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Contribution of collagen-binding protein Cnm of *Streptococcus mutans* to induced IgA nephropathy-like nephritis in rats

Shuhei Naka[1,8], Daiki Matsuoka[1,8], Taro Misaki[2,3], Yasuyuki Nagasawa[4], Seigo Ito[5], Ryota Nomura[6], Kazuhiko Nakano[7] & Michiyo Matsumoto-Nakano[1]

IgA nephropathy (IgAN), the most common primary glomerulonephritis, is considered an intractable disease with unknown pathogenic factors. In our previous study, *Streptococcus mutans*, the major causative bacteria of dental caries, which expresses Cnm, was related to the induction of IgAN-like nephritis. In the present study, the Cnm-positive *S. mutans* parental strain, a Cnm-defective isogenic mutant strain, its complementation strain, and recombinant Cnm (rCnm) protein were administered intravenously to Sprague Dawley rats, and the condition of their kidneys was evaluated focusing on the pathogenicity of Cnm. Rats treated with parental and complement bacterial strains and rCnm protein developed IgAN-like nephritis with mesangial proliferation and IgA and C3 mesangial deposition. Scanning immunoelectron microscopy revealed that rCnm was present in the electron-dense deposition area of the mesangial region in the rCnm protein group. These results demonstrated that the Cnm protein itself is an important factor in the induction of IgAN in rats.

IgA nephropathy (IgAN), the most common primary glomerulonephritis, is considered an intractable disease with various pathogenetic factors[1–3]. Over approximately 20 years from the onset of disease, 30–40% of patients develop terminal renal failure[1–4]. Common clinical findings include proteinuria and hematuria, but a renal biopsy is considered essential to confirm a diagnosis[5–7]. The diagnostic hallmark of IgAN is the predominance of IgA deposits, either alone or with IgG, IgM, or both, in the glomerular mesangial regions[8]. More than 90% of IgAN patients have complement C3 deposition in their glomeruli[9]. Most C3 is present in the mesangial and paramesangial regions including some vascular endothelial cells[2,10]. Typical histopathological findings in patients with IgAN include increased numbers of mesangial cells and matrix in the mesangial region as well as the deposition of immune complexes[11]. Patients with IgAN sometimes present with macroscopic hematuria when they have an upper respiratory tract infection, such as tonsillitis[12]. Several bacterial species have been reported to be potential factors involved in the pathogenesis of IgAN[13–17], including dental caries-related bacteria[18–25] and periodontitis-related bacteria[26–28].

Streptococcus mutans, the major causative bacteria of dental caries, is a Gram-positive, facultative anaerobic bacteria[29], which can induce infective endocarditis by invading the bloodstream during invasive dental procedures, such as tooth extractions[30]. A collagen-binding protein (Cnm) of approximately 120 kDa is expressed on the surface of some *S. mutans* strains[31] and is involved in adhesion to and invasion of vascular endothelial cells, indicating it might be an important factor that causes infective endocarditis[32–34]. It was reported that *S. mutans* expressing Cnm was detected more frequently in patients with IgAN than in healthy subjects[18]. Indeed, the intravenous administration of *S. mutans* expressing Cnm isolated from the oral cavity of IgAN patients induced IgAN-like nephritis in rats[21]. In addition, when Cnm-positive *S. mutans* was inoculated into the oral cavity of a rat dental caries model, IgAN-like lesions developed with severe dental caries extending to the pulp space, which can cause bacteremia[22].

These results led us to consider that Cnm protein on the surface of *S. mutans*, but not *S. mutans* itself, might be an important virulence factor involved in *S. mutans*-related IgAN. In the present study, we focused on the ability of Cnm on *S. mutans* to cause IgAN-like nephritis in rats using Cnm-deficient mutant strains and recombinant Cnm (rCnm) protein.

[1]Department of Pediatric Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Okayama, Japan. [2]Division of Nephrology, Seirei Hamamatsu General Hospital, Hamamatsu, Shizuoka, Japan. [3]Department of Nursing, Faculty of Nursing, Seirei Christopher University, Hamamatsu, Shizuoka, Japan. [4]Department of General Internal Medicine, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan. [5]Department of Internal Medicine, Japan Self-Defense Force Iruma Hospital, Saitama, Japan. [6]Department of Pediatric Dentistry, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan. [7]Department of Pediatric Dentistry, Graduate School of Dentistry, The University of Osaka, Suita, Osaka, Japan. [8]These authors contributed equally: Shuhei Naka, Daiki Matsuoka. e-mail: mnakano@okayama-u.ac.jp

