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CASE REPORT

A Pediatric Case of Anaphylaxis Caused by Matsutake Mushroom (*Tricholoma Matsutake*) Ingestion

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ABSTRACT

Background: Anaphylaxis is one of the severest forms of allergic diseases. Some kinds of mushroom are known as causative allergens in food anaphylaxis. Matsutake mushroom (*Tricholoma matsutake*) is a typical edible mushroom available in autumn in Japan. We encountered an 8-year-old Japanese girl who developed anaphylaxis after ingesting matsutake mushrooms.

Methods: We studied the case in detail, by measuring specific IgE antibodies and conducting skin tests, to confirm the diagnosis. We also detected seven cytokines and chemical mediators in the blood in order to study the pathophysiology of the anaphylaxis.

Results: We diagnosed anaphylaxis caused by ingestion of matsutake mushrooms based on the following. A skin prick test showed a positive reaction to matsutake mushroom, and specific IgE antibody for matsutake mushroom extract was detected in the patient's serum by fluorometric ELISA. Blood levels of chemical mediators including histamine, ECP, tryptase and cytokines such as IL-6, IL-5 and IL-10 but not IFN-γ also increased significantly during the allergic episode.

Conclusions: We demonstrated that chemical mediators including histamine, tryptase and ECP as well as several cytokines were involved significantly during the episode of anaphylaxis. In addition, eosinophils as well as mast cells played significant roles in the anaphylaxis. Furthermore, $CD4^+CD25^+$ T regulatory cells that released IL-10 were likely activated during the anaphylaxis. Matsutake mushroom should be considered as a causative allergen in food anaphylaxis.

KEY WORDS

anaphylaxis, chemical mediators, cytokines, IgE, matsutake mushroom

INTRODUCTION

Matsutake mushroom (*Tricholoma matsutake*) is a typical edible mushroom, available in autumn, in Japan. Some kinds of mushroom spores are known as inhalation allergens and contact allergens that cause allergic reactions like bronchial asthma,[1] and contact dermatitis.[2] There has to date only been a small number of reports describing food anaphylaxis or anaphylactoid reaction caused by mushrooms, in particular by matsutake ingestion.[3-7] In food allergies, it is very important to confirm the causative food in order to prevent relapses.

We report here on a young Japanese girl who developed anaphylaxis due to matsutake mushroom, which was confirmed by the results of a skin prick test and fluorometric ELISA, and the measuring of a specific IgE antibody for matsutake mushroom. We also detected significant increases in the blood levels of chemical mediators and cytokines during the episode.

CLINICAL SUMMARY

CASE

An 8-year-old Japanese girl was admitted to our hospital because of anaphylaxis, swelling of her face and dyspnea, which set on one hour after having a dinner containing matsutake mushrooms at a Japanese res-

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taurant. She has been suffering from bronchial asthma, atopic dermatitis, and pollinosis to Japanese cedar pollen. In her family her sister has atopic dermatitis, and her father has a peach allergy and Japanese cedar pollen allergy.

One hour after having approximately 14 *g* of matsutake mushrooms, the patient experienced nausea, swelling of her face and dyspnea. She was therefore transferred to our emergency unit by ambulance. On arrival, she had difficulty breathing and severe stridor. Angioedema of her face, eyelids and fingers, and skin erythema of her extremities were also evident. Based on such a typical clinical course and symptoms, we diagnosed an anaphylactic reaction due to food allergy, most likely matsutake mushroom. The patient was treated with subcutaneous epinephrine at a dose of 0.005 mg/kg, 200 mg of intravenous hydrocortisone, and inhalation therapy of dexamethasone and epinephrine. We also treated her with intravenous prednisolone at a daily dose of 2 mg/kg for two days. With these treatments, her symptoms gradually improved and she was discharged on the fourth hospital day without any sequelae.

Her laboratory data on admission revealed no abnormalities, except for mild elevation of white blood cell count (12,000/μL), and serum IgE level (504 IU/mL), (PRIST, Sweden Diagnostic, Uppsala, Sweden). CAP System FEIA RAST (Sweden Diagnostic) data showed positivity against the following environmental and inhaled allergens:house dust mite, class 6, and Japanese cedar pollen, class 3. Further studies were carried out and we diagnosed anaphylaxis due to matsutake mushroom.

None of the allergen extracts, including matsutake mushroom, used in this study were commercially available. Therefore, we prepared our own extracts. Of each mushroom, 14 *g* of flesh was homogenized. Next, 350 mL of 0.1 M phosphate buffered saline (PBS, pH7.4) containing 700μ L of protease inhibitor cocktail (Sigma, St. Louis, MI, USA) was added and the resultant substance was homogenized and mixed for 30 minutes at 4℃. The mixture was then centrifuged at 2000 *g* for 10 minutes. The supernatants were collected, dialyzed and filtered. These were then stored as original allergens at -80℃ until used.

Fresh-food prick tests using the prick + prick technique[8,9] were performed with a matsutake mushroom and other mushrooms. All skin prick tests (SPTs) were performed with steel lancets. Responses were read at 15 minutes and graded according to the standard method recommended by the European Academy of Allergy and Clinical Immunology.[10] Grade + is defined as 25% of the area of wheal induced by the positive histamine control (10 mg/mL). Grade ++ is defined as 50%, Grade +++ as 100% and Grade ++++ as 200%. Grade + or larger is considered positive provided the response to the test solution is also larger than that to the negative control.

The specific IgE antibody levels against each mushroom such as matsutake mushroom in the patient and healthy control sera were measured by the method of fluorometric enzyme-linked immunosorbent assay (ELISA) as previously reported.[11] The fluorescence intensity was read as fluorescence units (FU) with a microplate reader (Spectroan FL-2575, Wako Laboratory Co, Tokyo, Japan), whereby 1 FU corresponds to 1 pM 4-MU/well.

We measured the serum levels of seven cytokines using the Cytometric Bead Array (BD PharMingen, San Diego, CA, USA) and Human IL-6 ELISA Ready-SET (eBioscience, San Diego, CA, USA) at different time points (days 1, 2, and 11).[12] We also measured chemical mediators, such as eosinophilic cationic protein (ECP) by Uni-CAP ECP[12](Sweden Diagnostic, normal range, < 5.0μ g/L), tryptase by Uni-CAP Tryptase[13] (Sweden Diagnostic, normal range, 2.1 to 9.0μ g/L) and plasma histamine by histamine radioimmunoassay kit[12] (Immunotech Ltd., Paris, France, normal range, 0.74 to 1.54 nMol/L).

PATHOLOGICAL FINDINGS

The SPT was performed using a fresh matsutake mushroom and other mushrooms including shiitake (*Lentinus edodes*), shimejidake (*Lyophyllum shimeji*), maitake (*Grifola frondosa*), enokidake (*Flammulina velutipes*), and nameko (*Pholiota nameko*). The results were positive grade ++ with the matsutake mushroom and grade + with enokidake at 15 minutes. However, the result was still positive with matsutake at 30 minutes.

