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Supporting information

The following supplementary material is available:
Fig. S1. Screening procedure for substrate sequences preferred by TGase 3 using a phage-displayed random peptide library.

This supplementary material can be found in the online version of this article.

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Letter to the Editor

ABCA12 dysfunction causes a disorder in glucosylceramide accumulation during keratinocyte differentiation

Harlequin ichthyosis (HI) is an autosomal recessive congenital ichthyoses, and patients of this disease frequently present with severe hyperkeratosis and scales over all of their epidermal surfaces. Recently, the gene encoding ABCA12 was identified as a causative gene for HI [1]. Since dysfunction in ABCA12 causes a decrease in epidermal ceramide (Cer) content concomitantly with loss of the skin lipid barrier, ABCA12 is thought to regulate epidermal generation of Cer. However, so far no direct target of ABCA12 has been identified. Thus, we attempted to study the substrate of ABCA12 by generating an ABCA12-deficient keratinocyte cell line and then comparing its sphingolipid metabolism to that of a normal keratinocyte cell line. Keratinocytes were isolated from a HI patient and a healthy donor [1], then were immortalized by expressing the T-antigen of SV40. After 15 passages, the morphology of the cells became uniform and we confirmed the expression of the SV40 T-antigen by Western blotting (Fig. 1A and B). The cell lines thus generated from the cells isolated from the HI patient and healthy donors were named HIKT and KT1, respectively. Genomic analysis revealed one mutation in the HIKT cells, from A to G adjacent a splice acceptor site of exon 24 (Fig. 1C). This mutation produced two splice variants of 674-bp and 513-pb (Fig. 1D). The 674-bp results from 9-bp lost from exon 24, and the 513-bp results from 170-bp lost from exon 24. This mutation has been reported to seriously affect the function of the ABCA12-protein [1]. We have successfully established an ABCA12-impaired keratinocyte cell line.

Several groups have demonstrated that the expression level of ABCA12 increases during the differentiation of keratinocytes, so that anaplastic keratinocytes express only low levels of ABCA12 [2,3]. As shown in Fig. 2A, anaplastic KT1 scarcely expresses any ABCA12. So, we attempted to induce differentiation of KT1 and HIKT cells, and thereby express high levels of ABCA12 in the cells. We induced differentiation in KT1 and HIKT cells and confirmed the differentiation by measuring the differentiation marker involcurin, which increased significantly in both cell lines during differentiation (3.1-fold increase in KT1 and 2.4-fold increase in HIKT) (Fig. 2A). We further found that ABCA12 also increased during differentiation, with especially high levels of expression at 7 days after induction in the HIKT cells. Following the induction of differentiation in KT1 and HIKT cells, their sphingolipid metabolism was examined at 7 days by labeling the cells with [^{3}H]dihydosphingosine or [^{14}C]galactose (Fig. 2B and C, respectively). Interestingly, apparent accumulations of [^{3}H]GlcCer (Fig. 2B) and [^{14}C]GlcCer (Fig. 2C) were observed in KT1 cells at 7 days post-induction compared to HIKT cells (203% increase at [^{3}H]GlcCer and 181% increase at [^{14}C]GlcCer, respectively). It is noteworthy that [^{14}C]gangliosides levels in KT1 cells were lower rather than equal to levels in HIKT cells (31% reduction), despite the accumulation of GlcCer. Since the ABCA12 is known to localize at lamellar bodies (LBs) in keratinocyte [4], this result indicates that differentiated KT1 cells aggressively accumulate GlcCer in LBs and not in the Golgi apparatus. Our results clearly demonstrate that ABCA12 deficiency impairs the GlcCer accumulation in LBs, thereby strongly indicating that ABCA12 transports GlcCer to the inner leaflet of LBs (Fig. 2D). Mass spectrometer analysis of accumulated

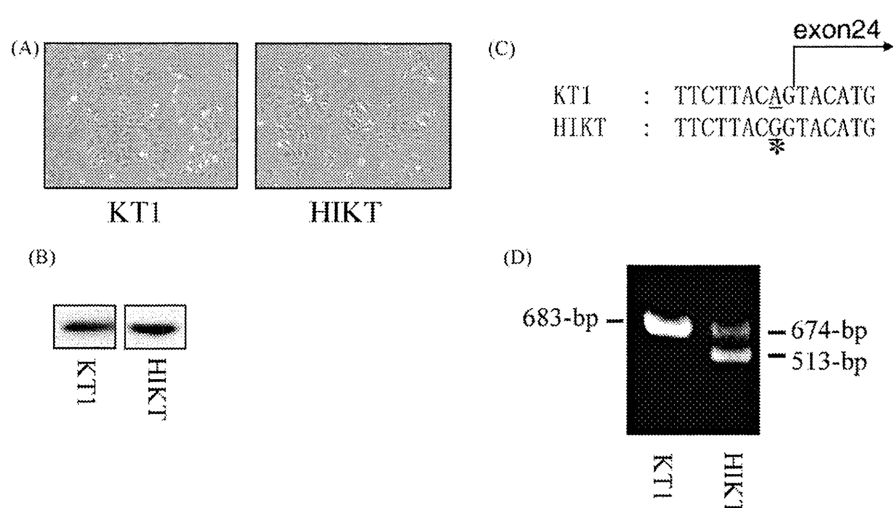

Fig. 1. KT1 and HIKT keratinocyte cell lines. KT1 and HIKT keratinocyte cell lines were generated by introducing the SV40 T-antigen into the cells using the culture supernatant of ΨCRIP-pMFGtsT (distributed from RIKEN cell bank, Tsukuba, Japan). After 15 passages, KT1 and HIKT cells exhibited similar morphology (A), and expected expression levels of T-antigen as examined by Western blotting using anti-SV40 LT (BD biosciences, NJ) (B). Genomic structures of KT1 and HIKT cells neighboring exon 24 (C). *A mutation from A to G in HIKT cells. RT-PCR analysis of mRNA fragments around the exon 23–24 boundary was performed as described previously [1] (D).