Results

Systemic condition of rats

We evaluated proteinuria, hematuria, and renal function, which are the clinical characteristics of IgAN, in rats. Animal experiments were performed using *S. mutans* strain SN74 (serotype *e*, Cnm-positive) isolated from the oral cavity of a patient with severe IgAN, SN74CND strain (CND), a Cnm deficient mutant of the SN74 strain, SN74CNDcomp strain (Comp), a Cnm complement SN74CND strain, and rCnm protein. Phosphate-buffered saline (PBS) was used as a control. Serum albumin (ALB), blood urea nitrogen (BUN), and creatinine (CRE) levels in all groups at the time of sacrifice were not significantly different (Table 1). Furthermore, there were no significant differences in serum IgA concentrations among the PBS, SN74, and rCnm groups (Supplementary Table 1). On the other hand, serum IgG concentrations were significantly higher in the rCnm group compared to the PBS group ($P < 0.0167$) (Supplementary Table 1). There was no significant difference in the urine protein/creatinine ratio in each group (Fig. 1a). Furthermore, hematuria was not observed in all rats in the PBS group and in 1 of 14 rats in the CND group. In contrast, 7 of 19 rats in the SN74 group, 3 of 20 rats in the Comp group, and 4 of 18 rats in the rCnm protein group developed hematuria. The hematuria positivity rate was significantly higher in the SN74 groups than in the PBS and CND groups ($P < 0.01$, $P < 0.05$) (Fig. 1b).

These results suggested no change in renal function or proteinuria in any of the experimental groups. However, hematuria was present in the Cnm-positive strain group, Comp group, and rCnm protein group, but not the CND group. These results suggest that Cnm protein or *S. mutans* with Cnm protein induced hematuria in the early stages of IgAN.

Histopathological analyses of kidney tissues

We evaluated mesangial cell and matrix proliferation, which are important features of IgAN. Histopathological analyses of period acid-Schiff (PAS)-stained sections revealed the prominent proliferation of mesangial cells and mesangial matrix in rats in the SN74, Comp, and rCnm protein groups (Fig. 2a). Mesangial proliferation scores were significantly higher in the SN74 and Comp groups than in the PBS and CND groups, and the score of the rCnm protein group was significantly higher than that of the CND group ($P < 0.01$) (Fig. 2b). These results suggest that the presence of Cnm protein induces mesangial cell and matrix proliferation. The mesangial proliferation score of the hematuria-positive rat group was higher than of the hematuria-negative rat group (Supplementary Fig. 1a). Furthermore, no obvious atrophy was seen in the tubules, though fibrosis was observed at the inner brush border of the tubules in Masson's trichrome (MT) stained images of rats in the SN74, Comp, and rCnm groups (Supplementary Fig. 2). In addition, α-SMA expression was observed in the immunofluorescent (IF) staining at the same area (Supplementary Fig. 3).

Table 1 | Serum levels of kidney markers in rats inoculated with PBS, SN74, SN74CND, SN74CNDcomp, or rCnm

Groups	Serum levels (mean ± SEM)		
	ALB (g/dL)	BUN (mg/dL)	CRE (mg/gL)
PBS (n = 14)	3.80 ± 0.06	19.29 ± 0.54	0.31 ± 0.01
SN74 (n = 19)	3.97 ± 0.03	20.72 ± 0.76	0.33 ± 0.01
SN74CND (n = 20)	3.96 ± 0.04	19.65 ± 0.68	0.31 ± 0.01
SN74CNDcomp (n = 20)	3.80 ± 0.04	17.07 ± 0.61	0.30 ± 0.01
rCnm (n = 18)	3.80 ± 0.05	20.80 ± 0.49	0.31 ± 0.01

Statistical significance was determined by analysis of variance with Bonferroni's correction. There were no significant differences in levels between the groups.
ALB albumin, *BUN* blood urea nitrogen, *CRE* creatinine, *SEM* standard error of the mean.