The specific IgE antibody levels against matsutake and other mushrooms in the patient's serum were measured by fluorometric ELISA as FU, which resulted in a score of 2.0 FU against matsutake mushroom and zero against other mushrooms. In healthy control serum we did not detect any IgE antibodies against any mushrooms tested (Fig. 1).

We measured the peripheral blood levels of seven cytokines and chemical mediators including ECP, tryptase and plasma histamine. Histamine, ECP, tryptase, IL-5, IL-6, and IL-10 were significantly higher at the time of anaphylaxis. After treatment with glucocorticoids, these levels went down immediately (Fig. 2). However, the IL-2, IL-4, IFN-γ, and TNF-α levels did not change during the episode. Surprisingly, at day 11, ECP and tryptase levels increased again and the IL-6 level was still high.

The results of SPT and specific IgE antibody testing were definitive in diagnosing this case as anaphylaxis to matsutake mushroom. The patient was therefore instructed to avoid eating matsutake mushrooms in order to prevent recurrence.

DISCUSSION

Food anaphylaxis or anaphylactoid reaction caused by matsutake mushrooms is rare even in Japan, with

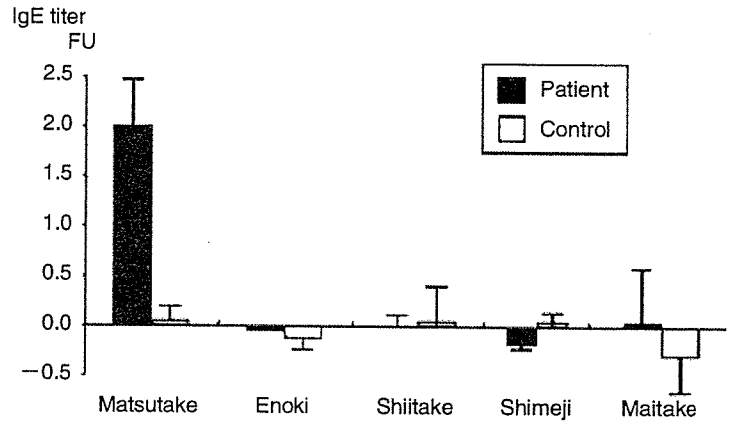

Fig. 1 Specific IgE antibodies for mushrooms in sera by fluorometric ELISA. Bars represent standard deviations of the triplicate data. Solid columns, patient's serum; open columns, negative control serum. 2.0 fluorescent units (FU), as an IgE titer to matsutake antigen, were detected in the patient's serum, but negative to any other kinds of mushrooms or the control serum.

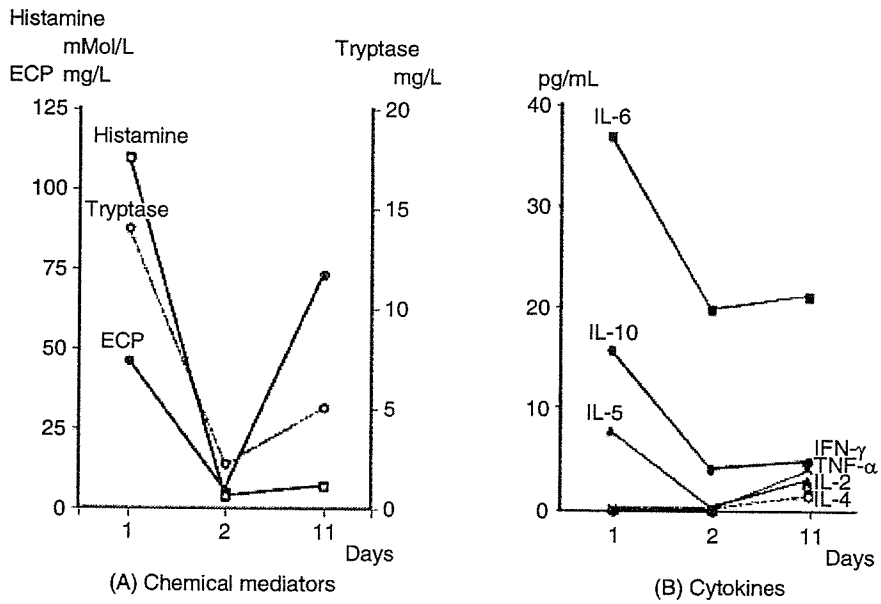

Fig. 2 Blood levels of the chemical mediators in the patient. (A) Histamine in plasma, ECP and tryptase in sera are shown. (B) IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-10 in sera are shown. Day 1 represents the day of the anaphylaxis episode. The normal ranges of all seven cytokines in the serum are <0.1 pg/mL.

few studies published about food anaphylactic reaction caused by the mushroom.[3-7] With food allergies, especially anaphylactic reactions, it is important to confirm the causative food or the component of food to prevent recurrence.

There have to date been five case reports about anaphylactic reactions caused by matsutake mushrooms.[3-7] Three cases were anaphylaxis, one case was anaphylactoid reaction after matsutake mushroom ingestion, and one case was food-dependent exercise-induced anaphylaxis due to matsutake mushroom ingestion followed by exercise. In four of

the five cases, positive reactions to skin tests or provocation tests were obtained, but the specific IgE antibody for matsutake mushroom was not detected in any case.

In the present case, the diagnosis of anaphylactic reaction was not difficult, because of its typical clinical course and symptoms. Based on the dinner menu of the patient before the episode, we suspected matsutake mushroom as a causative allergen. Therefore, the patient was screened and tested for all food allergens in the menu, and showed a positive reaction to matsutake mushroom on the SPT. The patient's serum specific IgE antibody levels against matsutake mushroom extract, measured by fluorometric ELISA, strongly suggested that her anaphylaxis was caused by matsutake mushroom.

The skin prick + prick test was thus useful to confirm the food allergen of the anaphylaxis. We considered a positive reaction against enokidake in SPT to be a non-specific reaction in our case because the patient had already eaten enokidake without any problems, and specific IgE antibody to enokidake was not detected. However, we could not rule out completely the possibility of cross-reactive epitopes between matsutake and enokidake. Measuring the specific IgE antibody by fluorometric ELISA was also useful to determine the causative food allergens, in comparison with CAP-RAST as reported previously.[3-7]

Furthermore, in our case higher blood levels of chemical mediators and cytokines including histamine, tryptase, ECP, IL-5, and IL-6[14] suggested that the immune balance tended to be T helper 2 (Th2) dominant and mast cells and eosinophils were activated at the time of the anaphylaxis. A similar finding was observed in patients with acute allergic reactions in an emergency study.[15] Interestingly, immunosuppressive/regulatory cytokine IL-10[16] also increased during the episode in our case. It is now known that $CD4^+CD25^+$ T regulatory cells release IL-10.[17] Our data suggest that T regulatory cells[16] are also activated to maintain an immunological balance such as between Th2 to Th1 cells and to inhibit inflammation during anaphylaxis. These responses were immediately suppressed by the administration of glucocorticoids. In addition, re-elevation of ECP and tryptase were shown on day 11. This might not be explained simply by a rebound phenomenon. Rather, the activations of eosinophils and mast cells might persist longer during anaphylaxis than first thought. Further studies are necessary to clarify this.