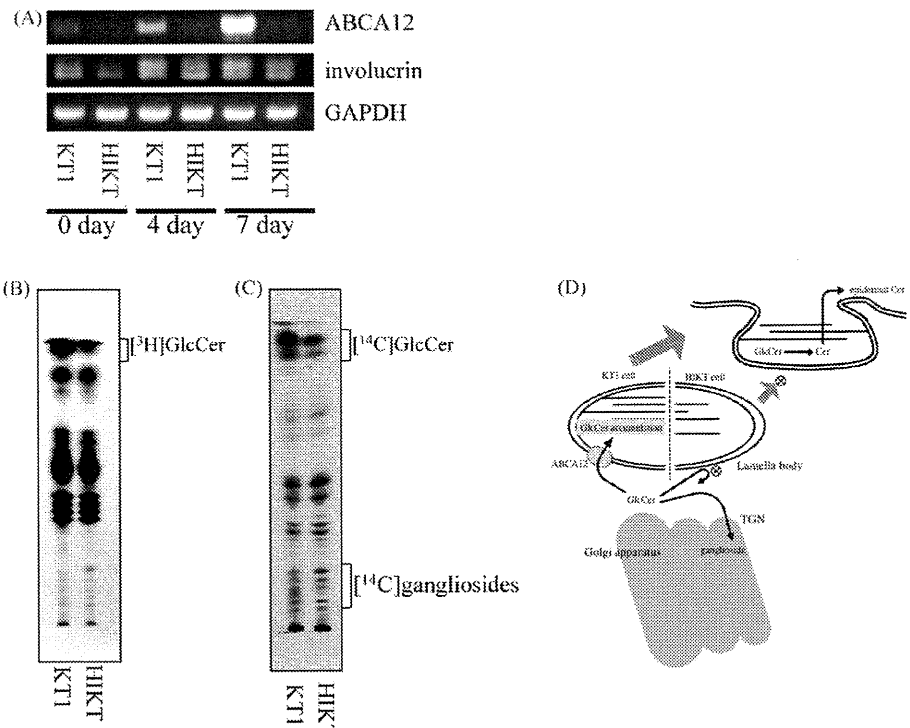

Fig. 2. Sphingolipids in KT1 and HIKT 4 days after induction of differentiation. (A) To induce differentiation, HIKT and KT1 cells were cultured in a DMEM/F-12 (2:1) mixture containing 10% fetal bovine serum, 1.2 mM Ca^{2+}, 10 μg/ml insulin, and 0.4 μg/ml ascorbic acid (differentiation medium) at 39 °C. Before (0 day) and 4 days and 7 days after induction, total RNA was isolated from each cell line, and semi-quantitative RT-PCR was performed using primers for ABCA12, 5′-GAATTGCAAACTGGAAGGAACTCCC-3′ and 5′-GAGTCAGCTAGGATTAGACAGC-3′; for involcurin, 5′-CTCCTCAAGACTGTTCCTCC-3′ and 5′-GCAGTCATGTGCTTTTCCTCTTGC-3′; for GAPDH, 5′-ATCACTGCCACCCAGAAGAC TGTGGA-3′ and 5′-GAGCTTGACAAAGTTGTCATTGAGAGC-3′. Seven days after induction of differentiation, HIKT and KT1 cells (10^6) were metabolically labeled with [^{3}H]dihydrosphingosine (2 μCi) (B) or [^{14}C]galactose (5 μCi) (C), then lipids were extracted and applied to HPTLC plates as described previously [5]. The HPTLC plates were developed with chloroform/methanol/0.05% $CaCl_2$ (60:35:8). The data are representative of tree independent experiments. (D) A possible mechanism of how to generate epidermal ceramides.

GlcCer will provide advanced information about substance preference of ABCA12. This remains for future study.

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LETTER TO THE EDITOR

Linear immunoglobulin A bullous dermatosis associated with herpes simplex virus infection and Kawasaki disease

Dear Editor,

Linear immunoglobulin (Ig)A bullous dermatosis (LAD) is an autoimmune subepidermal bullous disease that involves IgA anti-basement membrane antibody and was suggested as an entity by Chorzelski and colleagues in 1979.[1] Its clinical characteristics are vesicles distributed annularly around erythema and/or sparsely distributed tense bullae. Direct immunofluorescence (DIF) shows linear deposition of IgA at the basement membrane zone. The pathogenesis of LAD remains unknown. LAD may occur following varicella zoster virus infection, may accompany malignancy and may be drug induced.[2,3] Kawasaki disease (KD) is an acute febrile eruptive disease that was described in 1967 by Kawasaki and preferentially affects younger children.[4] It is characterized by various kinds of skin rash and systemic vasculitis. The pathogenesis of this disease has been suggested to be a viral infection, such as Epstein–Barr virus or herpes simplex virus (HSV), toxic shock syndrome toxin-1 (TSST-1) and endotoxin from Gram-negative cocci; however, it remains unknown.[5] HSV infection could be causative for both KD and LAD, but their coexistence has not been reported. Recently, Rowley and coworkers[6,7] reported that IgA plasma cell infiltration is observed at the involved vascular wall in KD patients, and such infiltrates are also seen in unaffected regions, such as the upper respiratory tract, pancreas and kidney. These are interesting findings when considering the coexistence of LAD and KD.

A 14-month-old boy was admitted to our division for pruritic erythematous plaques and blisters scattered across his body. His past medical history was not remarkable and neither did he have a history of atopic dermatitis, chickenpox and HSV infection. At first, he presented to the Division of Pediatrics due to fever of unknown origin, and he was admitted the next day. When cervical lymphadenopathy, reddening of the lips, skin rash, conjunctivitis and indurative edema of extremities appeared, he was diagnosed with KD 3 days after the onset of symptoms. i.v. immunoglobulin (IVIG) and urinastatin were administrated, and his condition including skin rash gradually improved. Acetylsalicylic acid was also administrated after 11 days to prevent cardiovascular complications. After 16 days, pruritic erythematous plaques and blisters appeared in the inguinal area, scrotum, perineum, face and neck (Fig. 1). Mucosal involvement was not seen. Blister was tense and large bullae without delle. Viral infection or autoimmune blister disease was suspected, and viral investigation was carried out. Blister fluid from a trunk blister was collected aseptically by inserting a sterile needle into the blister and aspirating the fluid. Spinal fluid was collected by a conventional technique. HSV DNA was detected in the spinal fluid taken after 9 and 20 days and also in the blister fluid after 20 days with the method described by Aurelius and colleages.[8] The serum HSV IgG titer was elevated (Table 1). There was no suggestive symptom of herpetic meningitis. Administration of i.v. acyclovir was not effective. The patient was referred to our division for further examination and treatment.

Blood tests showed increased white blood cell count (20 980/μL) and C-reactive protein of 0.52 mg/dL (normal <0.24). Liver function, renal function and serum Ig were all within normal limits. Biopsy from a blister on his back showed subepidermal blister formation and mild inflammatory infiltration that consisted of lymphocytes, neutrophils and partially of eosinophils in the upper dermis (Fig. 2). An

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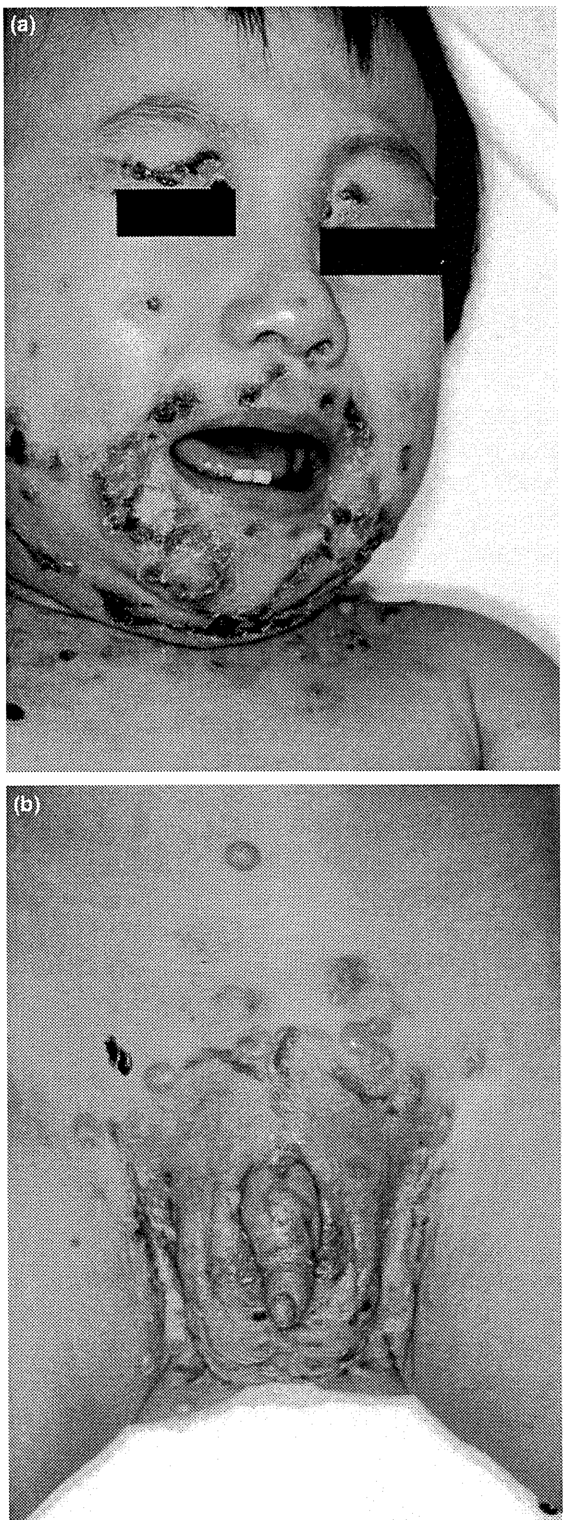

Figure 1. Pruritic erythematous plaques and blisters are seen on the (a) face, neck, (b) inguinal area, scrotum, and perineum.