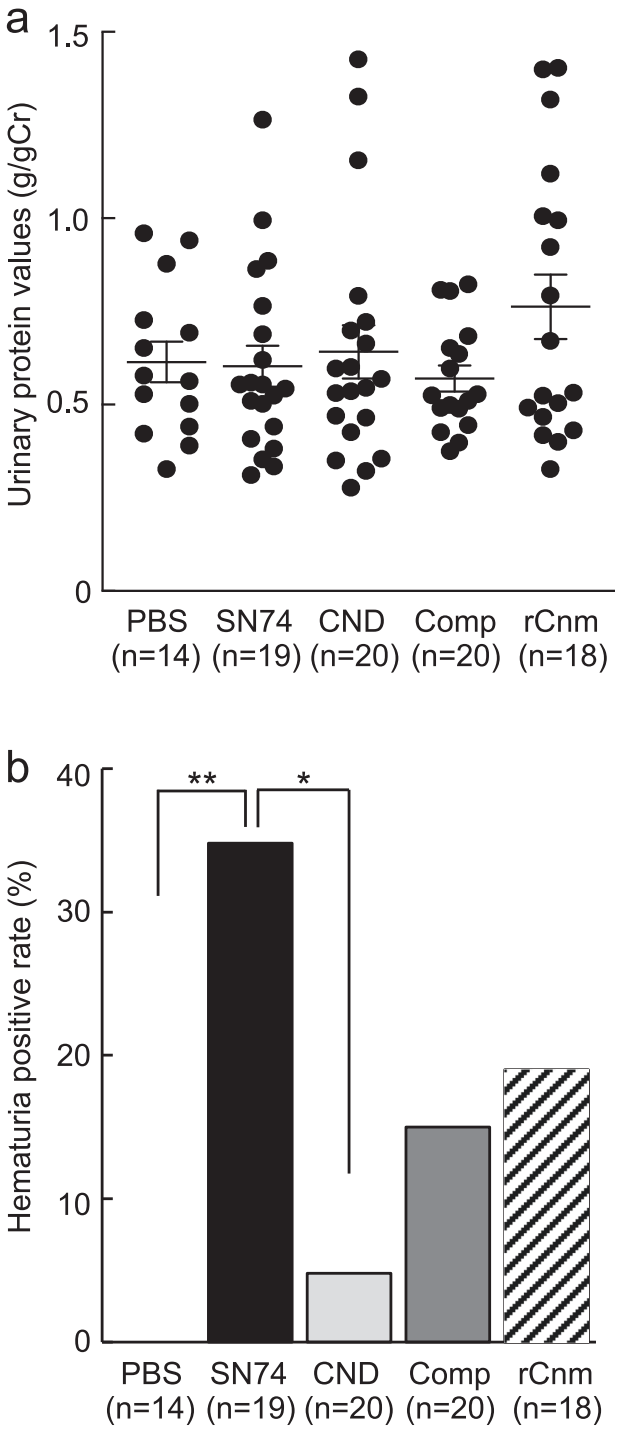

Fig. 1 | Analysis of urine components in rats. a Urinary protein ratio (urinary protein/urinary creatinine). **b** Hematuria. Each column represents the mean ± standard errors of the means of the PBS group ($n = 14$), SN74 group ($n = 19$), SN74CND group ($n = 20$), SN74CNDcomp group ($n = 20$), and rCnm group ($n = 18$). **a** Statistical significance was determined by analysis of variance with Bonferroni's correction. **b** Statistical significance was determined using Fisher's exact test. $^{*}P < 0.05$, $^{**}P < 0.01$.

Immunohistochemical analyses of kidney tissues

We evaluated the deposition of IgA in the mesangial region, the most important and defining finding of IgAN. We also evaluated C3 and IgG which is highly stained in tissues from patients with IgAN. Immunohistochemical analyses using IgA-, C3-, and IgG specific antibodies showed prominent positive reactions in the mesangial regions of rats inoculated

with SN74, Comp, or rCnm protein groups (Figs. 3, 4). In contrast, these deposits were not observed in the PBS and SN74CND groups. Immunochemical analysis using IgG-specific antibodies demonstrated IgG deposition in some rats in the rCnm group (Fig. 5). The positive rate of IgA deposition was significantly higher in the SN74, Comp, and rCnm groups than in the PBS and CND groups (Fig. 6a). The positive rate of C3 deposition was significantly higher in the SN74 group than in the PBS and CND groups, and significantly higher in the rCnm protein group than in the PBS group (Fig. 6b). The positive rate of IgG deposition was 5 out of 18 rats in the rCnm protein group, but there was no significant difference between each group (Fig. 6c). The positive rates for IgA and C3 deposition were significantly higher in the SN74 and Comp groups than in the PBS and CND groups, and significantly higher in the rCnm protein group than in the PBS group (Fig. 6d). These results suggest that the presence of Cnm protein induces the deposition of IgA, C3, and IgG in the mesangial region of the renal glomerulus. The positive rate of deposition of IgA, C3, and/or IgG in the hematuria-positive rat group was higher than in the hematuria-negative rat group (Supplementary Fig. 1b).