In conclusion, an SPT and specific IgE antibody testing using fluorometric ELISA were useful methods to determine that the causative food in a school girl who developed anaphylaxis was matsutake mushroom. We also demonstrated that many kinds of chemical mediators and cytokines were involved in the anaphylaxis. We believe that this case has shed further light on the understanding of the pathophysiology of food anaphylaxis.

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BRIEF REPORT

Intravenous gamma globulin for thrombotic microangiopathy of unknown etiology

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Abstract We encountered the case of a 4-year-old boy with thrombotic microangiopathy (TMA) of unknown etiology. Verotoxin-induced hemolytic uremic syndrome (HUS), *Streptococcus-pneumoniae*-related HUS, factor H deficiency, drug-induced thrombotic thrombocytopenic purpura (TTP), and ADAMTS13 (von Willebrand factor-cleaving protease; a disintegrin-like and metalloprotease with thrombospondin type 1 repeats)-related TTP were excluded. His condition was refractory to anticoagulants and plasma exchange, and his clinical course was catastrophic, with central nervous system symptoms and progressive renal failure. However, factual treatment of intravenous gamma globulin (IVIG) ended the hemolysis and resulted in a rise in platelet count. He fully recovered except for end-stage renal failure, but he underwent a successful renal transplant after peritoneal dialysis. He has not suffered a relapse of TMA or an allograft rejection for 4 years. IVIG might be an option for some patients with TMA of unknown etiology refractory to conventional treatment.

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Keywords Thrombotic microangiopathy · Gamma globulin · Thrombotic thrombocytopenic purpura · Hemolytic uremic syndrome · Plasma exchange · ADAMTS13

Introduction

Thrombotic microangiopathy (TMA) and thrombotic thrombocytopenic purpura–hemolytic uremic syndrome (TTP/HUS) are acute syndromes characterized by thrombocytopenia, microangiopathic hemolytic anemia, neurological abnormalities, and acute renal failure. TTP was associated with high mortality before plasma exchange (PE) was introduced as initial therapy. The survival rate increased from 10% to more than 80%, thanks to PE [1–5]. However, patients who do not have decreased activity of von Willebrand factor-cleaving protease [a disintegrin-like and metalloprotease with thrombospondin type 1 repeats (ADAMTS13)] or autoantibody to ADAMTS13 are frequently refractory to PE, and their prognosis is poor [6]. Additionally, atypical HUS is known to be associated with a more unfavorable prognosis compared with verotoxin-induced HUS [2]. We experienced the case of a 4-year-old boy with TMA of unknown etiology, which showed a catastrophic course in spite of PE and pulsed steroid therapy, and he underwent intravenous gamma globulin (IVIG) treatment. IVIG treatment might be effective in some patients with TMA of unknown etiology refractory to PE and steroid therapy.

Case report

A 4-year-old boy was referred to our hospital because he had proteinuria. He had had Kawasaki disease at the age of

10 months and bronchial asthma when he was 2 years old. Kawasaki disease was treated with gamma globulin. He had no cardiac complications. Additionally, he presented no altered parameter indicative of renal dysfunction at the time of recovery from Kawasaki disease. On admission, his body temperature was 36.6°C, his heart rate was 90 beats/min, his blood pressure was 156/118 mmHg, and his respiratory rate was 20 breaths/min. No abnormality, except for hypertension, was detected during chest, abdominal, and neurological examinations. The results of urinary examinations were as follows: protein (3+: 565 mg/dl), β2-microglobulin (MG) 12,610 ng/ml (normal<250), N-acetyl-{beta}-D-glucosaminidase (NAG) 39.6 U/l, red blood cells (RBC) 30–50/high power field (HPF), white blood cells (WBC) 1–5/HPF, granular casts (+) and fatty casts (+). The urine protein/creatinine ratio was 8.2. Blood examinations demonstrated a hemoglobin concentration of 12.6 g/dl (126 g/l), leukocytes count 9,750/μl, platelet count 59×10^3/μl, PT (INR) 1.01, activated partial thromboplastin time (APTT) 35.8 s, fibrinogen 338 mg/dl (3.38 g/l), fibrin degradation product E (FDP)-E 282 ng/dl (normal<60), total protein 6.8 g/dl (68 g/l), serum albumin 4.5 g/dl (45 g/l), lactate dehydrogenase (LDH) 671 U/l (normal value 120–200 U/l), total bilirubin 2.1 mg/dl (35.9 μmol/l), direct bilirubin 1.6 mg/dl (27.4 μmol/l), blood urea nitrogen (BUN) 31 mg/dl (11 mmol/l), serum creatinine 0.97 mg/dl, urate 6.6 mg/dl (458 μmol/l), C-reactive protein (CRP) 0.1 mg/dl (10 mg/l), immunoglobulin G (IgG) 858 mg/dl (8.58 g/l), antinuclear antibodies (ANA) (-), anti-double-stranded DNA (anti-dsDNA) antibody (-), platelet-associated (PA) IgG (-), cytoplasmic/perinuclear antineutrophil cytoplasmic antibodies (C/P-ANCA) (-), anticardiolipin antibody (-), and direct and indirect Coombs' test (-). The results of the microbiological examinations were negative for antiverotoxin lipopolysaccharide IgM. Verotoxin-producing *Escherichia coli* or *Streptococcus pneumoniae* was not detected in the throat or stool cultures. On examination of peripheral blood smears, a small number of fragmented red cells was noted, but the platelet size was normal. A bone marrow smear showed an increased number of megakaryocytes, but other marrow elements were normal. Impaired renal function, together with the presence of thrombocytopenia, anemia, fragmented erythrocytes, and increased LDH and bilirubin suggested TMA. To reduce the risk of complications due to thrombocytopenia, we treated the child with methylprednisolone pulse therapy (MPT) before renal biopsy. As a result, the platelet count increased to 179×10^3/μl, and the serum level of creatinine decreased (Fig. 1). The renal biopsy showed vascular thrombi, mesangial cell lysis, and glomerular sclerosis, which are characteristic features of TMA (Fig. 2).

However, thereafter, the second MPT was ineffective, and his symptoms flared up. Renal function started to deteriorate again and thrombocytopenia recurred. Then, on the 23rd hospital day, he presented generalized convulsions and disturbance of consciousness (Fig. 1). Brain magnetic resonance imaging (MRI) was normal, but technetium-99m-methylene diphosphonate hexamethylpropyleneamine oxime (99mTc-HAMPAO) brain single proton emission computed tomography (SPECT) showed a diffuse decrease of brain blood flow. The electroencephalogram (EEG) showed diffuse slow waves and paroxysmal discharges. Hemolysis requiring transfusion of red blood cells occurred. Anticoagulants and further therapy with pulse methylprednisolone had no effect. Therefore, we started PE with frozen fresh plasma (FFP) every day, but PE proved ineffective. The possibility of heparin-induced thrombocytopenia (HIT) was also ruled out. On the 40th hospital day, laboratory data showed normal ADAMTS13 activity and absence of the inhibitory autoantibody to ADAMTS13, which was examined before PE and FFP transfusion. The clinical usefulness of PE has been demonstrated only for ADMATS13-related TMA [6]. Therefore, we discontinued PE and tried treatment with IVIG (1 g/day for 3 days) from the 55th hospital day. Surprisingly, the platelet count normalized 7 days after the start of IVIG (Fig. 1). The patient's level of consciousness also improved dramatically. Brain blood flow, as assessed by brain SPECT and the EEG, also normalized. He fully recovered except for end-stage renal failure. Thus, he received a renal transplant 1 year and 7 months after the beginning of peritoneal dialysis. He has suffered no relapse of TMA or rejection of the allograft during the two and a half years that has elapsed since the renal transplant.