Table 1. Serum viral titer

Days after KD onset	0	7	20
HSV IgM (<0.80)	0.21	0.16	0.31
HSV IgG (<2.0)	<2.0	**≥128.0**	**55**

Blister appeared 16 days after onset. The virus titer that comes off from the reference range is indicated by the bold. HSV, herpes simplex virus; Ig, immunoglobulin; KD, Kawasaki disease.

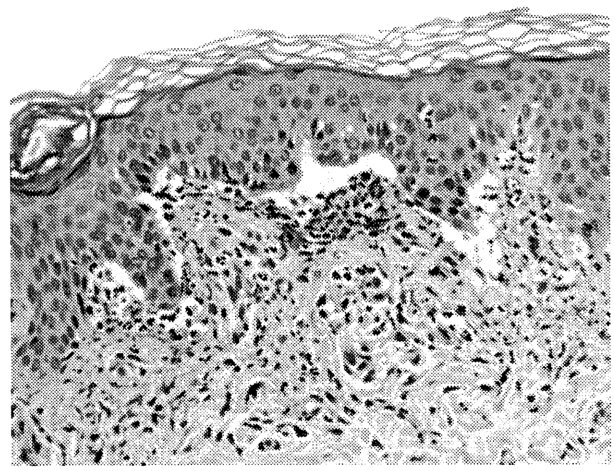

Figure 2. Subepidermal blister formation and mild inflammatory infiltration consisting of lymphocytes and partially of eosinophils in the upper dermis (hematoxylin–eosin stain, original magnification ×200).

intraepidermal neutrophilic microabscess was also observed. Vasculitis, balloon cell or intranuclear inclusion body were not seen. Tzanck test was not performed. DIF revealed linear deposition of IgA at the basement membrane zone (Fig. 3a). Indirect immunofluorescence of 1 mol/L NaCl-split human skin demonstrated that the epidermal side was positive for IgA antibodies at a titer of 1/40 (Fig. 3b). Immunoblotting studies identified that the patient's serum reacted with the 120-kDa antigen (linear IgA bullous dermatosis antigen, LAD-1) (Fig. 4). Finally we diagnosed this case as LAD, lamina lucida type. Acetylsalicylic acid was discontinued due to suspected drug-induced LAD; however, the symptoms did not improve. Oral prednisolone (PSL) 10 mg/day and 4,4-diamino-diphenyl-sulfone (DDS) 10 mg/day were administrated, and blisters and erosions rapidly epithelialized. Then, both were gradually tapered, PSL to 2.5 mg/day and DDS to 2.5 mg/day, and the boy showed persistent high grade fever for more than 5 days,

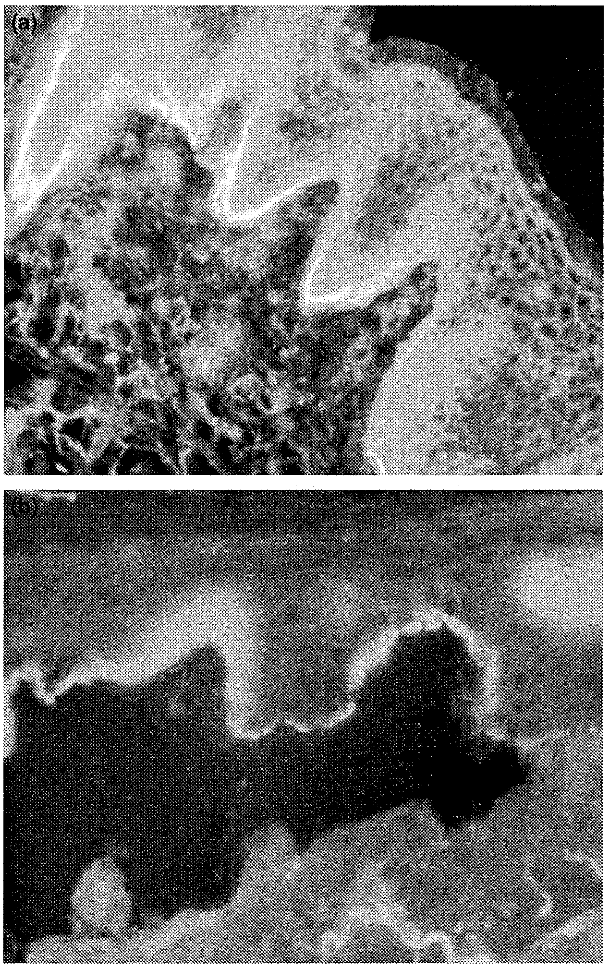

Figure 3. Direct immunofluorescence revealed linear deposition of immunoglobulin (Ig)A at the basement membrane zone (a). Indirect immunofluorescence on 1 mol/L NaCl-split human skin demonstrated that IgA antibodies were positive for the epidermal side at a titer of 1/40 (b).

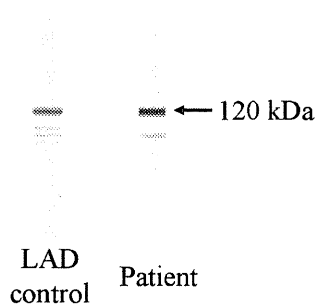

Figure 4. Immunoblotting studies identified that the patient's serum reacted with the 120-kDa antigen. LAD, linear immunoglobulin A bullous dermatosis.

cervical lymphadenopathy, reddening of the lips and tongue, skin rash and conjunctivitis. He was diagnosed as KD again. PSL and DDS were discontinued immediately. IVIG was effective for KD as well as at first onset. Acetylsalicylic acid was also administrated again and resurgence of LAD was not seen. To date, 32 months after KD settled, the patient has been well, and there have been no additional symptoms of KD and LAD.

The trigger of LAD is unknown. Post-chicken pox and drug-induced LAD have been reported.[2] In our case, HSV DNA was detected in both the spinal fluid and the blister fluid, and the HSV IgG titer was elevated, suggesting systemic HSV infection. Blister was clinically considered due to LAD but not HSV because they were tense and large bullae without delle. In addition, we could not find the unique histological features of herpes simplex from skin biopsy, hence there is a possibility that detection of HSV DNA from the blister fluid may have been by contamination of systemic infection of HSV. Although serum HSV IgG titer may also have been influenced by the administration of IVIG, we considered that HSV infection actually existed because HSV DNA was detected from spinal fluid. To our knowledge, this is the first case of LAD following HSV infection. Acetaminophen-induced LAD has been reported.[3] We ruled out this case from drug-induced LAD, because urinastatin was discontinued before the onset of LAD and symptoms did not recur when IVIG and acetylsalicylic acid were reinstituted.

