stem cells by bone morphogenetic protein-2: effect of cofactors on differentiating lineages. BMC Dev Biol 2005;5:1.

RHEUMATOLOGY

Rheumatology 2014;53:1783–1787
doi:10.1093/rheumatology/keu180
Advance Access publication 11 May 2014

Concise report

Early progression of atherosclerosis in children with chronic infantile neurological cutaneous and articular syndrome

Kenichiro Yamamura[1], Hidetoshi Takada[1], Kiyoshi Uike[1], Yasutaka Nakashima[1], Yuichiro Hirata[1], Hazumu Nagata[1], Tomohito Takimoto[1], Masataka Ishimura[1], Eiji Morihana[1], Shouichi Ohga[2] and Toshiro Hara[1]

Abstract

Objective. Chronic inflammation plays a key role in the development of atherosclerosis. Early progression of atherosclerosis has been reported in patients with RA. Cryopyrin-associated periodic syndromes (CAPS) are autosomal dominant autoinflammatory disorders caused by heterozygous *NLRP3* gene mutations. Chronic infantile neurological cutaneous and articular (CINCA) syndrome is the most severe form of CAPS and patients display early onset of rash, fever, uveitis and joint manifestations. However, there has been no previous report on atherosclerosis in patients with CAPS. The objective of this study is to assess the development of atherosclerosis in patients with CINCA syndrome.

Methods. Intima–media thickness (IMT) of the carotid arteries, stiffness parameter β, ankle brachial index (ABI) and pressure wave velocity (PWV) were evaluated by ultrasonography in 3 patients with CINCA syndrome [mean age 9.0 years (S.D. 5.3)] and 19 age-matched healthy controls [9.3 years (S.D. 4.3)].

Results. The levels of carotid IMT, stiffness parameter β and PWV in CINCA syndrome patients were significantly higher than those in healthy controls [0.51 mm (S.D. 0.05) *vs* 0.44 (0.04), $P = 0.0021$; 6.1 (S.D. 1.7) *vs* 3.9 (1.0), $P = 0.0018$; 1203 cm/s (S.D. 328) *vs* 855 (114), $P = 0.017$, respectively].

Conclusion. Patients with CINCA syndrome showed signs of atherosclerosis from their early childhood. The results of this study emphasize the importance of chronic inflammation in the development of atherosclerosis. Further analysis on atherosclerosis in young patients with CINCA syndrome may provide more insights into the pathogenesis of cardiovascular disease.

Key words: ankle–brachial index, atherosclerosis, chronic infantile neurologic cutaneous and articular syndrome, cryopyrin-associated periodic syndromes, intima-media thickness, pulse wave velocity.

CLINICAL

Introduction

It is well known that chronic inflammation is a predisposing factor for atherosclerosis. There has been considerable interest regarding the possible causal role of inflammation in the development of atherosclerosis in adult patients with RA, SLE and familial Mediterranean fever (FMF). Patients with SLE, APS or RA have increased mortality rates related to early atherosclerosis. Relative risk of 5 for myocardial infarction, 6–10 for stroke in SLE patients and 3.6 for cardiovascular deaths in RA patients has been reported [1]. Furthermore, the American Heart Association has reported that chronic inflammatory disease is one of the eight high-risk factors for atherosclerosis, even in children [2].

Cryopyrin-associated periodic syndromes (CAPS), including chronic infantile neurological cutaneous and articular (CINCA) syndrome, Muckle–Wells syndrome and familial cold autoinflammatory syndrome, are autosomal dominant autoinflammatory syndromes caused by heterozygous mutations of the *NLR family pyrin domain*

[1]Department of Pediatrics and [2]Department of Perinatal and Pediatric Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

Submitted 25 November 2013; revised version accepted 11 March 2014.

Correspondence to: Kenichiro Yamamura, Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582 Japan.
E-mail: yamamura@pediatr.med.kyushu-u.ac.jp

containing 3 (*NLRP3*) gene. It has been reported that disease associated *NLRP3* mutation causes IL-1β oversecretion by caspase-1 activation. CINCA syndrome, the most severe form among them, is characterized by chronic systemic inflammation manifested as early onset of rash, fever, uveitis, chronic meningitis and joint symptoms [3]. However, there has been no previous report evaluating atherosclerosis in patients with CAPS.

Several physiological examinations are applied to assess atherosclerosis. Carotid intima–media thickness (cIMT) is known to be an indicator of atherosclerosis for adults and children [4]. In fact, increased cIMT has been shown in children with obesity, hyperlipidaemia and diabetes mellitus [5]. It has been reported that stiffness parameter β is more useful in detecting atherosclerotic changes in earlier stages than cIMT [6]. Also, pulse wave velocity (PWV) and ankle–brachial index (ABI) are simplified parameters of the severity of atherosclerosis and predictors of prognosis in adult patients with cardiovascular disease [7, 8]. The objective of this study is to assess the development and progression of atherosclerosis in young patients with CINCA syndrome by measuring cIMT, stiffness parameter β, PWV and ABI.