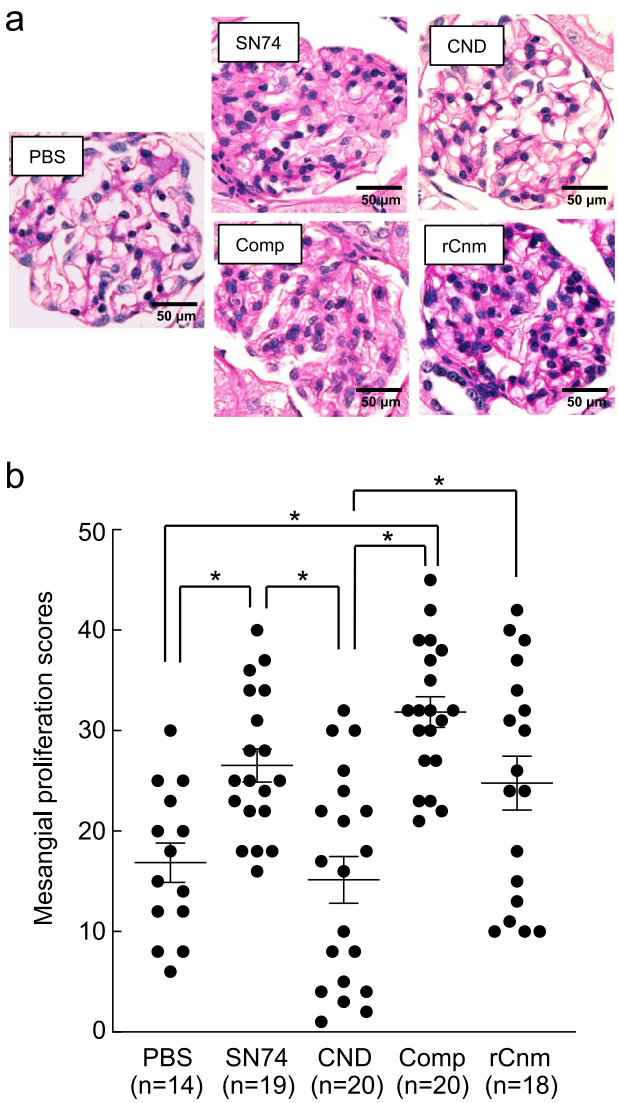

Fig. 2 | Histopathological appearance of kidney tissues following PAS staining. **a** Representative image of PAS staining. Scale bars, 50 μm (all panels). **b** Mesangial proliferation scores. Each column represents the mean ± standard errors of the means of the control group ($n = 14$), SN74 group ($n = 19$), SN74CND group ($n = 20$), SN74CNDcomp group ($n = 20$), and rCnm group ($n = 18$). Statistical significance was determined by analysis of variance with Bonferroni's correction. $^*P < 0.01$.

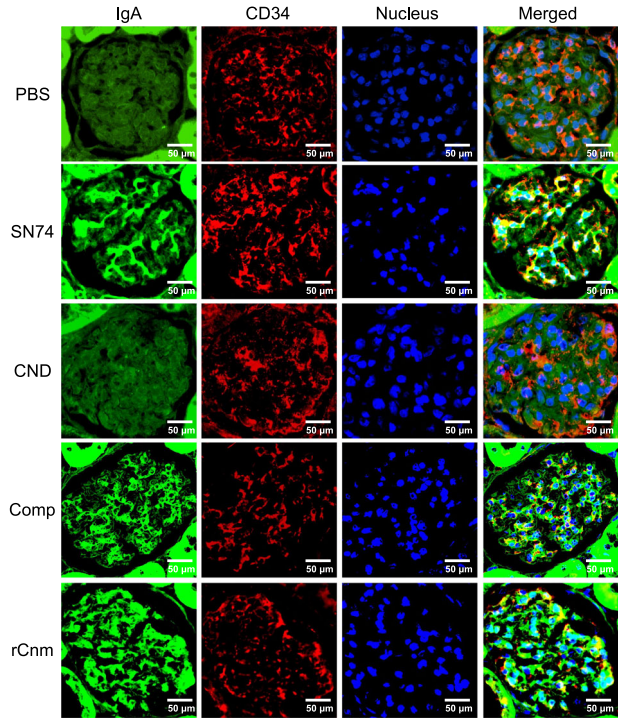

Fig. 3 | Representative histopathological appearance of kidney tissues by immunohistochemistry with IgA-specific antibodies. The first image shows staining with an anti-IgA antibody. The second image shows staining with an anti-CD34 antibody. The third image shows nuclear staining. The fourth row of images shows the left three images superimposed. Scale bars, 50 μm (all panels).

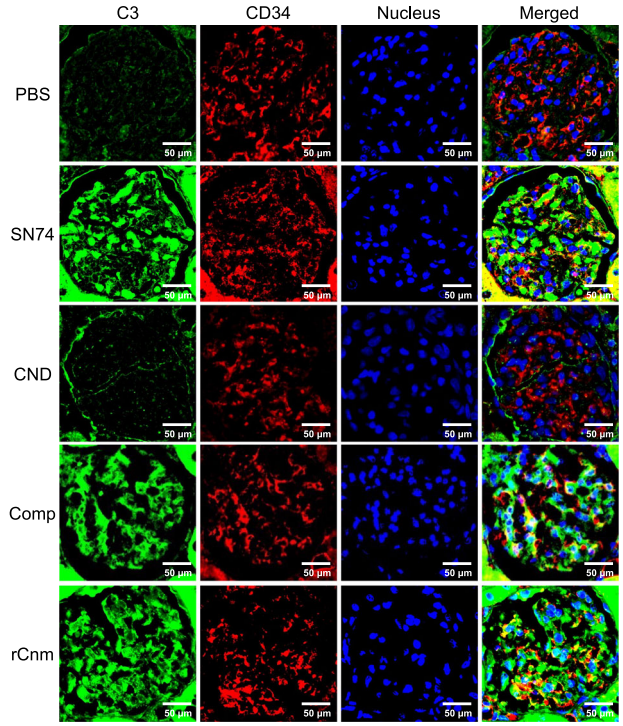

Fig. 4 | Representative histopathological appearance of kidney tissues by immunohistochemistry with C3-specific antibodies. The first image shows staining with an anti-C3 antibody. The second image shows staining with an anti-CD34 antibody. The third image shows nuclear staining. The fourth row of images shows the left three images superimposed. Scale bars, 50 μm (all panels).