Kawasaki disease involves systemic vasculitis, especially in the coronary arteries, resulting in aneurysm formation that is often fatal.[4] Although multiple infectious agents and toxins including HSV have been considered as the implication of the etiology of KD and many studies were investigated, none have been identified so far.[5] Various kinds of skin rash (so-called undefined skin rash) may be observed in patients with KD, even pustule and chicken pox-like eruptions.[9,10] LAD-like eruptions in KD have not been described so far. Recently, IgA plasma cell infiltration has been observed at the vascular wall,[6] and also in the upper respiratory tract, pancreas and kidney.[7] These findings suggest that an IgA immune response may spread to various organs, even in patients without vasculitis, by entry of the etiological agent through the upper respiratory tract.[7,11,12] Other investigators have reported that patients with KD show increasing serum levels of IgA class antibodies to several antigens.[13,14] In our case, there is the possibility that antecedent KD and HSV infection may have triggered the onset of LAD, which involves anti-basement membrane IgA antibody. Because the onset of KD was earlier than the appearance of HSV infection, we considered that KD and HSV were independent events. LAD did not relapse upon the resurgence of KD. Our hypothesis is that in this patient, LAD was triggered by two components, KD and HSV infection. We believe that this is a unique and interesting case to consider the etiology of LAD.

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LETTER TO THE EDITOR

A missense mutation c.G2747A (p.R916Q) of *ADAR1* gene in dyschromatosis symmetrica hereditaria is not a novel mutation

Masahiro Hayashi · Tamio Suzuki

Received: 26 January 2010 / Accepted: 29 January 2010 / Published online: 19 February 2010

Dear Editor,

Dyschromatosis symmetrica hereditaria (DSH: OMIM no. 127400) is a rare pigmentary genodermatosis predominantly seen in Asia, including Japan, Taiwan and China. It is inherited in autosomal dominant manner and has high penetrance. Its clinical manifestations are intermingled hypo- and hyperpigmented macules on the dorsal aspects of hands, feet and the face, and they appear in infancy or early childhood [3].

We have shown that adenosine deaminase acting on RNA1 (*ADAR1*), also known as the double-stranded RNA-specific adenosine deaminase (*DSRAD*), is the responsible gene for DSH [1] and around 90 of various mutations have been reported. We have been making the database of *ADAR1* gene mutations reported to date. Figure 1 shows the schematic mutations.

We have read an article described by Xu et al. [4] that they reported two "novel" mutations of *ADAR1*, c.G2747A leading to p.R916Q in exon 9 and c.C3124T leading to p.R1042C in exon 12, respectively. The latter c.C3124T (p.R1042C) is certainly a novel mutation; however, former mutation c.G2747A (p.R916Q) has already been reported by our group [2].

We would be very grateful if the editor will check them and let the author correct the statement. We really appreciate your kindly cooperation.

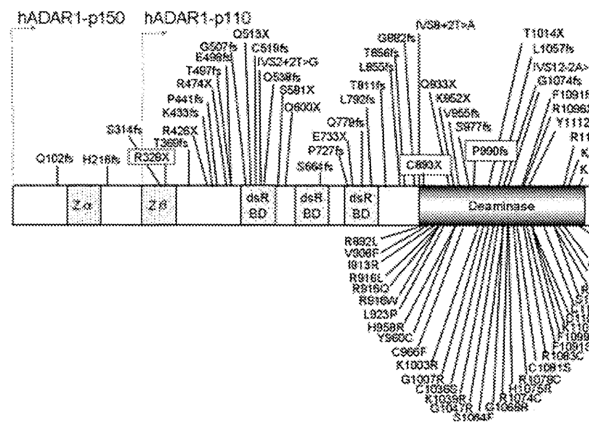

Fig. 1 Mutations of the *ADAR1* gene reported in patients with DSH. Mutations above the *horizontal axis* are nonsense, frameshift and splicing mutations and those *below* are missense mutations; *zα and zβ* Z-DNA-binding domains, *dsRBD* double-stranded RNA-binding domains, *deaminase* deaminase domain

Although many mutations of *ADAR1* have been detected, the pathogenesis and mechanism of onset of DSH have not been elucidated yet. Further investigations are needed to clarify them.

Conflict of interest statement The authors declare that they have no conflict of interest.

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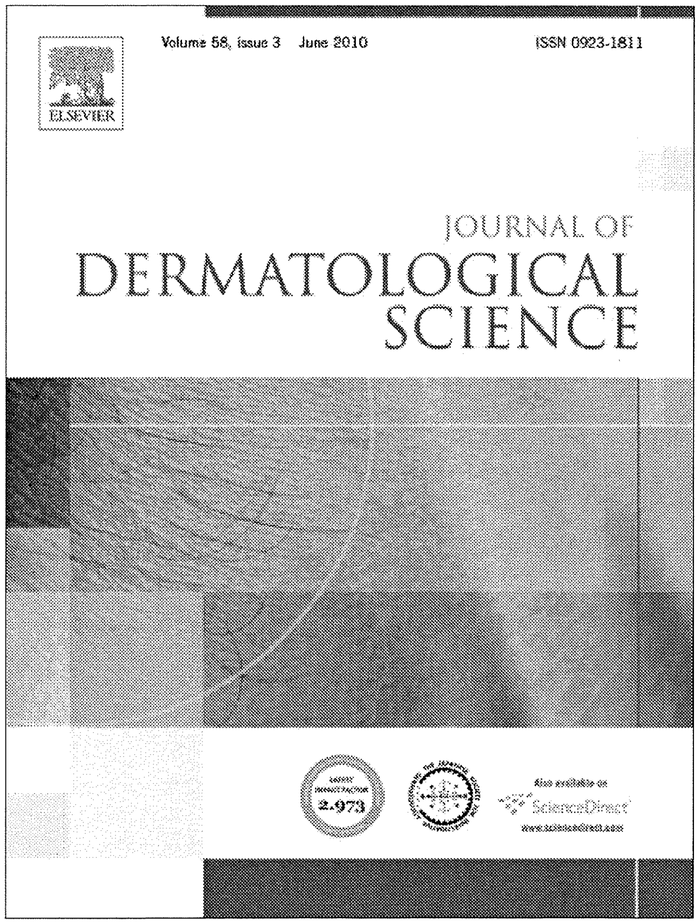
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reported in another unrelated family too [6]. Whether are these two mutations hotspots that needs to be further validated.

To date, more than 90 different mutations have been reported. Clinical manifestations observed in the patients of all study, showed no obvious phenotypic variations and no evident genotype–phenotype correlations between affected individuals. We hope more findings of novel mutations would helpful for revealing the mechanism leading to DSH and further clarifying the relation between genotype and phenotype.

In conclusion, our results provide a significant addition to the DSH mutation database and will contribute further to the understanding of DSH genotype/phenotype correlations and to the pathogenesis of this disease.

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Letter to the Editor

Mutation analyses of patients with dyschromatosis symmetrica hereditaria: Five novel mutations of the *ADAR1* gene

Dyschromatosis symmetrica hereditaria (DSH: MIM#127400) shows an autosomal dominant pattern of inheritance with high penetrance, which is characterized by hyperpigmented and hypopigmented macules on the face and dorsal aspects of the extremities. The phenotypes appear in infancy or early childhood [1]. In Asia, the condition occurs predominantly among Japanese and Chinese individuals. Miyamura et al. have clarified that a heterozygous mutation of the adenosine deaminase acting on RNA 1 (*ADAR1*, formerly *DSRAD*) gene causes DSH in 4 Japanese DSH families [2]. Subsequently, more than 90 mutations have been reported from East Asian countries so far [3,4,5], which confirmed that the *ADAR1* gene is responsible for DSH not only in Japanese but also in other ethnic groups.