Discussion

The etiology of TMA in this patient was uncertain. His clinical course was atypical compared with that of ADMTS13-related TTP or verotoxin-induced HUS. The clinical course was relatively slower than the typical course of these of diseases. At the initial presentation, our patient showed only modest thrombocytopenia and mild renal insufficiency. He responded to the first MPT, but the effect was transient and limited. Later on, he developed neurological manifestations and overt anemia. Plasma exchange with FFP and anticoagulant therapy were not effective. However, he was rescued by IVIG in the end. It is difficult to give a correct diagnosis to him. However, the diagnosis should be atypical HUS or TMA of unknown origin because his main clinically indicated manifestation was renal dysfunction, and his medical condition was refractory to PE. Autoimmune hemolytic anemia and TTP were also ruled out.

The underlying cause and the platelet consumption mechanism in some patients with TMA, especially in TTP, have been elucidated. Von Willebrand factor (vWF), which is synthesized in endothelial cells and assembled in

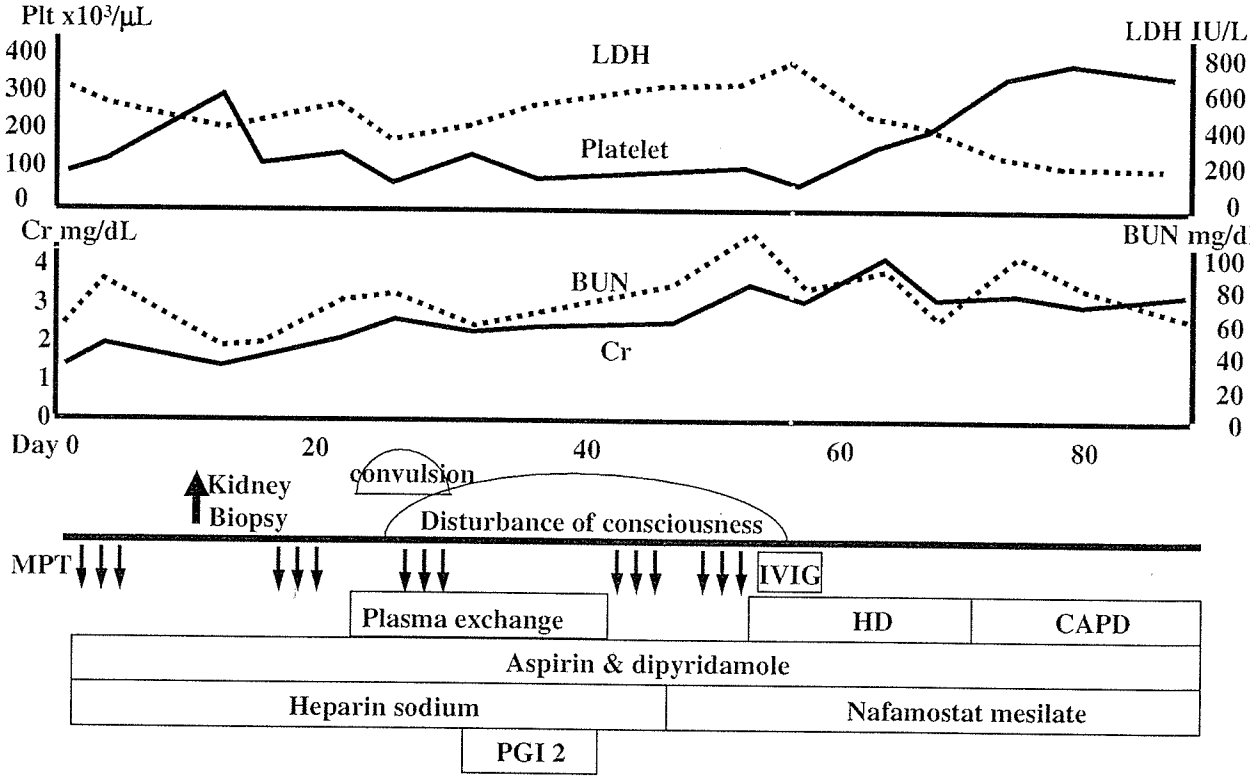

Fig. 1 Clinical course of the patient: *IVIG* gamma globulin, *CAPD* continuous ambulatory peritoneal dialysis, *HD* hemodialysis, *PGI2* prostaglandin, *MPT* methyl prednisolone pulse therapy.

Note: To convert serum creatinine in mg/dl to μmol/l, multiply by 88.4; to convert urea nitrogen in mg/dl to mmol/l, multiply by 0.357. Normal value of lactate dehydrogenase (LDH): 120–200 U/l

larger multimers, is present in normal plasma. The larger multimers are rapidly degraded in the circulation to a size within the normal range for vWF multimers by ADAMTS13 [7–9]. Deficiency of or an autoantibody directed against ADMTS13 could lead sequentially to the accumulation of vWF multimers, platelet aggregation, and the platelet clumping characteristic of TTP [10–12].

Plasma exchange serves to remove autoantibodies and high molecular weight vWF multimers [12, 13]. PE and potent immunosuppressive therapy are effective in patients with TTP, which is associated with 90% mortality in adults [5, 14, 15]. However, 10–20% of patients either show a transient or incomplete response to plasma therapy or do not respond to it. Mori et al. reported that patients with a

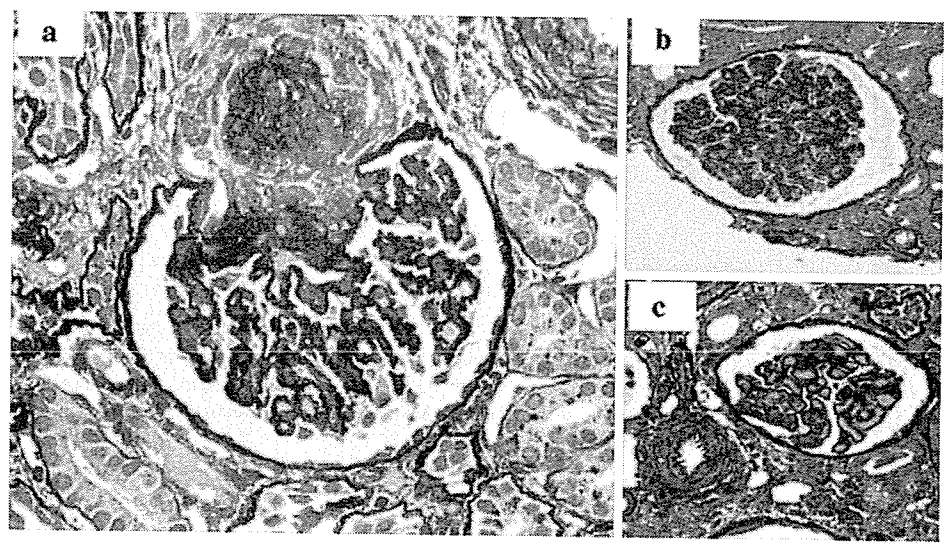

Fig. 2 Renal biopsy findings. a Thrombus in arteriole at the glomerular hilus [periodic acid-methenamine silver (PAM) stain ×400]. b Advanced diffuse glomerular sclerosis in the glomeruli (PAM stain ×400). c Mesangiolysis in the glomeruli (PAM stain ×400)