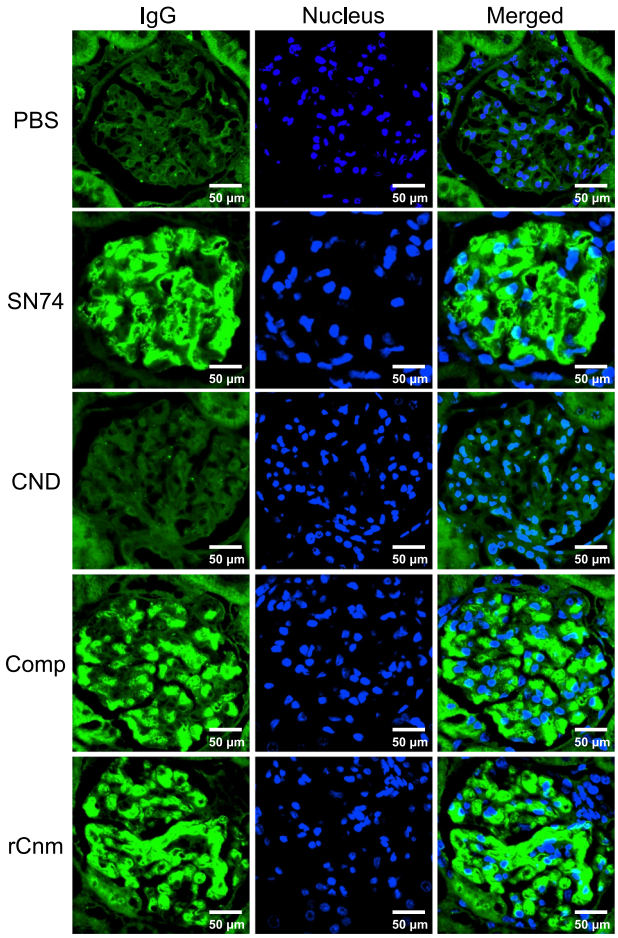

Fig. 5 | Representative histopathological appearance of kidney tissues by immunohistochemistry with IgG-specific antibodies. The first image shows staining with an anti-IgG antibody. The second image shows nuclear staining. The third row of images shows the left two images superimposed. Scale bars, 50 μm (all panels).

Immunoelectron microscopy of Cnm protein in renal glomeruli

We evaluated the deposition of electron dense deposits (EDD) in the mesangial region, which are an important feature of IgAN. Furthermore, the presence of Cnm protein in renal glomeruli was evaluated by immunoelectron microscopy using Cnm antibodies. Electron microscopic analysis showed EDD in the rCnm protein groups. Furthermore, immunoelectron microscopy analysis using Cnm antibodies demonstrated colloidal deposition in the EDD region in the rCnm protein groups (Fig. 7). These results suggest that Cnm protein is associated with the initiation of EDD deposition in renal glomeruli.

Discussion

Our previous studies examined the onset of IgAN-like renal lesions using two rat animal models[21,22]. First, Cnm-positive *S. mutans* strain SN74 was administered intravenously to 4-week-old specific pathogen-free Sprague Dawley rats, in which bacteremia induced by invasive dental procedures, such as tooth extractions, was simulated[21]. Second, the SN74 strain was inoculated into the oral cavities of 2-week-old specific pathogen-free Sprague Dawley rats fed a high-sucrose diet for 32 weeks, which produced severe dental caries extending to the pulp space leading to the intravenous entry of oral bacteria from the lesion[22]. Both models developed IgAN-like glomerulonephritis, which led us to consider that Cnm protein on the surface of *S. mutans*, not *S. mutans*, might be an important factor in the development of IgAN-like glomerulonephritis.

For the present study, it was considered important to determine the number of bacteria to be administered to the IgA model rats. It has been estimated that following invasive dental procedures, approximately 60% of treated patients possess greater than 1×10^4 CFU/mL of bacteria in systemic blood[35]. When converted to rats, that value is more than 1×10^8 CFU, which was an important factor for determining the amount of bacteria administered in the present study.

In rat jugular vein administration models used in our previous studies, following administration of 1×10^7 CFU of an *S. mutans* MT8148 strain, bacteria were isolated from blood obtained up to 24 h later[36], while bacteria were isolated up to 48 h later after administration of 1×10^9 CFU[37,38]. In a rat model of infective endocarditis, 1×10^8 CFU of *S. mutans* TW295 was administered and then the bacteria were isolated from blood obtained up to 7 days later[39]. Nevertheless, there is a possibility that such bacteria could be isolated after longer periods in these models with a heart valve injury induced beforehand as compared to normal rats. Furthermore, the strains used in those studies are different from *S. mutans* SN74 used in this study, a PA- and Cnm-positive strain, while MT8148 is PA-positive and Cnm-negative, and TW295 is PA-negative and Cnm-positive.

In our preliminary experiments conducted for a prior study, IgA model rats died when treated with 1×10^9 CFU of *S. mutans* SN74, whereas no clear findings related to development of IgAN were observed at concentrations below 1×10^7 CFU[21]. Those results were referred to when deciding the dose of *S. mutans* SN74 at 1×10^8 CFU for the present experiments.

In addition, though only one intravenous administration was used in this study, it has been reported that 1 mg of dental plaque contains more than 1×10^7 bacteria and the organisms can enter the bloodstream at a high frequency when invasive dental procedures are performed[40]. Therefore, a single administration was considered adequate to allow the bacteria to enter the bloodstream.