ADAR1 protein catalyzes the deamination of adenosine to inosine in double-stranded RNA substrates [6,7], which results in the creation of alternative splicing sites or alternations of the codon and thus leads to functional changes in the protein. The *ADAR1* gene is expressed ubiquitously, but the target RNA(s) in the skin still remain unknown. Although it has been reported that DSH may be caused by *ADAR1* haploinsufficiency [8,9], further molecular pathogenesis of DSH has not been clarified yet.

In this study, we describe mutation analyses of a Chinese and 9 Japanese families with DSH. We detected 5 novel and 4 known mutations, and failed to find any mutation in 1 Japanese family. Five novel mutations included 3 missense mutations (c.T2878A, p.Y960N; c.A3116C,p.K1039T; c.A3182G,p.Y1061C), 1 nonsense mutation (c.C1190A,p.S397X), and 1 two-nucleotide deletion mutation (c.1096-1097delAA,p.K366fs) in 1 family and 4 sporadic patients (Table 1). The mutational analysis of the *ADAR1* gene was performed as previously described [10]. Three novel missense mutations that altered amino acids conserved among all known species, including pufferfish, zebrafish, frog, rat, mouse, cow, and human within the deaminase domain of ADAR1 protein. These mutations were not detected in the control blood samples of the surveyed 100 unrelated, normally pigmented Japanese adults. Therefore, we consider these mutations pathologic with no functional activity. The patients originally consulted us for their skin conditions. All patients phenotypically presented typical macules on the dorsal aspects of the hands, feet, lower arms and lower legs, and freckle-like macules on the face. Informed consent and blood samples of patients were obtained under protocols approved by the Ethics Committee of Yamagata University School of Medicine.

The missense mutations at codon 960, tyrosine to cysteine and codon 1039, lysine to arginine have been already reported [5]. Here, we also reported same codon changes not to same amino acids but to asparagine (patient no. 2) and threonine (patient no. 1), respectively. So far, all known missense mutations are located within the deaminase domain [5], which encompasses amino acids 886–1221, suggesting that this domain is critical for enzyme

Table 1
Novel mutations of the *ADAR1* gene.

Patient no.	Patient's pedigree			Mutation			Onset year-old	Polymorphism	Novel or known
	Incidence	Affected individuals	Unaffected individuals	Nucleotide change[a]	Amino-acid change	Position			
1	Sporadic	1	–	c.A3116C	p.K1039T	Exon 12	3	c.A1151G, p.K384R in Exon 2, IVS4-20C>T, c.G2682A, p.V894V in Exon 9, IVS14+8A>G	Novel
2	Sporadic	1	–	c.T2878A	p.Y960N	Exon 10	1	c.G2682A, p.V894V in Exon 9	Novel
3	Familial	3	2	c.C3286T	p.R1096X	Exon 13	2	c.A1151G, p.K384R in Exon 2, c.G2682A, p.V894V in Exon 9 IVS14+8A>G	Known
4	Sporadic	1	–	c.1096-1097delAA	p.p.K366fs	Exon 2	1	c.G2682A, p.V894V in Exon 9	Novel
5	Familial	4	3	c.C1190A	p.S397X	Exon 2	3	IVS14+8A>G	Novel
6	Familial	2	1	c.C2746T	p.R916W	Exon 9	2	c.A1151G, p.K384R in Exon 2, IVS14+8A>G	Known
7[b]	Familial	3	6	c.C3247T	p.R1083C	Exon 13	6	None	Known
8	Sporadic	1	–	c.3169delC	p.L1057fs	Exon 12	3	c.G2682A, p.V894V in Exon 9, IVS14+8A>G	Known
9	Sporadic	1	–	c.A3182G	p.Y1061C	Exon 12	3	c.A1151G, p.K384R in Exon 2, c.G2682A, p.V894V in Exon 9, IVS14+8A>G	Novel
10	Familial	3	2	No mutation	–	–	2	c.A1151G, p.K384R in Exon 2, c.G2682A, p.V894V in Exon 9,	–

[a] GenBank accession no. NM_001111.3. Position 1 is A of the translation initiation codon.
[b] Chinese family.

function. The result in this study suggests that these codons might be hot spot for mutation within the deaminase domain.

The family of no. 10 revealed the typical phenotypes of DSH, hyperpigmented and hypopigmented macules on the face and dorsal aspects of the extremities, as shown in Fig. 1A–C. However, we could find any pathological mutation neither in SSCP nor sequencing of all exons and flanking regions. Furthermore, we examined *ADAR1* gene expression in peripheral blood cells from the affected individuals of no.10 family using RQ-PCR method [9], and then, confirmed that an amount of the expression was similar to that of a healthy control (Fig. 1D), indicating that there might be no mutation in the promoter region of *ADAR1* gene. These results might suggest locus heterogeneity for the disease. We planned to obtain experimental evidence for the possibility with tests for genetic linkage analysis of the family of patient 10. However, we could not test it, because DNA only from the affected, not the unaffected could be available. So far, there has been no report on the ADAR1 enzymatic activity in blood or skin tissue of the patient with DSH. If a convenient assay system for the ADAR1 protein using the patient's sample is established and available to us, we suppose that the enzymatic activity of patient no. 10 may be similar to those of healthy controls, on the other hand those of other patients in whom we could detect mutations of the *ADAR1* gene may decrease by one half, demonstrating the possibility for the locus heterogeneity.

In conclusion, we have found 5 novel mutations in the *ADAR1* gene of 1 DSH pedigree and 4 sporadic individuals, 4 recurrent mutations in 3 pedigrees and 1 sporadic individuals, and no mutation in a pedigree with typical phenotypes of DSH. These results may provide new insight into the pathogenesis of DSH.

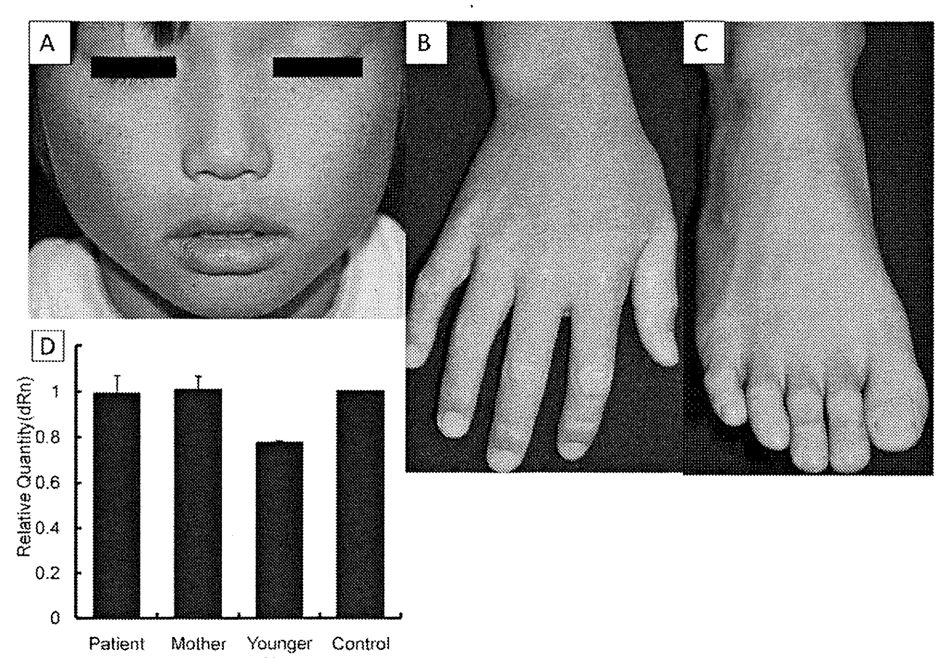

Fig. 1. Clinical phenotypes of patient no. 10 with freckle-like macules on the face (A) and a mixture of hyperpigmented and hypopigmented macules distributed on the back of her hand (B) and the top of her feet (C). Relative quantity of *ADAR1* transcripts of the affected individuals of family no. 10 (D). Data derived from real-time quantitative RT-PCR is expressed as mean ± S.D. of 3 independent experiments performed in triplicate. Mother and the younger sister of the patient no.10 family were affected.