clinical diagnosis of TTP who had moderate deficiency of the vWF cleaving protease without detectable antibodies against ADMTS13 might have refractory TTP, and their prognosis is poor [6]. Actually, our patient did not have the inhibitory autoantibody to ADAMTS13.

Historically, IVIG was tried for TTP before PE became the first-line treatment. There have been some successful reports of IVIG therapy for TTP, including PE- or plasma-infusion-refractory cases: two children and 12 adults [16–20]. In summary, ten of those 14 patients responded to IVIG and achieved complete remission. Interestingly, nine of the ten patients resisted or responded transiently to PE or FFP infusion, which may suggest that these patients had some atypical features. Among them there were two pediatric cases. One of the two patients was successfully treated with IVIG alone. The other patient did not respond to IVIG but responded to FFP infusion. Unfortunately, the presence of an ADMTS13 inhibitor or ADMTS13 activity was unknown in these reports because these cases were described before the discovery of ADMTS13. On the other hand, we could not find any case report showing the efficacy of IVIG for atypical HUS. The mechanism of efficacy of IVIG in patients with TMA refractory to PE or FFP infusion is still unknown and should be elucidated. Our patient was repeatedly subjected to PE, which could have served to remove autoantibodies. Therefore, the mechanism of the effectiveness of IVIG in our patient could not be explained by an interference of the autoantibody with Fc receptor activity. IVIG may neutralize a platelet-aggregating factor of unknown origin or eliminate unknown responsible pathogens causing TTP [21, 22]. In any case, it may be of value to choose IVIG for patients with TMA refractory to PE or FFP infusion who show normal activity of ADAMTS13 or absence of an ADAMTS13 inhibitor. Although measuring ADAMTS13 activity and its inhibitor is recently becoming a more common practice, it has not yet been broadly standardized. A rapid and widely applicable laboratory test is strongly desired because of the poor prognosis of patients with TMA refractory to conventional therapy, such as PE, steroids, and anticoagulants.

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LETTER TO THE EDITOR

Runx1 transcription factor is involved in the regulation of KAP5 gene expression in human hair follicles

KEYWORDS
Keratin-associated proteins;
Overexpression;
Real-time PCR;
Runt-related transcription factor;
Transforming growth factor-beta

In mammalian hair fibers, keratin intermediate filaments are embedded in the interfilamentous matrix containing keratin-associated proteins (KAPs). The KAP5 subfamily, an ultrahigh sulfur KAP (>30 mol% content), was recently characterized in human species, and consists of 11 genes located on human chromosome 11p15.5 and 11q13.5 [1]. The expression site of KAP5 genes is mostly restricted to the cuticle layer of anagen hair follicles [2]. The runt-related protein (Runx) transcription factor family plays important roles not only in hematopoiesis, osteogenesis and neurogenesis, but also in developmental processes [3]. Recently, it was reported that Runx3 is predominantly expressed in the dermal compartment of hair follicles during mouse morphogenesis and hair cycling [4]. A DNA microarray analysis revealed that Runx1 expression was up-regulated during anagen development induced by wax depilation in mice [5]. Whereas numerous binding motifs of Runx1 are predicted in the promoter region of human KAP10 and 12 family genes expressed in the cuticle [6], the roles of the Runx family in human hair follicles have not been explored. In this study, we examined the site of Runx1 expression in human hair follicles by an immunohistochemical procedure, and the effect of Runx1 on KAP5.1 gene expression by an overexpression study in the cell culture system.

Human tissue specimens from scalp skin were obtained during plastic surgery with the informed consent of donors. Paraffin sections after antigen unmasking in 10 mM citrate buffer (pH 6.0) were incubated with rabbit polyclonal anti-Runx1 antibody (Abcam, Cambridge, UK). Runx1 immunoreactivity was visualized using an anti-rabbit staining kit and 3-amino-9-ethylcarbazole (AEC) solution (HISTFINE, Nichirei, Tokyo, Japan). Immortalized outer root sheath (IORS) cells[1] cultured in keratinocyte serum-free medium (K-SFM: Invitrogen, Carlsbad, CA) were transfected with mouse Runx1 expression plasmid (GenBank accession no. BC069929: Open Biosystems, Huntsville, AL) or β-galactosidase (β-gal) expression plasmid (Invitrogen) using the FuGENE6 reagent (Roche Diagnostics, Indianapolis, IN). *hKAP5.1* gene expression in IORS cells was quantified by real-time RT-PCR using the LightCycler system combined with SYBR Green (Roche Diagnostics, Indianapolis, IN). PCR primers were used for the hKAP5.1 gene (KRTAP5-7: GenBank accession no. AB126076), 5′-tctcttcccaagtcaactgc-3′ and 5′-agagtgttggacaggcaaag-3′ (206 bp); for the human glyceraldehyde-3-phosphate dehydrogease gene (GAPDH), 5′- gagtcaacggatttggtcgt -3′ and 5′-tgggatttccattgatgaca -3′ (201 bp). The site of *hKAP5.1* expression in human hair follicles was performed by in situ hybridization (ISH) as described previously [2].

The cell nucleus of the hair shaft was strongly positive for Runx1 immunostaining at the keratinizing level in anagen hair follicles (Fig. 1a). The nucleus of the inner root sheath (IRS) and the outer root sheath (ORS) cells also showed intense Runx1 immunoreactivity but not bulb matrix cells (Fig. 1b). The cuticle layer of the hair shaft was intensely stained in the upper portion of the keratinizing area (Fig. 1d) where the expression of KAP5 genes was initiated as shown in Fig. 1e. The outermost cell layer of ORS showed intense Runx1 immunoreactiv-