Patients and methods

Study population

Three patients (a 5-year-old boy [9], a 7-year-old girl [10] and a 15-year-old boy [11]) with CINCA syndrome and 19 age-matched healthy controls were enrolled in this study. *NLRP3* mutations were observed in all three patients. The parameters of atherosclerosis were investigated in these three patients who were in remission for 1 year after the initiation of canakinumab treatment. The Institutional Review Board of Kyushu University Hospital approved the study and informed consent was obtained from each subject.

Sonographic study

Carotid artery US was performed with an iE33 ultrasound machine (Philips, Amsterdam, The Netherlands) using an 11 MHz probe. Measurements were obtained with subjects in the supine position by experienced sonographers blinded to the subjects' clinical status. Ultrasonographic images of the right and left common carotid arteries (CCAs) of each subject at the lower third cervical region proximally and 1 cm above the carotid bulb distally in the longitudinal plane were obtained. CCA IMT measurements of the distal CCA posterior wall were done manually by the distance measurement system of the sonography device after magnification of the images. Three measurements were made in a non-neighbouring fashion within an ~1 cm segment from both the left and right CCA proximal and distal portions. The IMT was measured during end diastole. Mean IMT was calculated as the average of three consecutive measurements of maximum far wall thickness obtained from the CCA. Measurement of the internal diameter of the CCA was performed for three consecutive heartbeats. Intraobserver variability was 1.7% for IMT and 3.1% for arterial wall diameter measurements. The stiffness parameter β was calculated from this formula [12]: $\beta = [\ln(SBP/DBP)]/(\Delta D/D)$, where SBP is the systolic blood pressure, DBP is the diastolic blood pressure, D is carotid artery diastolic diameter and ΔD is the change in artery diameter during systole.

PWV and ABI

PWV and ABI were measured using a BP-203RPEIII (Omron Colin, Tokyo, Japan). PWV, ABI, the blood pressure of the extremities, ECG and heart sounds were synchronously measured and then automatically recorded. Electrodes were contacted on both wrists and a microphone was attached to the left margin of the sternum. The extremities were then wrapped by cuffs that were connected to a pulse monitor. The volume wave and time difference emitted from the pulse monitor were recorded. The pulse wave was defined as the value obtained by dividing the distance between the two points by the time spent in transferring the pulse. In the current study, the pulse wave was measured in the brachial artery and ankle (baPWV). The ABI was defined as the ratio between the systolic pressure measured in the ankle and that measured in the brachial artery.

Laboratory evaluation

In the morning, after an overnight fast, venous blood was sampled for the measurement of serum concentrations of glucose, total cholesterol, triglycerides and standard CRP.

Statistical analysis

Data are expressed as mean (S.D.). Differences between data were studied using the Student's *t* test. Analytical statistics of data between group comparisons of categorical data parameters were performed by using the chi-square test. Statistical significance was taken as $P < 0.05$. All statistical analyses were performed using JMP8 (SAS Institute, Tokyo, Japan).

Results

Clinical characteristics of the study group are presented in Table 1. Age, sex and triglyceride levels were similar between patients with CINCA syndrome and control subjects ($P = 0.65$, 0.53 and 0.17, respectively). Total cholesterol levels in CINCA syndrome patients were significantly lower than those in healthy controls, although they were within normal ranges in both groups. CRP concentrations in the patient group were significantly higher than in healthy controls [5.76 mm (S.D. 2.05) *vs* 0.08 (0.16), $P < 0.0001$].

All subjects tolerated the sonographic examination well. Sonographic study results and normal values of the parameters for the age of the patients [13, 14] are summarized in Table 2. Carotid artery analysis revealed that the IMT and stiffness parameter β of patients with CINCA syndrome were significantly higher than those of healthy controls [0.51 mm (S.D. 0.05) *vs* 0.44 (0.04), $P = 0.0021$, and 6.1 (S.D. 1.7) *vs* 3.9 (1.0), $P = 0.018$, respectively).

TABLE 1 Clinical and laboratory characteristics of the subjects

	Patient 1	Patient 2	Patient 3	CINCA syndrome ($n=3$), mean (S.D.)	Controls ($n=19$), mean (S.D.)	*P*-value
Gender, male/female	Male	Female	Male	2/1	9/10	0.53
Age, years	5	7	15	9.0 (5.3)	9.3 (4.3)	0.65
BMI, kg/m^2	16.0	15.5	16.8	16.1 (0.6)	17.3 (2.9)	0.51
Systolic blood pressure, mmHg	91	96	128	105 (20)	99 (8)	0.38
Diastolic blood pressure, mmHg	45	50	68	54 (12)	53 (4)	0.73
Total cholesterol, mg/dl	123	122	131	125 (5)	159 (17)	0.0046
Triglycerides, mg/dl	61	79	157	99 (51)	70 (28)	0.17
Glucose, mg/dl	93	85	102	94 (3)	94 (6)	0.95
CRP, mg/dl	0.26	1.62	5.55	2.48 (2.75)	0.08 (0.16)	<0.0001

CINCA syndrome: chronic infantile neurological cutaneous and articular syndrome.