In the present study, the rate of hematuria and IgA and complement C3 deposition in the mesangial regions of the SN74 and Comp groups were significantly higher than in the CND and PBS groups; however, there were no significant differences in proteinuria or the levels of any serum parameter between the groups. These results suggest that rats in the SN74 and Comp groups reached the early stage of IgAN. Furthermore, the PAS proliferation scores the SN74 and Comp groups were significantly higher than those of the PBS and CND groups. These histopathological findings suggest that the typical important features of IgAN were present in the SN74 and Comp groups, but not the CND group. Therefore, the present study demonstrated that the Cnm protein has a strong relationship with the development of IgAN.

The mesangial proliferation score and the positive rate of IgA, C3, and/or IgG deposition in the hematuria-positive group were higher than in the hematuria-negative group. Hematuria was suggested to be associated with mesangial proliferation and deposition of IgA, C3, and/or IgG in glomerular mesangial regions. In clinical practice, hematuria is an important indicator of IgAN, but it has become clear that hematuria may also be an important indicator in a model IgAN-like nephritis.

To clarify whether IgAN was caused by Cnm, we administered rCnm protein intravenously. Surprisingly, when the rCnm protein was administered directly to rats, they developed IgAN-like nephritis based on clinical and histopathological findings. Immunoelectron microscopy images of the rCnm protein groups clearly demonstrated Cnm deposition in the EDD region. These findings indicated that Cnm deposits in the mesangial region were related to the deposition of IgA or C3.

Infection of the mucosal epithelium of the upper respiratory tract of IgAN patients by bacteria or viruses is common. Antigenic proteins from viruses, including herpes simplex virus[41], adenovirus[42], hepatitis B virus[43], cytomegalovirus[44], or bacteria, including *Escherichia coli* strain 07[45], *Pseudomonas aeruginosa*[46], *Haemophilus parainfluenzae*[13], MRSA[47–49] are associated with IgAN. However, there have been no reports of the protein

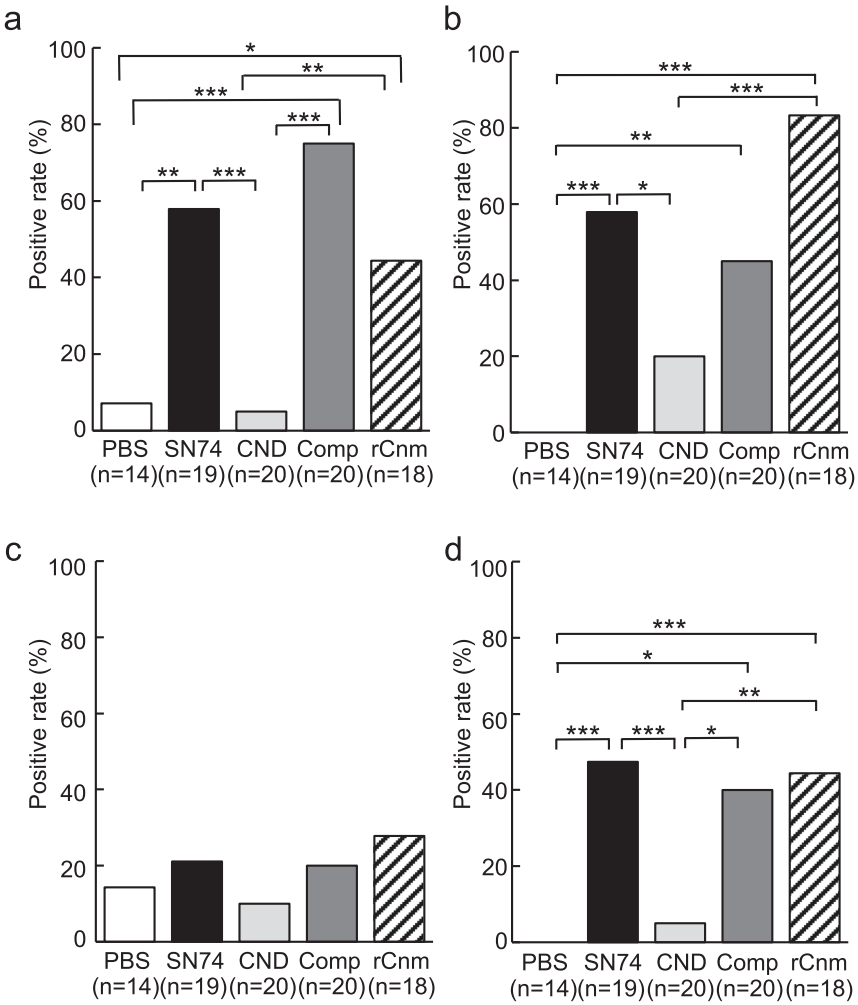

Fig. 6 | Frequencies of positive immunochemical staining. a Frequencies of positive immunochemical staining with anti-IgA, (**b**) anti-C3, (**c**) anti-IgG, and (**d**) anti-IgA and anti-C3. *P*-values of less than 0.05 were considered to indicate significant differences. Statistical significance was determined using Fisher's exact test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

deposited in the lesions. This is the first report to show the deposition of Cnm protein in a lesion.