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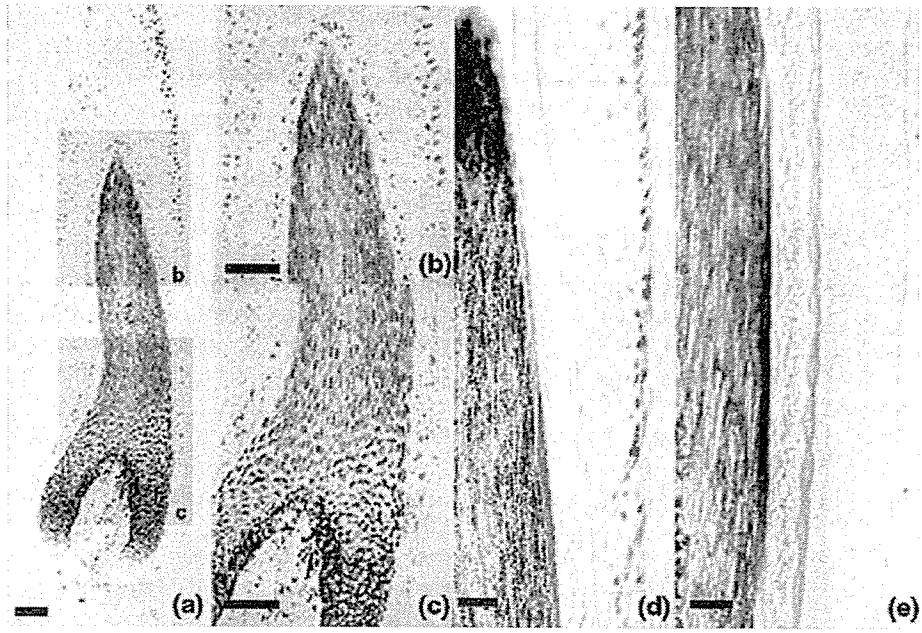

Fig. 1 RUNX1 immunoreactivity in human anagen hair follicles. (a) Runx1 immunoreactivity was detected in the nuclei of the hair shaft, IRS, and ORS but not the lower bulb matrix. Scale bar: 50 μm. (b) Higher magnification of the upper portion in the keratinizing area. The inner layers showed lower Runx1 immunoreactivity compared to the outer layer in the ORS. Scale bar: 50 μm. (c) Higher magnification of the lower portion in the keratinizing area. The bulb matrix cells just above the DP were completely negative for Runx1 immunostaining. Scale bar: 50 μm. (d) Runx1 immunoreactivity (red colour) was apparent in the cuticle layer of the hair shaft before its detachment from the IRS. At this portion, the outermost cell layer of the ORS was also intensely stained by Runx1 immunostaining. Scale bar: 20 μm. (e) *hKAP5.1* transcripts (blue colour) were predominantly observed in the superior or the same area of the Runx1 expression site in the cuticle layer.

ity (Fig. 1d) compared to the other inner layer. Both the duct and the secretary portion of the sweat gland were also intensely stained by anti-Runx1 antibody (data not shown). Putative binding motifs of RUNX1/AML1 (TGTGGT) were found in the upstream genomic sequence of human KAP5 genes by bioinfomatic analysis (data not shown) as well as human KAP10 and 12 genes [6]. At least three RUNX1/AML1 motifs were predicted within the region 1.2 kb upstream of the initiation codon (Fig. 2a) of the *hKAP5.1* gene. We examined the expression of *hKAP5.1* in cultured normal ORS cells as a representative of follicular keratinocytes. Residual genomic DNA was thoroughly removed by DNase I treatment before reverse transcription. The expression of the *hKAP5.1* gene was detected in normal ORS cells at the RT-PCR level (Fig. 2b, lane 2). The amplified PCR products were identified as a proper fragment of the *hKAP5.1* gene by direct sequencing (data not shown). We also confirmed that the PCR products were not artificially amplified from genomic DNA contamination using non-reverse transcribed samples as a template (Fig. 2b, lane 3). To examine the regulation of *hKAP5.1* gene expression by Runx1 in follicular epithelial cells, the *Runx1* gene was overexpressed in IORS cells, followed by quantification using real-time PCR. The expression level of the *hKAP5.1* gene was significantly up-regulated by overexpression of the *Runx1* gene in a dose-dependent manner while the expression level of the GAPDH gene was almost identical, as shown in Fig. 2c.

Several transcription factors such as GATA-3, Hox13, and Lef-1 are responsible for the differentiation of follicular epithelial cells, and are predominantly expressed in the keratinizing area of anagen hair follicles [7]. Here, we have showed that Runx1 immunoreactivity is intense in the keratinizing area of anagen hair follicles, the observation of which is consistent with Runx1 expression in the epithelial compartments of mice developing whiskers [8]. Runx3 is predominantly expressed in the dermis and DP, and Runx3-deficient mice display an aberrant fur shape [4]. Loss of Runx function leads to cancer in several tissues, and Runx is regulated by the transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) pathway [9]. TGF-β superfamily signalling is involved in morphogenesis in mammalian hair follicles. Furthermore, both BMP and TGF-β ligands

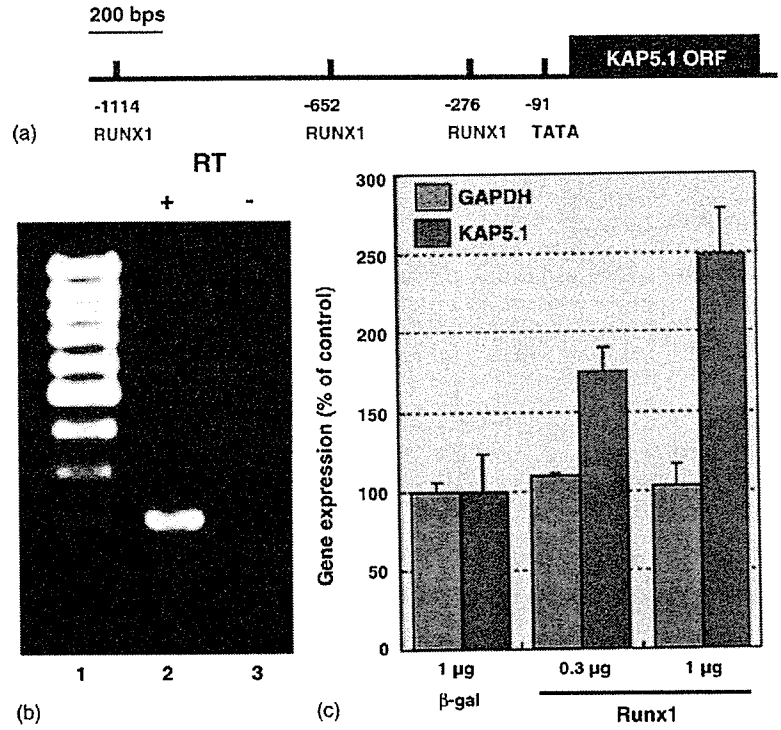

Fig. 2 Involvement of Runx1 in the regulation of *hKAP5.1* gene expression. (a) The putative binding sites of RUNX1/AML1 in the *hKAP5.1* gene. Three RUNX1/AML1 motifs were expected within the 1.2 kb upstream of the *hKAP5.1* gene by bioinfomatic analysis. (b) *hKAP5.1* gene expression in ORS cells. Total RNA was prepared from ORS cells cultured in keratinocyte serum-free medium (Invitrogen), followed by reverse transcription. The cDNA fragment of the KAP5.1 gene (206 bp) was amplified by the PCR reaction only when reverse-transcribed templates were used (see lanes 2 and 3). Lane 1: 100 bp ladder marker; lane 2: RT plus; lane 3: RT minus. (c) Up-regulation of *hKAP5.1* gene expression by Runx1. Cultured IORS cells were transfected with Runx1 or β-gal expression plasmid by FuGENE6, followed by the quantification of *hKAP5.1* gene expression by real-time RT-PCR. *hKAP5.1* gene expression (purple columns) was elevated by Runx1 overexpression in a dose-dependent manner while GAPDH expression (blue columns) was not affected by either Runx1 or β-gal overexpression.