TABLE 2 Ultrasonographic examination, baPWV and ABI in CINCA syndrome patients and control subjects

	Patient 1	Patient 2	Patient 3	CINCA syndrome ($n=3$), mean (S.D.)	Controls ($n=19$), mean (S.D.)	*P*-value
Intima-media thickness, mm (normal value for each age) [13]	0.47 (0.40)	0.5 (0.40)	0.57 (0.50)	0.51 (0.05)	0.44 (0.04)	0.0021
Systolic diameter, mm	5.5	5.8	5.8	5.7 (0.2)	6.2 (0.8)	0.30
Diastolic diameter, mm	4.8	5.2	5.4	5.1 (0.3)	5.3 (1.7)	0.63
Stiffness parameter β (normal value for each age) [14]	4.8 (3.4)	5.7 (3.7)	7.6 (4.5)	6.1 (1.7)	3.9 (1.0)	0.018
Right baPWV, cm/s	1068	920	1566	1185 (338)	850 (114)	0.0025
Left baPWV, cm/s	1053	1022	1587	1221 (318)	859 (114)	0.0014
Averaged baPWV, cm/s (normal value for each age) [15]	1061 (<941)	971 (<919)	1577 (1041)	1203 (328)	855 (114)	0.0017
Right ABI	1.15	0.91	0.98	1.00 (0.10)	1.04 (0.10)	0.67
Left ABI	1.16	0.95	0.92	0.99 (0.10)	1.06 (0.10)	0.48
Averaged ABI (normal value for each age) [15]	1.16 (>1.00)	0.93 (>1.00)	0.95 (>1.00)	0.99 (0.10)	1.05 (0.10)	0.54

CINCA syndrome: chronic infantile neurological cutaneous and articular syndrome; baPWV: brachial artery pulse wave velocity; ABI: ankle-brachial index.

The averaged baPWV of the patients was significantly higher than that of controls [1203 cm/s (S.D. 328) *vs* 855 (114), $P=0.017$) (Table 2). There was no significant difference in ABI between the two groups, although the values of two patients were lower than the normal range [15].

Discussion

In the present study we found that patients with CINCA syndrome develop atherosclerosis from early childhood. There have been many previous studies describing atherosclerosis associated with inflammatory diseases such as RA, SLE and FMF [1]. However, this is the first report showing the youngest group of patients who developed atherosclerosis associated with inflammatory disorders.

It has been shown that inflammation plays an important role in the development of atherosclerosis. The presence of macrophages and activated lymphocytes within the plaques supports the nature of an immune system-mediated inflammatory disorder of atherosclerosis. It has been shown that higher disease activity representing higher inflammatory burden is associated with increased cardiovascular events in patients with RA and SLE [16]. It may be induced by elevated inflammatory cytokines, which can cause the development of endothelial dysfunction in atherosclerotic processes. In addition, changes in lipid metabolism and a wide variety of immune and inflammatory alterations that directly affect the endothelium, vascular smooth muscle cells and inflammatory cellular components of the atherosclerotic plaque may also play important roles in the development and progression of atherosclerosis in patients. CINCA syndrome is the most severe form of CAPS, and patients display severe systemic inflammation from the neonatal period [3].

Therefore it is reasonable to assume that the progression of atherosclerosis from childhood in three patients with CINCA syndrome is closely related to chronic systemic inflammation. It was reported that the incidence of atherosclerosis could be reduced by aggressive disease-modifying therapies in patients with RA and SLE [16]. In patients with CINCA syndrome, we can investigate the association between inflammation and atherosclerosis without any effect of classical risk factors such as obesity, smoking, hyperlipidaemia or diabetes. This may provide a novel clue to clarify the role of inflammation in the development of atherosclerosis.

In patients with FMF and SLE, age and disease duration were reported to be associated with the severity of atherosclerosis [17]. In the present study we found that the oldest patient (patient 3) with the longest disease duration had the most advanced atherosclerosis, which is in line with this report. Early diagnosis and effective treatment for chronic inflammation in these patients have been emphasized in preventing cardiovascular disease because a negative correlation between the duration of anti-inflammatory treatment and IMT has been observed in SLE patients [18].

Interestingly, improvements in PWV and cIMT [19] were reported in patients with RA after sufficient infliximab treatment. In patients with CINCA syndrome, canakinumab was reported to induce rapid and sustained remission of symptoms [20]. It is possible that a significant improvement in atherosclerosis will be observed in our patients with CINCA syndrome after canakinumab treatment in the near future.