It is considered possible that of *S. mutans* Cnm proteins may be separated from the bacterium surface, then bind to IgA and other immunoglobulins in blood, leading to induction of glycosylated abnormal IgA (Gd-IgA) expression. On the other hand, the present results revealed that Cnm proteins exist in the dens-deposit region of the kidney. Therefore, those proteins may have an auxiliary role in binding of IgA to the mesangial region or are possibly related to formation of immune complexes by IgA attached with IgG and C3. In a future study, we intend to examine binding of Cnm protein to other immunoglobulins, including IgA, to clarify the mechanism of IgAN development related to Cnm.

In summary, the present study clearly demonstrated that the Cnm protein itself is an important factor in the development of the IgAN. Further studies are needed to clarify the novel potential mechanism of the onset of IgAN to the Cnm protein.

Materials and methods

Bacterial strains

S. mutans strain SN74 (serotype *e*) was isolated from the oral cavity of a patient with severe IgAN[21]. Strain SN74CND, a Cnm-inactivated isogenic mutant strain of SN74, and SN74CND-comp, a Cnm-complemented strain of SN74CND, were constructed in our previous study[50]. In addition, we used rCnm, a recombinant protein extracted after the transformation of *Escherichia coli* BL21 strain with a plasmid containing the *cnm* gene inserted into a pGEX 6P-1 vector[50].

Animal experiments

All rats were treated humanely, in accordance with National Institutes of Health and AERI-BBRI Animal Care and Use Committee guidelines. All procedures used in the present study were approved by the Animal Care and Use Committee of Okayama University (approval number: OKU2020864). The effects of the intravenous administration of *S. mutans* were analyzed in a rat model, as described previously, with some modifications[21,51]. Briefly, specific pathogen-free Sprague–Dawley rats (male, 4-week-old; Japan CLEA, Tokyo, Japan) were randomly divided into PBS, SN74, CND, Comp, and rCnm groups. Rats were allowed free access to water and food throughout the experimental period. Rats were fed an MF diet (ORIENTAL YEAST Co., Ltd, Tokyo, Japan). Rats received intravenous injections (through the jugular vein) of each *S. mutans* type (1×10^8 colony-forming units) suspended in 100 µl PBS or PBS alone (i.e., without the addition of bacteria) or 200 µg of rCnm.

The rats were euthanized 45 days after infection and their kidneys were removed. Urinary levels of protein and CRE were measured by Nagahama Lifescience (ORIENTAL YEAST Co., Ltd.). Serum levels of CRE, ALB, and BUN were measured by Nagahama Lifescience. Serum IgA concentrations were measured using the Rat IgA ELISA Kit (BETHYL Laboratories Inc, Texas, USA), and serum IgG concentrations were measured using the Rat IgG ELISA Kit (BETHYL Laboratories Inc). Hematuria was defined as more than five red blood cells per field of view at ×400 magnification[22].

Histological analyses of kidneys

Histological evaluations of kidneys were performed by using the following methods[20,21]. Excised kidney samples were fixed in 3.7% formaldehyde

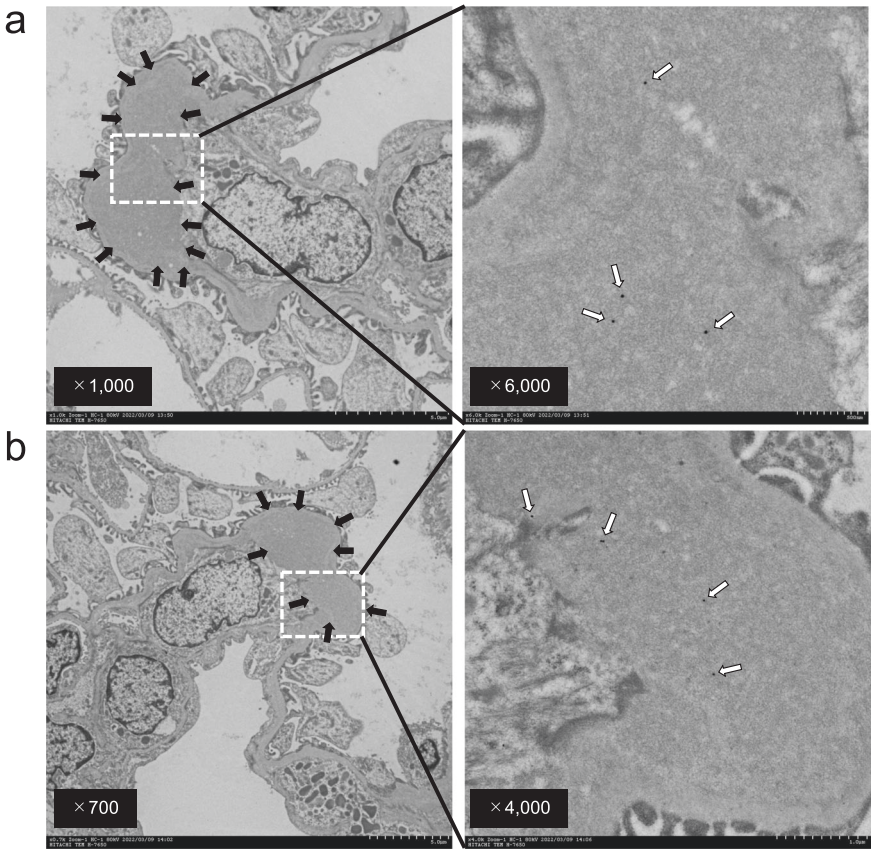

Fig. 7 | Identification of rCnm proteins in the mesangial region by immunoelectron microscopy. a, **b** Representative images of the kidneys of rats administrated with rCnm. Black arrows indicate electron-dense deposits. White arrows indicate colloidal deposition.