are abundant in the keratinizing area of growing hair follicles in mature hair cycling [10], in which area Runx1 immunoreactivity is extremely high, as shown in this study. It is possible that the Runx family may play crucial roles in the maturity of normal hair follicles through direct or indirect transcriptional regulation by the TGF-β/BMP pathway. Since *hKAP5.1* gene expression was observed in the ORS cells under culture conditions, we evaluated the influence of Runx1 on *hKAP5.1* expression by a transfection study using IORS cells. The up-regulation of *hKAP5.1* expression by Runx1 is well consistent with the results of bioinfomatic and ISH analysis on the *hKAP5.1* gene. This data also primarily demonstrate that Runx1 is involved in the transcriptional regulation of differentiation molecules in follicular epithelial cells. The broader spectrum of Runx1 immunoreactivity not restricted in the cuticle layer possibly shows Runx1 involvement in the regulation of other KAP genes and/or hair keratin genes.

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LETTER TO THE EDITOR

Epiregulin, a member of the EGF family, is over-expressed in psoriatic epidermis

KEYWORDS
Epiregulin; EGF family; Psoriasis; TGF-α; Amphiregulin; Northern blot; *In situ* hybridization

Psoriasis is characterized by the hyperproliferation of keratinocytes, altered epidermal differentiation, dermal angiogenesis, and a dense lesional infiltrate in the dermal and epidermal component, consisting mainly of macrophages, lymphocytes, and neutrophils. To date, the expression of many regulatory molecules has been well clarified in psoriatic epidermis. Previous reports have shown that various cytokines and growth factors are over-expressed in psoriatic epidermis [1]. Keratinocytes are the main component cells of the epidermis, and their growth is regulated by both positive and negative mediators [2]. Of these mediators, the most important mechanism for the proliferation of keratinocytes is the signal from the epidermal growth factor (EGF) receptor. The EGF family consists of EGF, transforming growth factor-α (TGF-α), heparin binding EGF-like growth factor (HB-EGF), amphiregulin, epiregulin, betacellulin, epigen, neuregulin (NRG)-1, NRG-2, NRG-3, and NRG-4, and the EGF receptor (EGFR) family consists of EGFR (also called ErbB1), ErbB2, ErbB3, and ErbB4 [2]. Previous reports have shown that TGF-α, amphiregulin and HB-EGF are over-expressed in psoriatic epidermis [3–5]. Given that epiregulin is a member of the EGF family and an autocrine growth factor for normal human keratinocytes [6], we speculated that epiregulin is over-expressed in psoriasis. To prove our hypothesis, we investigated the expression of epiregulin as well as TGF-α and amphiregulin in psoriatic epidermis.

All procedures that involved human subjects received prior approval from the Ethics Committee of Ehime University School of Medicine, Toon, Ehime, Japan, and all subjects provided written informed consent. Twelve psoriatic lesional skin samples and 10 normal healthy skin samples were obtained. To exclude the RNA from the dermis, we separated the epidermis from the dermis by a heat-separation technique. The specimens were heated at 60 °C in sterile saline for 1 min. The epidermis was then separated from the dermis, immediately frozen in liquid nitrogen. We confirmed that heat-separation did not influence on the results. Total RNA was extracted from snap-frozen epidermis by using Isogen (Nippon Gene, Tokyo, Japan). Northern blot analysis was performed as previously described [6]. TGF-α mRNA was detected in all the psoriatic

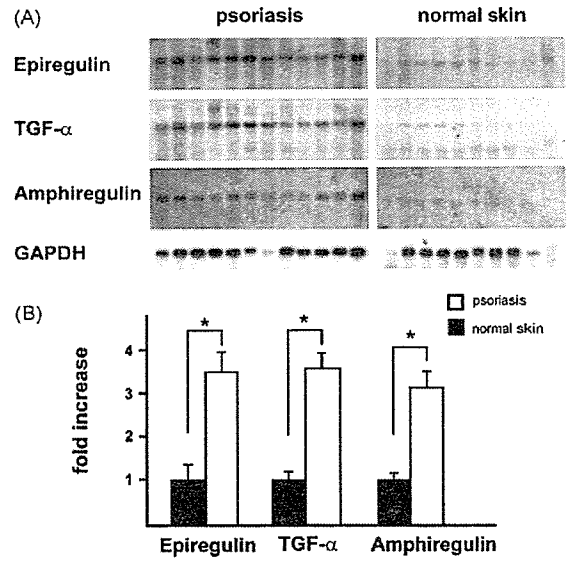

Fig. 1 mRNA expression of epiregulin, TGF-α, and amphiregulin in psoriatic epidermis. (A) The mRNA expression levels of epiregulin, TGF-α, and amphiregulin were analyzed by Northern blotting in psoriatic and normal epidermis. (B) Densitometric analysis of Northern blotting. Quantification of the signals was performed by using a densitometer and analyzed by ImageQuant software. Normal epidermis signal values were taken as 100% control. The signals were adjusted by GAPDH as an internal standard. $^{*}p < 0.05$.