However, there are some limitations in the present study. First, our study contains only a small number of patients because of the extremely rare incidence of this disease. Second, the parameters investigated in this study are considerably variable with the age of the subjects. It is also possible that the values of the parameters change because of the measurement equipment. Multicentre and long-term follow-up analysis with standardized procedures and tools on a larger number of the patients are necessary to provide more precise information on the pathogenesis of atherosclerosis.

Conclusion

Patients with CINCA syndrome developed atherosclerosis from early childhood. Atherosclerosis in CINCA syndrome patients may be a prototype of cardiovascular disease predominantly induced by chronic inflammation.

Rheumatology key messages

- Patients with CINCA syndrome develop atherosclerosis from early childhood.
- This report shows the youngest group of patients who developed atherosclerosis associated with inflammatory disorders.
- Early treatment with anti-IL-1β antibody might be beneficial in preventing atherosclerosis in CINCA syndrome.

Acknowledgements

We thank Junji Kishimoto (Digital Medicine Initiative, Kyushu University Hospital, Fukuoka, Japan) for the contribution of statistical analysis.

Funding: This work was supported by a grant for Research on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan to T.H. and a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (24791072) to K.Y.

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

References

1 Tyrrell PN, Beyene J, Feldman BM *et al*. Rheumatic disease and carotid intima-media thickness: a systematic review and meta-analysis. Arterioscler Thromb Vasc Biol 2010;30:1014–26.

2 Kavey RE, Allada V, Daniels SR *et al*. Cardiovascular risk reduction in high-risk pediatric patients: a scientific statement from the American Heart Association Expert Panel on Population and Prevention Science; the Councils on Cardiovascular Disease in the Young, Epidemiology and Prevention, Nutrition, Physical Activity and Metabolism, High Blood Pressure Research, Cardiovascular Nursing, and the Kidney in Heart Disease; and the Interdisciplinary Working Group on Quality of Care and Outcomes Research: endorsed by the American Academy of Pediatrics. Circulation 2006;114:2710–38.

3 Kubota T, Koike R. Cryopyrin-associated periodic syndromes: background and therapeutics. Mod Rheumatol 2010;20:213–21.

4 Peters SA, den Ruijter HM, Bots ML, Moons KG. Improvements in risk stratification for the occurrence of cardiovascular disease by imaging subclinical atherosclerosis: a systematic review. Heart 2012;98:177–84.

5 Jarvisalo MJ, Putto-Laurila A, Jartti L *et al*. Carotid artery intima-media thickness in children with type 1 diabetes. Diabetes 2002;51:493–8.

6 Hirai T, Sasayama S, Kawasaki T, Yagi S. Stiffness of systemic arteries in patients with myocardial infarction. A noninvasive method to predict severity of coronary atherosclerosis. Circulation 1989;80:78–86.

7 Hirata K, Kawakami M, O'Rourke MF. Pulse wave analysis and pulse wave velocity: a review of blood pressure interpretation 100 years after Korotkov. Circ J 2006;70:1231–9.

8 Vlachopoulos C, Aznaouridis K, Terentes-Printzios D, Ioakeimidis N, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with brachial-ankle elasticity index: a systematic review and meta-analysis. Hypertension 2012;60:556–62.

9 Takada H, Ishimura M, Inada H *et al*. Lipopolysaccharide-induced monocytic cell death for the diagnosis of mild neonatal-onset multisystem inflammatory disease. J Pediatr 2008;152:885–7.

10 Takada H, Kusuhara K, Nomura A *et al.* A novel CIAS1 mutation in a Japanese patient with chronic infantile neurological cutaneous and articular syndrome. Eur J Pediatr 2005;164:785–6.

11 Saito M, Nishikomori R, Kambe N *et al.* Disease-associated CIAS1 mutations induce monocyte death, revealing low-level mosaicism in mutation-negative cryopyrin-associated periodic syndrome patients. Blood 2008;111: 2132–41.

12 Mitsnefes MM, Kimball TR, Witt SA *et al.* Abnormal carotid artery structure and function in children and adolescents with successful renal transplantation. Circulation 2004; 110:97–101.

13 Jourdan C, Wuhl E, Litwin M *et al.* Normative values for intima-media thickness and distensibility of large arteries in healthy adolescents. J Hypertens 2005;23: 1707–15.

14 Sato A, Ichihashi K, Momoi M. Stiffness parameter B: changes with age in normal children. Neurosonology 2007;20:124–8.

15 Niboshi A, Hamaoka K, Sakata K, Inoue F. Characteristics of brachial-ankle pulse wave velocity in Japanese children. Eur J Pediatr 2006;165:625–9.

16 Roman MJ, Shanker BA, Davis A *et al.* Prevalence and correlates of accelerated atherosclerosis in systemic lupus erythematosus. N Engl J Med 2003;349: 2399–406.

17 Ugurlu S, Seyahi E, Cetinkaya F *et al.* Intima-media thickening in patients with familial Mediterranean fever. Rheumatology 2009;48:911–5.