(diluted in PBS), embedded in paraffin, and cut into 3 μm-thick sections for histopathological analysis. PAS staining was performed to evaluate increases in the numbers of mesangial cells and mesangial matrix in glomeruli. Mesangial proliferation scores were then calculated based on the proportion of glomeruli with mesangial cells and matrix proliferation among 50 glomeruli in PAS-stained sections[21,22]. MT staining was performed to evaluate fibrosis in the tubules and interstitial area. Additionally, alterations in IgA, C3, IgG, CD34, and α-SMA expression patterns in tissue samples were detected using standard immunohistochemical techniques with IgA-, C3-, IgG-, CD34 (vascular endothelial cell marker), α-SMA -specific antibodies. The primary antibodies used were Purified Mouse Anti-Rat IgA (BD Biosciences, Franklin Lakes, NJ, USA), anti-C3 (B-9) (sc-28294; Santa Cruz Biotechnology, Dallas, TX, USA), anti-rat IgG (H + L), (Alexa Fluor 488 Conjugate) (#4416; Cell Signaling TECHNOLOGY, MA, USA), anti-CD34 (EP373Y) (ab81289; Abcam, Cambridge, MA, USA), and α-Smooth Muscle Actin (D4K9N) XP Rabbit mAb (#19245; Cell Signaling TECHNOLOGY) antibodies. Secondary antibodies were Donkey Anti-Mouse IgG H&L (Alexa Fluor 488) preadsorbed (ab150109; Abcam) and Donkey Anti-Rabbit IgG H&L (Alexa Fluor 647) (ab150075; Abcam). Fluorescence immunostaining was performed using these antibodies. Stained sections were observed using a semi-motorized fluorescence microscope (BX53; OLYMPUS, Tokyo, Japan).

Immunogold transmission electron microscopy

Transmission electron microscopy was performed in accordance with the method of Naka et al.[21]. For pre-fixation, excised kidney tissue specimens were immersed in a solution of 2% glutaraldehyde and 2% paraformaldehyde in PBS (0.1 M, pH 7.4) for 16–18 h. Post-fixation was performed in 2% osmium tetroxide for 1.5 h. After specimens were washed with PBS, they were dehydrated through a graded ethanol series and embedded in low-viscosity resin (Spurr resin; Polysciences, Warrington, PA, USA). Sections (80 nm) were mounted on a 100-mesh nickel grid, incubated with PBS containing 10% goat serum (GEMINI Bio, San Carlos, CA, USA) and 1% BSA. Sections were incubated with anti-Cnm antibody overnight at 4 °C and washed with PBS containing 0.1% BSA more than five times. Then, they were incubated with gold colloid [Anti-IgG (H + L), Rabbit, Goat-Poly, Gold 15 nm, EM; BBI solutions, Crumlin, UK] conjugate goat anti-rat IgG antibody (BioLegend), washed three times with PBS containing 0.1% BSA, and then washed once with distilled water. Specimens were finally fixed with 2% glutaraldehyde. Specimens were observed under a transmission electron microscope (H-7650; HITACHI, Tokyo, Japan).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 Statistics Software (GraphPad, Inc., La Jolla, CA, USA). All results are presented as the mean ± standard error. Differences in whole-body weight serum levels, urine components, and mesangial proliferation scores were assessed by analysis of variance with Bonferroni's correction. *P*-values less than 0.01 were considered statistically significant. Differences in serum IgA and IgG levels were assessed by analysis of variance with Bonferroni's correction. *P*-values less than 0.0167 were considered statistically significant. Positive immunohistochemical staining results and relationship between hematuria and IgA, C3, and/or IgG positive rate results were compared using Fisher's exact test. *P*-values less than 0.05 were considered statistically significant. Relationship hematuria and mesangial proliferation scores results were compared using Student *t*-test. *P*-values less than 0.05 were considered statistically significant.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon request. The source data underlying Figs. 1, 2, 6,

Supplementary Fig. 1, and Table 1, Supplementary Table 1. These source data are listed in Supplementary Data 1.

Materials availability

Correspondence and requests for materials should be addressed to Michiyo Matsumoto-Nakano.

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Author contributions

S.N. and D.M. designed the study under the supervision of M.M.N. and K.N. S.N., D.M., T.M., Y.N., S.I. and M.M.N. performed the animal experiments. Statistical analyses and data interpretation were performed by S.N., D.M., T.M., S.I., R.N. and K.N. S.N., D.M., T.M., K.N. and M.M.N. wrote the manuscript and all authors approved the final version.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Michiyo Matsumoto-Nakano.

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