Electronic database information

Accession numbers and URLs for data in this paper are as follows: GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for the cDNA of human *ADAR1* [accession number NM_001111.3]).

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Letter to the Editor

Schöpf-Schulz-Passarge syndrome resulting from a homozygous nonsense mutation in *WNT10A*

Schöpf-Schulz-Passarge syndrome (SSPS; OMIM 224750) is an autosomal recessive inherited form of ectodermal dysplasia characterized by eyelid cysts (apocrine hidrocystomas), palmoplantar keratoderma, hypodontia, hyperhidrosis, hypotrichosis and onychodystrophy, as well as other, often variable, ectodermal developmental anomalies [1,2]. SSPS shows clinical overlap with odonto-onycho-dermal dysplasia (OODD; OMIM 277980), but the eyelid cysts are a typical feature of SSPS [1].

In 2007, the molecular pathology of OODD was shown to involve a loss-of-function mutation in the *WNT10A* gene, which encodes the wingless-type MMTV integration site family member 10A [3], and a further pathogenic mutation in *WNT10A* was subsequently shown in another family with OODD [4]. In 2009 and 2010, additional nonsense and missense mutations in *WNT10A* were delineated, not only in OODD, but also in individuals with severe oligodontia and various other manifestations of ectodermal dysplasia, including a family with SSPS [5]. To date, five nonsense mutations (p.Trp9X, p.Cys107X, p.Arg128X, p.Glu233X, and p.Cys376X) and three missense mutations (p.Arg128Gln, p.Ala131-

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ARTICLE

Extract of Passion Fruit (*Passiflora edulis*) Seed Containing High Amounts of Piceatannol Inhibits Melanogenesis and Promotes Collagen Synthesis

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The effect of passion fruit, the fruit of *Passiflora edulis*, on melanin inhibition and collagen synthesis was studied using cultured human melanoma and fibroblast cells. Passion fruit was divided into three parts, rind (PF-R), pulp (PF-P), and seed (PF-S), and each part was extracted using 80% ethanol. The concentration of polyphenols was higher in PF-S than in PF-R or PF-P. Treatment of melanoma cells with PF-S led to inhibition of melanogenesis. In addition, the production of total soluble collagen was elevated in dermal fibroblast cells cultured in the presence of PF-S. PF-R and PF-P did not yield these effects. Furthermore, the removal of polyphenols from PF-S led to the abolishment of the effects described above. We discovered that piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene) is present in passion fruit seeds in large amounts and that this compound is the major component responsible for the PF-S effects observed on melanogenesis and collagen synthesis.

KEYWORDS: ***Passiflora edulis*; piceatannol; passion fruit seed; melanin; collagen**

INTRODUCTION

Tropical fruits and vegetables are beneficial for human health. Passifloraceae is a well-known tropical plant from the South American tropical forests, and its leaves, vines, and flowers are used as medicinal herbs. There are many reports on the actions of Passifloraceae herbs, which include its anti-anxiety effect in humans (*1*) and its anti-inflammatory and cough-suppressant effects in mice (*2, 3*). However, studies on the fruit of *Passiflora edulis* from the Passifloraceae family, which is known as passion fruit, are limited.

Passion fruit is usually eaten in its natural state, with the seeds, or processed as tropical juice. Passion fruit contains many phytochemicals, such as polyphenolic compounds (*4*) and carotenoid families (*5*), and vitamin C (*6*), which are known as being beneficial for the skin. The health effects of the polyphenols contained in many natural plants, including tropical fruits, have been increasingly and energetically studied. The content of polyphenols in various tropical fruits (*7*), as well as the bioactive effects of tropical fruits, such as acai and mangosteen (*8, 9*), have been reported.

Skin abnormalities, such as aging, are caused by genetic and environmental factors. In addition, the damage to skin by UV exposure, mental and environmental stress, eating habits, etc., leads to skin pigmentation, wrinkles, and even skin cancer. Melanin is the pigment that colors the skin. Although melanin is important for protection from UV rays, production to an excessive degree leads to skin erythema, freckles, and other skin disorders. Many bioactive substances from naturally occurring plants are studied for the prevention of melanogenesis. In those reports, the effect of polyphenols from safflower seed, grape seed (*10, 11*), and many other edible plants are described. Collagen, which is another molecule that is essential for skin health, plays many important roles in the body, including cell−cell adhesion, cell proliferation, and cell differentiation. The functional properties of the skin considerably depend upon the quantity and condition of the collagen present in the dermis. Some foods and food components contribute to the maintenance of the collagen condition in the skin or inhibit collagen-degrading factors, e.g., royal jelly (*12*) and polyphenols, such as catechin and flavonoids (*13, 14*). To prevent skin damage and maintain its protective potency against environmental agents, there is an increased research focus on compounds that target the skin. As described above, polyphenols play important roles in dermal cells; thus, we predicted the effectiveness of passion fruit in the promotion and maintenance of skin health.

The aim of this paper was to investigate the effect of passion fruit on melanogenesis and collagen synthesis in dermal cells and identify the component responsible for these effects.

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MATERIALS AND METHODS

Materials. Piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene), resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), and polyvinylpolypyrrolidone

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(PVPP) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Culture reagents, such as Dulbecco's modified Eagle's medium (DMEM), Medium 106S, phosphate-buffered saline (PBS), low-serum growth supplement (LSGS), glutamine, penicillin, streptomycin, gentamicin, amphotericin B, and trypsin−ethylenediaminetetraacetic acid (EDTA), were purchased from Invitrogen (Carlsbad, CA), and fetal bovine serum (FBS) was purchased from Biowest (Maine-et-Loire, France). Synthetic melanin was purchased from Nacalai Tesque (Kyoto, Japan), and the Sircol soluble collagen assay kit was purchased from Biocolor Life Science Assays (Newtownabbey, U.K.). Other reagents were purchased from Wako Pure Chemicals (Tokyo, Japan).

Sample Preparation. Commercial whole passion fruit (product of Kagoshima, Japan) was divided into three parts: rind, pulp, and seed. Each part was freeze-dried, milled, and extracted twice using 10-fold amounts of 80% (v/v) ethanol, by shaking at room temperature. The extract solution was centrifuged at 3000*g* for 10 min to obtain the supernatant, which was filtered through a paper filter to remove the sediment. Aliquots containing the extracts were evaporated, freeze-dried, and dissolved in distilled water at the concentration of 100 mg/mL (for rind and pulp) or 20 mg/mL (for seeds). The extract solutions were filtered using a hydrophilic membrane filter (Kanto Kagaku, Japan), with a pore size of 0.45 μm. The rind, pulp, and seed of passion fruit extracts were termed PF-R, PF-P, and PF-S, respectively.

Polyphenol Quantification. The polyphenol concentration was measured according to a modified Folin−Ciocalteu method, as described previously (*15*), using (−)-epicatechin to obtain the standard curve for polyphenol. To examine whether polyphenols contribute to the inhibition of melanogenesis and collagen synthesis, polyphenols were removed from PF-S via absorption with PVPP because the amide bonds of PVPP form hydrogen bonds with the hydroxyl groups of polyphenols. PVPP was swollen in distilled water, added to 20 mg/mL PF-S, and stirred for 30 min. The solution was centrifuged at 12000*g* for 15 min to obtain PVPP-treated PF-S. The polyphenol concentration was measured again using the Folin−Ciocalteu method, to confirm that the polyphenols were removed. The experiment was carried out in triplicate.