18 Ristic GG, Lepic T, Glisic B *et al.* Rheumatoid arthritis is an independent risk factor for increased carotid intima-media thickness: impact of anti-inflammatory treatment. Rheumatology 2010;49:1076–81.

19 Del Porto F, Lagana B, Lai S *et al.* Response to anti-tumour necrosis factor alpha blockade is associated with reduction of carotid intima-media thickness in patients with active rheumatoid arthritis. Rheumatology 2007;46: 1111–5.

20 Kone-Paut I, Lachmann HJ, Kuemmerle-Deschner JB *et al.* Sustained remission of symptoms and improved health-related quality of life in patients with cryopyrin-associated periodic syndrome treated with canakinumab: results of a double-blind placebo-controlled randomized withdrawal study. Arthritis Res Ther 2011;13: R202.

別冊日本臨牀　新領域別症候群シリーズ　No. 27（2014年3月20日発行）別刷

神経症候群（第2版）

—その他の神経疾患を含めて—

II

IV　自己免疫性疾患

その他の炎症性疾患

中條–西村症候群

金澤伸雄

その他の炎症性疾患

中條-西村症候群

Nakajo-Nishimura syndrome

金澤伸雄

Key words：中條-西村症候群，遺伝性自己炎症疾患，脂肪筋肉萎縮，免疫プロテアソーム，*PSMB8*

1. 概念・定義

1939年に東北帝国大学皮膚科泌尿器科の中條により，血族婚家系に生じた兄妹例が'凍瘡ヲ合併セル続発性肥大性骨骨膜症'として報告されたのが，本疾患の最初の記載とされる[1]．凍瘡と骨膜肥厚を伴うばち状指を特徴とし，心不全に基づく末梢循環障害が原因として想定された．更に，1950年に血族婚の2家系に生じた3症例も同じ疾患だとして中條の報告を引用し，原発性の遺伝性疾患である可能性を指摘したのが，和歌山県立医科大学皮膚科泌尿器科の西村らである[2]．その後も主に関西の皮膚科からの報告が続き，1985年に大阪大学皮膚科の喜多野らが自験4症例を含む8家系12症例をまとめ，我が国以外で報告のない新疾患'a syndrome with nodular erythema, elongated and thickened fingers, and emaciation'として，初めて英文で報告した[3]．

内科領域からも，膠原病類似疾患あるいは特殊なリポジストロフィーとして関東・東北の症例が報告され，皮膚科領域からの報告例と合わせ，新しい疾患'hereditary lipo-muscular atrophy with joint contracture, skin eruptions and hyper-γ-globulinemia'として，新潟大学神経内科の田中らが，1991年に日本医事新報，更に，1993年にInternal Medicine誌に発表した[4,5]．一方，小児例を集めた報告は1986年の和歌山県立医科大学小児科の杉野らの学会報告のみであったが，その後彼らは本疾患の周期性発熱と地域的偏りに着目し，家族性地中海熱(familial Mediterranean fever)を模した'familial Japanese fever'という疾患名を2006年に提唱している[6,7]．

中條-西村症候群との疾患名は，2009年に本疾患が稀少難治性疾患として厚生労働科学研究費補助金難治性疾患克服研究事業の研究奨励分野177疾患の一つに採択された際に初めて正式に用いられ，*PSMB8*遺伝子変異の同定を報告した米国科学アカデミー紀要の論文により国際的に認められた[8]．

2. 疫　　学

中條の報告以来，東北・関東に5家系7症例(宮城，秋田，新潟，東京)，関西に20家系23症例(大阪，奈良，和歌山)あり，そのうち現在も継続してフォローされている症例は関西の10数例のみである[9]．なかには，診断がつかないまま全身型若年性特発性関節炎に準じて抗IL-6受容体抗体製剤を投与したところ奏効したと報告されていたが，遺伝子解析にて本疾患であることが最近明らかとなった奈良の小児例もある[10]．性別では男性20例，女性10例で，男性が多い．生年別では，1940年前後と1970年前後に生まれた集団に加え，2000年前後に生まれた患児が2例ある．

3. 病　　因

近親婚や家族内発症が多くみられることから常染色体劣性遺伝性と予想されていたが，複数

Nobuo Kanazawa: Department of Dermatology, Wakayama Medical University　和歌山県立医科大学 皮膚科