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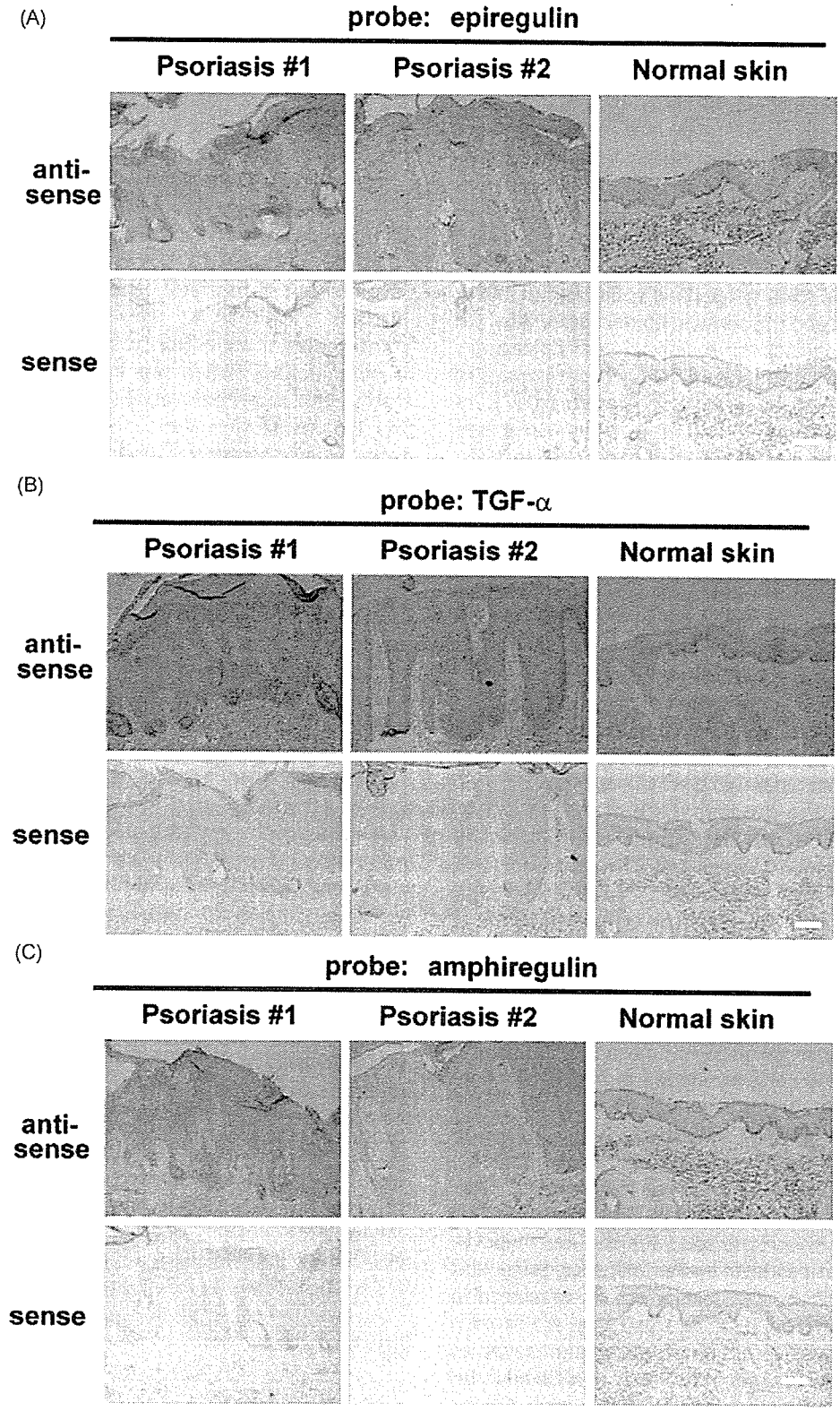

Fig. 2 *In situ* hybridization of epiregulin, TGF-α, and amphiregulin in psoriatic epidermis. *In situ* hybridization was performed using an automated *in situ* hybridization protocol. (A) Epiregulin; (B) TGF-α; (C) amphiregulin. Bar: 100 μm.

epidermis samples and was expressed at higher levels than in the normal epidermis. Amphiregulin mRNA was also detected in all the psoriatic epidermis samples and was expressed at higher levels than in the normal epidermis. The 4.8-kb transcript of epiregulin was detected in both normal and psoriatic epidermis, although the expression level was higher in the psoriatic epidermis than in the normal epidermis (Fig. 1A). Densitometric analysis revealed that TGF-α and amphiregulin mRNA were elevated by 3.6- and 3.2-fold, respectively. Epiregulin mRNA was also increased in psoriatic epidermis by 3.5-fold (Fig. 1B). To identify the localization of epiregulin, TGF-α, and amphiregulin mRNA, we performed *in situ* hybridization as previously described [7]. In normal skin, we found that epiregulin mRNA was expressed in the basal layer and that its expression was very faint. However, epiregulin mRNA was over-expressed in the spinous layer, mainly in the uppermost region of the spinous layer, but not in the basal layer of the psoriatic lesional epidermis (Fig. 2A). TGF-α and amphiregulin mRNA were also over-expressed in the spinous layer, but not in the basal layer, of the psoriatic lesional epidermis (Fig. 2B and C).

Considerable evidence currently exists to support the concept that psoriasis is mediated by many inflammatory cytokines. There is a predominance of Th1 cytokines, mainly interferon gamma (IFN-γ), in contrast to the predominance of Th2 cytokines found in atopic dermatitis. This immune-mediated aspect of psoriasis pathogenesis has been confirmed by the use of cyclosporine or targeted therapies against T cells, CD11a, or TNF-α [1]. With regard to keratinocytes, psoriatic plaques represent a hyperproliferative condition and deregulated differentiation [1]. Keratinocytes are the main component cells of the epidermis, and the important mechanism for keratinocyte growth is the EGF receptor-ligand system. In this EGF receptor-ligand system, the signal from ErbB1 (EGFR) is important for keratinocyte growth. TGF-α, HB-EGF, amphiregulin and epiregulin are autocrine growth factors and all bind to ErbB1 [2]. Previous reports have shown that TGF-α and amphiregulin mRNA are over-expressed in psoriatic lesional skin and play important roles for the keratinocyte proliferation of psoriatic epidermis, suggesting that all members of the EGF family are up-regulated in psoriatic epidermis [3–5]. In this study, we investigated the mRNA expression of epiregulin as well as TGF-α and amphiregulin in psoriatic and normal epidermis. The mRNA expression of TGF-α and amphiregulin was elevated in psoriatic epidermis, as compared to normal skin, as previously reported. The mRNA expression of epiregulin was also increased in psoriatic epidermis. Over-expressed epiregulin mRNA was localized in the spinous layer, but not in the basal layer, of the psoriatic epidermis. TGF-α and amphiregulin mRNA localization in psoriatic epidermis is within the spinous layer. This deregulated growth factor signaling may contribute to the hyperproliferative condition of psoriasis. In fact, a transgenic mouse with the amphiregulin gene revealed a psoriasis-like phenotype [8]. Based on our results and previous reports, a cross-induction mechanism of the keratinocyte-derived EGF family is involved in the development of psoriatic hyperproliferative epidermis.

Intracellular signaling systems such as Erk/JNK or MAP kinase, which are activated by the EGF family, are up-regulated in psoriatic epidermis [9]. Recent studies have shown that transgenic mice over-expressing a constitutive active form of STAT3 develop a psoriasis-like phenotype [10]. Because these molecules are downstream of EGFR signaling, an over-expressed EGF family may contribute to the pathogenesis of psoriasis by activating signal transduction molecules. Because topical treatment will probably remain the mainstay of psoriasis therapy for most patients, and aberrant expression of epiregulin as well as TGF-α and amphiregulin could facilitate the development of proliferative pathological conditions of psoriasis, additional targeting of such signal transduction molecule and/or growth factors may enhance anti-psoriatic therapy.

In conclusion, we have demonstrated the over-expression of epiregulin in psoriatic epidermis. Autocrine EGF-related growth factors may play an important role in the pathogenesis of psoriasis.

Acknowledgments

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Ⅴ.　平成18年度班会議プログラム

厚生労働省科学研究費補助金、難治性疾患克服事業「難治性皮膚疾患（重症多形滲出性紅斑（急性期）を含む）の画期的治療法に関する研究」

平成１８年度第１回班会議プログラム

主任研究者：愛媛大学医学部皮膚科、橋本公二

日時：　平成１８年８月５日（土）
　　９：３０から１７：００まで

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