Fractionation of PF-S. High-performance liquid chromatography (HPLC) analysis was carried out using a Shimadzu system controller SCL-10A, a UV detector RF-10A, and a fraction collector FRC-10A (Shimadzu, Kyoto, Japan). Samples were fractionated via reverse-phase HPLC using an octa decyl silyl (ODS) column (Mightysil RP-18 GP250-10, 250 × 10 mm inner diameter, 5 μm, Kanto Kagaku, Tokyo, Japan). A total of 4 mL of 20 mg/mL PF-S was injected into the HPLC apparatus using a linear gradient of 0−30% at 0−10 min from solvent B to solvent A, where solvent A consisted of a water/acetonitrile/acetic acid (400:10:1, v/v/v) mix and solvent B consisted of a solvent A/methanol (2:1, v/v) mix. The temperature of the column was maintained at 40 °C. Measurements were carried out at a flow rate of 3 mL/min, and the wavelength used for UV detection was 280 nm. The absorbance of each fraction was measured with a spectrophotometer (V-630, JASCO Corporation, Tokyo, Japan). The HPLC chromatogram was divided into three fractions. Fraction 1 (tubes 1−17; 0−30 min), fraction 2 (tubes 18−23; 31−40 min), and fraction 3 (tubes 23−39; 41−70 min). The solvents used for HPLC were eliminated from each fraction by evaporation and freeze-drying, and the remaining extracts were dissolved in distilled water to maintain a concentration of 100 μg/mL. Each fraction and 100 μg/mL of PF-S served as samples for the measurement of melanin and soluble collagen, as described below.

Qualitative Analysis and Determination of Piceatannol. Ground passion fruit seeds were extracted with 70% (v/v) acetone 3 times, with shaking at room temperature. Samples were evaporated and freeze-dried to obtain crude extracts. A total of 100 mg of crude extract was suspended in 50 mL of 50% methanol solution and centrifuged at 1500*g* for 5 min. This supernatant was separated via reverse-phase liquid chromatography using a linear-gradient mode, as follows. Chromatographic measurements were carried out using an Agilent 1100 series liquid chromatography/mass spectrometry (LC/MS) system (Agilent Technologies, Tokyo, Japan) that included a photodiode array (PDA) detector. The HPLC column used in this study was Inertsil ODS-3 (150 × 2.1 mm inner diameter, 5 μm, GL Science, Tokyo, Japan). The mobile phase consisted of (A) water and (B) acetonitrile (v/v) using an initial gradient elution of 10% B and a gradient of 10−45% B at 0−25 min. The column temperature was maintained at 45 °C. All measurements were carried out at a flow rate of 0.25 mL/min using a detector wavelength of 280 nm. The mass spectrometric data were collected in full-scan mode, from m/z 200 to 1000.

Cells and Cell Culture. Melanin-producing MNT-1 human melanoma cells (a gift from Dr. V. J. Hearing, Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD) were cultured in DMEM containing 10% FBS, 4 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.

SF-TY human dermal fibroblast cells (Health Science Research Resources Bank, Tokyo, Japan) were cultured in Medium 106S supplemented with 5% LSGS, 10 μg/mL gentamicin, and 0.25 μg/mL amphotericin B. Melanoma and fibroblast cells were incubated at 37 °C under 5% CO_2 and 95% air.

Measurement of Melanin Content. MNT-1 cells were seeded at a density of 7.0×10^4 cells/well in 12-well culture plates and were cultured for 24 h. Subsequently, the medium was replaced with fresh DMEM containing various concentrations of PF-R, PF-P, PF-S, and kojic acid [5-hydroxy-2-(hydroxymethyl)-4-pyrone], which was used as a positive control. After 72 h of culture, cells were washed with PBS and trypsinized with 0.25% trypsin containing 0.02% EDTA. The number of cells harvested was counted. To measure the melanin produced in these cells, harvested cells were washed twice with PBS and centrifuged at 300*g* for 5 min at 4 °C, to obtain cell pellets. The cell pellets were dissolved in 500 μL of 1 N NaOH and quantified for melanin content using spectrophotometry at a wavelength of 415 nm. The concentration of the melanin produced was calculated from the standard curves using synthetic melanin dissolved in 1 N NaOH. The melanin produced was expressed as a concentration ratio relative to control MNT-1 cells that were not treated with any of the extracts or kojic acid.

Measurement of Soluble Collagen Content. SF-TY cells were seeded at a density of 1.7×10^4 cells/well in 48-well culture plates and were cultured for 24 h. Subsequently, the medium was replaced with fresh medium containing various concentrations of PF-R, PF-P, PF-S, and ascorbate, which was used as a positive control. After 72 h of culture, the medium was collected and assayed for soluble collagen content using the Sircol soluble collagen assay kit, according to the instructions of the manufacturer. Briefly, supernatants of SF-TY cells were centrifuged at 12000*g* for 4 min, and 100 μL of each supernatant was mixed with 1 mL of Sircol dye and shaken for 30 min. The aliquot was then centrifuged at 12000*g* for 10 min to pellet the collagen−dye complex. After the suspension was decanted, droplets were dissolved in 0.75 mL of Sircol alkali reagent. The concentration of collagen was measured using spectrophotometry at a wavelength of 540 nm, using soluble collagen for the standard curve. The value of the blank (i.e., the medium alone) was subtracted from each sample, to remove the contribution of the collagen contained in FBS. Total soluble collagen was expressed as a concentration ratio relative to control SF-TY cells that were not treated with any of the extracts or ascorbate. Adherent cells were washed twice with PBS and harvested using trypsin, for cell counting.

Statistical Analyses. Data represent the mean ± standard deviation (SD) for the indicated number of experiments. Statistical significance of the difference between the corresponding control was carried out using the paired t test, where $*$, $**$, and $***$ represented $p < 0.1$, $p < 0.05$, and $p < 0.01$, respectively.

RESULTS

Total Polyphenol Content. Total polyphenol content in freeze-dried rind, pulp, and seed was measured using the Folin−Ciocalteu method. The results showed that PF-S contained a much larger amount of polyphenols compared to PF-R and PF-P, because polyphenols represented 33% of the freeze-dried seeds. The rind and pulp contained only 4 and 0.2% polyphenols, respectively. The part ratio occupying raw passion fruit was as follows: rind, 40%; pulp, 48%; and seed, 12%, and the freeze-drying process reduced the weight of each part by 14, 18, and 45%, respectively. This means that the calculated polyphenol content in the raw fruit was 0.22, 0.02, and 1.8%, respectively. Although the seed represents only 12% of the whole fruit, in weight, 88% of the total polyphenol content was found in the seed.

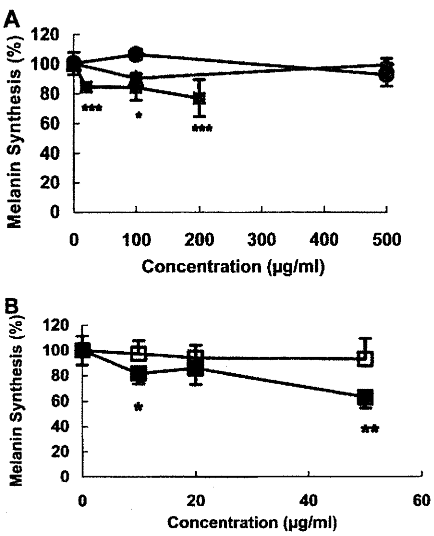

Figure 1. Comparison of melanin synthesis associated with (A) 80% ethanol extracts of passion fruit rind (PF-R; ●), pulp (PF-P; ▲), and seed (PF-S; ■). (B) PF-S (■) and PVPP-treated PF-S (□). Data are expressed as means ± SD (n = 4). Statistical analyses were performed using the paired t test: ∗, $p < 0.1$; ∗∗, $p < 0.05$; ∗∗∗, $p < 0.01$.