0047-1852/14/¥60/頁/JCOPY

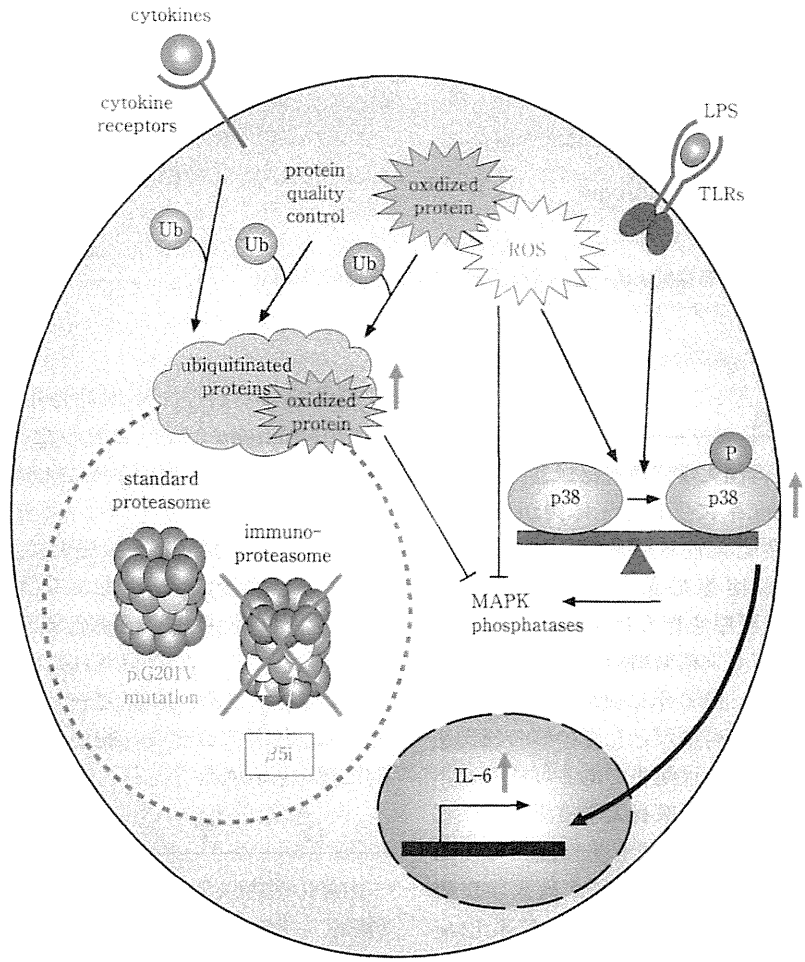

図1　中條-西村症候群において想定される炎症惹起メカニズム

*PSMB8*変異のために免疫プロテアソームの機能が低下することによって免疫担当細胞をはじめ各種細胞にユビキチン化・酸化タンパク質が蓄積し，これがストレスとなってp38 MAPKが活性化し，IL-6に代表されるサイトカイン産生が亢進する．

の家系の患者とその兄弟のゲノムを用いたホモ接合部マッピングによって，染色体6p21.31-32上にある*PSMB8*遺伝子の，602番目のグアニンのチミンへの変異(602G>T)に伴う201番目のグリシンのバリンへの置換(G201V)が原因として同定された[8,11]．検索したすべての症例に同じ変異がホモで存在し，強い創始者効果が認められている．

4. 病　　態

プロテアソームはポリユビキチン鎖によってラベルされた分子を選択的に分解する巨大分子複合体であり，不要なタンパク質を除去するだけでなく，細胞周期やシグナル伝達など多彩な細胞機能にかかわる．酵素活性をもつβ1，β2，β5サブユニットが誘導型のより活性の高いβ1i，β2i，β5iサブユニットに置き換わったものは免疫プロテアソームと呼ばれ，免疫担当細胞で恒常的に発現し，また体細胞においても炎症時などにinterferon(IFN)γによって誘導される．*PSMB8*遺伝子はこのβ5iサブユニットをコードし，G201V変異によって，β5iがもつ

キモトリプシン様活性が著しく低下するだけでなく，隣接サブユニット(β4，β6)との接合面の変化のために複合体の形成不全が起こり，成熟した免疫プロテアソームの量が減少するとともに，β1iとβ2iがもつトリプシン様，カスパーゼ様活性も大きく低下する．その結果，患者の炎症局所に浸潤するマクロファージなど各種細胞内にユビキチン化・酸化タンパク質が蓄積する[8]．

炎症発現に重要な転写因子の中で，nuclear factor(NF)-κBの活性化にはプロテアソームによるinhibitor of κBの分解が必須とされるが，患者細胞におけるNF-κBの活性化には明らかな差異はなく，むしろmitogen-activated protein kinase(MAPK)経路にあるリン酸化p38の核内貯留があり，p38 MAPKの活性化が認められた．患者血清と培養線維芽細胞培養上清中におけるinterleukin(IL)-6の過剰産生と合わせ，本来分解されるべき不要・不良タンパク質の貯留に対するストレス応答として，炎症や組織変性をきたすと考えられる(**図1**)．

2010年にポルトガル・メキシコから報告されたJMP(joint contractures，muscular atrophy，microcytic anemia and panniculitis-associated lipodystrophy)症候群と，スペイン・アメリカから報告されたCANDLE(chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature)症候群も，中條-西村症候群と臨床的に酷似し，*PSMB8*遺伝子の異なる変異が原因として同定されたことから，免疫プロテアーゼ機能不全という共通の病態を有する同一疾患群と考えられる[12,13]．

5. 診断と鑑別診断

代表的な1症例における臨床像を**図2**に示す．幼少児期，特に冬期に手足や顔面の凍瘡様皮疹にて発症し(**図2-a**)，その後四肢・体幹の結節性紅斑様皮疹や弛張熱を不定期に繰り返すようになる(**図2-h**)．ヘリオトロープ様の眼瞼紅斑を認めることもある(**図2-e**)．組織学的には，真皮から筋層に至るまで巣状にリンパ球・組織球を主体とした稠密だが多彩な炎症細胞の浸潤を認め，内皮の増殖を伴う血管障害を伴う(**図2-f**)．筋炎を伴うこともあるが，筋力は比較的保たれる(**図2-i**)．早期より大脳基底核の石灰化を認めるが，精神発達遅滞ははっきりしない(**図2-c**)．次第に特徴的な長く節くれだった指と顔面・上肢を主体とする脂肪筋肉萎縮・やせが進行し，手指や肘関節の屈曲拘縮をきたす(**図2-g**)．LDH，CPK，CRP，AAアミロイドのほか，γ-グロブリン，特にIgGが高値となる．IgEが高値となる例もある．更に進行すると抗核抗体や各種自己抗体が陽性になることがある．早期より肝脾腫を認めるが，脂質代謝異常は一定しない．胸郭の萎縮を伴う拘束性呼吸障害や，心筋変性や心電図異常を伴う心機能低下のために早世する症例がある一方，著明な炎症所見を認めず病院を受診しない症例もある[9]．

これらの特徴を踏まえた臨床診断基準案を**表1**に示す．進行性の脂肪筋肉萎縮は本疾患の最大の特徴であるが，皮疹や発熱などの炎症症状が強い乳幼児期は脂肪萎縮が目立たず，大脳基底核石灰化が決め手になることもある[10,14]．ほかの遺伝性自己炎症疾患との鑑別に時に遺伝子解析が必要となり，特に寒冷刺激で症状が増悪する点はクリオピリン関連周期熱症候群と似る．凍瘡様皮疹と大脳基底核石灰化はAicardi-Goutieres症候群(家族性凍瘡様ループス)と共通だが，明らかな神経精神症状がない点で鑑別される．JMP症候群とCANDLE症候群は同一疾患群と考えられるが，前者では発熱がなく，強い萎縮・拘縮に加えて痙攣も報告されている[12]．CANDLE症候群は中條-西村症候群と非常によく似るが，皮疹への浸潤細胞で好中球が目立ち，患者血液のmRNA発現アレイによってIFNγシグナルの活性化が示されたことから，免疫プロテアソームの機能不全に対して，その誘導シグナルであるIFNγが過剰に作用しているという病態が想定されている[13]．

6. 治療と予後

副腎皮質ステロイド(ステロイド)全身投与は発熱，皮疹などの炎症の軽減には有効だが，減量により容易に再燃し，また脂肪筋肉萎縮には

図2　代表的な中條-西村症候群症例の臨床像

a. 右足外側縁の凍瘡様紅斑(5歳時). b. 下腿筋炎による尖足位. c. CTでの大脳基底核石灰化(24歳時). d. 骨シンチにおける関節部異常集積像. e. ヘリオロープ様眼瞼紅斑を伴うやせて骨ばった顔貌(27歳時). f. 手掌の結節性紅斑様皮疹の病理組織像. g. 長く節くれだった指. h. 右手首, 左手掌に結節性紅斑様皮疹を認める. i. 大腿筋のMRI像(左よりT1, T2, ガドリニウム強調T1像, 24歳時).

表1 中條-西村症候群臨床診断基準案

1. 常染色体劣性遺伝(血族婚や家族内発症あり)
2. 手足の凍瘡様紫紅色斑(乳幼児期から冬期に出現)
3. 強い浸潤・硬結を伴う結節性紅斑が出没(環状のこともある)
4. 繰り返す弛張熱(周期熱:必発ではない)
5. 手足の長く節くれだった指・関節拘縮
6. 進行性の限局性脂肪筋肉萎縮・やせ(顔面・上肢に著明)
7. 肝脾腫
8. 大脳基底核石灰化

8項目中5項目以上陽性で他疾患を除外できれば確定

無効である.むしろ幼少児期からのステロイド全身投与は,長期内服による成長障害,中心性肥満,緑内障,骨粗鬆症など弊害も多く,慎重な投与が必要である[9].ほかの自己炎症疾患と同様,抗tumor necrosis factor α製剤や抗IL-1β製剤などの生物学的製剤,特に病態から抗IL-6受容体抗体製剤が有効である可能性があり,小児例において全身型若年性特発性関節炎に準じた投与が奏効しquality of life(QOL)の回復に寄与したと報告されている[10].JMP症候群やCANDLE症候群では,関節リウマチに準じてメトトレキサートや各種生物学的製剤が投与されているが,いずれもステロイドと同様,炎症には有効だが脂肪筋肉萎縮には無効と報告されている[12,13].最近では,IFNγシグナルの過剰活性化を抑制するJanus kinase阻害薬の有効性も報告されている[15].

脂肪萎縮に関しては,PSMB8が脂肪細胞の分化に関与するという報告があるが,患者由来induced pluripotent stem細胞から脂肪細胞への正常な分化も確認されており(未発表データ),今後更なる検討が必要と思われる[11].糖や脂質の代謝異常は一定しないが,最近脂肪萎縮症に対してレプチン補充療法が承認されたことから,今後試みてよい治療の一つと思われる.

皆同じ変異をもつにもかかわらず,発症時の炎症の程度や萎縮の進行の速さ,程度には個人差が認められる.心肺のほか肝臓などの障害がゆるやかに進み,60歳代で亡くなる例が多いようであるが,早期から拘束性呼吸障害や心機能低下をきたして30歳代で突然死する重症例もあり,注意が必要である.また脂肪筋肉萎縮に伴う手の拘縮のほか,咬合不全や重度の鶏眼などによりQOLが低下する.

遺伝子変異に連なる病態の解明に基づいた有効かつ副作用の少ない治療法が開発され,発症早期に正確に診断し治療を開始することで,反復性炎症のみならず萎縮や拘縮の進行が抑制できる日が一日も早く実現することが望まれる.

■文　献

1) 中條　敦:凍瘡ヲ合併セル続発性肥大性骨骨膜症.皮膚科泌尿器科雑誌 **45**: 77-86, 1939.
2) 西村長應ほか:2家族に発生した凍瘡様皮膚病変を併発した続発性肥大性骨骨膜症.皮膚科性病科雑誌 **60**: 136-141, 1950.
3) Kitano Y, et al: A syndrome with nodular erythema, elongated and thickened fingers, and emaciation. Arch Dermatol **121**: 1053-1056, 1985.
4) 田中正美ほか:リポジストロフィーの新しい病型の提唱—関節拘縮,結節性紅斑,高γ-グロブリン血症を伴う常染色体劣性遺伝形式を示す脂肪筋萎縮症—.日本医事新報 **3495**: 32-34, 1991.
5) Tanaka M, et al: Hereditary lipo-muscular atrophy with joint contracture, skin eruptions and hyper-γ-globulinemia: a new syndrome. Intern Med **32**: 42-45, 1993.
6) 杉野禮俊ほか:Partial lipodystrophyに酷似した外観を呈し,凍瘡様皮疹,長く節くれ立った指,基底核石灰化を伴い,炎症反応陽性を示す遺伝性疾患の4小児例.疾患単位と考えたい.日小

児会誌 **90**: 727, 1986.
7) 杉野禮俊ほか：Familial Japanese fever: partial lipodystrophy に酷似した外観を呈し，凍瘡様皮疹，長く節くれ立った指，関節拘縮，基底核石灰化などを伴う遺伝性発熱症候群．日小児科医会報 **32**: 106, 2006.
8) Arima K, et al: Proteasome assembly defect due to a proteasome subunit beta type 8(PSMB8) mutation causes the autoinflammatory disorder, Nakajo-Nishimura syndrome. Proc Natl Acad Sci USA **108**: 14914-14919, 2011.
9) 金澤伸雄ほか：中條-西村症候群．日臨免疫会誌 **34**: 388-400, 2011.
10) 石川智朗ほか：ヒト型抗IL-6受容体抗体(トシリズマブ)が著効した慢性炎症性疾患の1例．日小児会誌 **113**: 310, 2009.
11) Kitamura A, et al: A mutation in the immunoproteasome subunit PSMB8 causes autoinflammation and lipodystrophy in humans. J Clin Invest **121**: 4150-4160, 2011.
12) Agarwal AK, et al: PSMB8 encoding the β5i proteasome subunit is mutated in joint contractures, muscle atrophy, microcytic anemia, and panniculitis-induced lipodystrophy syndrome. Am J Hum Genet **87**: 866-872, 2010.
13) Liu Y, et al: Mutations in PSMB8 cause CANDLE syndrome with evidence of genetic and phenotypic heterogeneity. Arthritis Rheum **64**: 895-907, 2012.
14) Kunimoto K, et al: A new infant case of Nakajo-Nishimura syndrome with a genetic mutation in the immunoproteasome subunit: An overlapping entity with JMP and CANDLE syndrome related to PSMB8 mutations. Dermatology **227**: 26-30, 2013.
15) Almeida de Jesus A, et al: A subset of up-regulated IFN regulated genes in CANDLE patients decrease with treatment with a JAK inhibitor. Arthritis Rheum **64**: S1124, 2012.