Inhibition of Melanogenesis by PF-R, PF-P, and PF-S in Melanoma Cells. Melanin synthesis was measured in melanin-producing human melanoma cells. Cells were cultured in the presence or absence of passion fruit extracts, and the melanin produced was compared to that of control cells, which were cultured in the absence of extracts. None of the samples inhibit cell growth at the concentrations examined (data not shown). As shown in **Figure 1A**, a significant decrease in melanin synthesis was observed when PF-S was applied to the melanoma cell culture at 20 μg/mL. In contrast, PF-R and PF-P did not inhibit melanin synthesis, although the concentration of these extracts was higher than that of PF-S. Kojic acid, which was applied for positive control, showed statistical significance ($p < 0.05$) at 10 μg/mL. To examine whether the polyphenols contained in PF-S are effective in the prevention of melanogenesis, polyphenols were removed from PF-S via PVPP treatment. More than 95% of the polyphenols were removed. PF-S inhibited melanin synthesis significantly, while PVPP-treated PF-S did not lead to inhibition of melanin synthesis (**Figure 1B**).

Soluble Collagen Production in Dermal Fibroblast Cells after PF-R, PF-P, and PF-S Treatment. Total soluble collagen was quantified in the culture medium of human dermal fibroblast cells (**Figure 2A**). Cells were cultured in the presence or absence of passion fruit extracts, and the collagen produced was compared to that of control cells, which were cultured in the absence of extracts. None of the samples inhibit cell growth at the concentrations examined (data not shown). Soluble collagen synthesis increased in a dose-dependent manner after the addition of PF-S to the cell culture. Ascorbate, which was applied for positive control, showed statistical significance ($p < 0.1$) at 25 μg/mL. However, similar to what was observed in melanoma cells, PF-R and PF-P did not increase collagen synthesis. Furthermore, PF-S led to a significant increase in the soluble collagen concentration in the medium (at 100 μg/mL) (**Figure 2B**), whereas the removal of polyphenols from PF-S via PVPP treatment did not lead to an increase of soluble collagen content in the culture medium.

Fractionation of PF-S and Measurement of Melanin and Soluble Collagen Synthesis. To determine the component of PF-S that was responsible for the inhibition of melanogenesis and soluble collagen synthesis, PF-S was fractionated using HPLC.

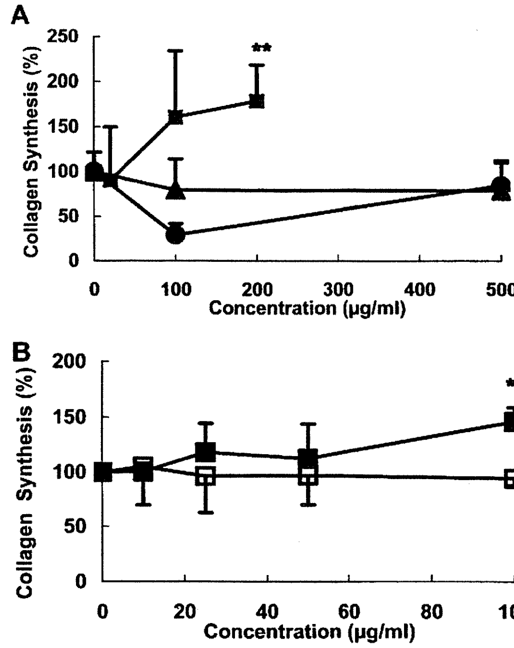

Figure 2. Soluble collagen synthesis associated with (A) 80% ethanol extracts of passion fruit rind (PF-R; ●), pulp (PF-P; ▲), and seed (PF-S; ■). (B) PF-S (■) and PVPP-treated PF-S (□). Data are expressed as means ± SD (n = 3). Statistical analyses were performed using the paired t test: ∗, $p < 0.1$; ∗∗, $p < 0.05$.

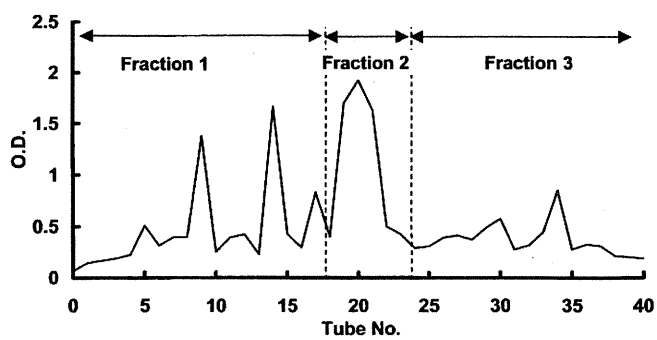

Figure 3. Absorbance of the fractioned 80% ethanol extracts of passion fruit seed (PF-S), obtained using reverse-phase chromatography with an ODS column. The three fractions were divided as fraction 1 (tubes 1−17; 0−18 min), fraction 2 (tubes 18−23; 19−22 min), and fraction 3 (tubes 23−39; 23−70 min).

Figure 3 shows the absorbance of each fraction of PF-S obtained by HPLC. One significant peak was observed at a retention time of ~20 min. PF-S was divided into three fractions, fractions 1−3, where fraction 2 contained the significant peak and fractions 1 and 3 were fractions with a retention time that was earlier and later, respectively, compared to fraction 2. The three fractions, with a concentration equivalent to 100 μg/mL, were added to MNT-1 and SF-TY cells to examine inhibition of melanogenesis and soluble collagen synthesis, as described previously. As shown in **Figure 4A**, fraction 2 inhibited melanin synthesis to a similar extent, as did PF-S (p = 0.103). Soluble collagen synthesis was also elevated in fraction-2-treated cells, which demonstrates that the components responsible for these effects are contained in fraction 2 (**Figure 4B**). However, only PF-S showed a significant increase in collagen synthesis.

Determination of Piceatannol. The polyphenol contained in passion fruit seed was determined by chromatographic measurements. The specific peak 1, as described in **Figure 5**, corresponded

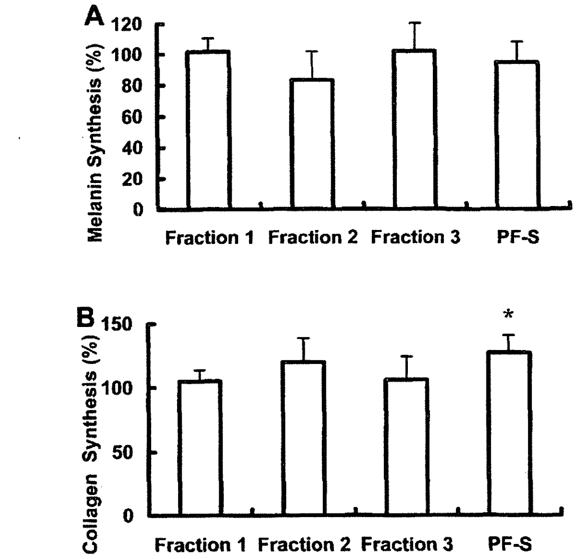

Figure 4. (A) Melanin synthesis associated with 100 μg/mL PF-S and 100 μg/mL PF-S fractionated using an ODS column. (B) Collagen synthesis associated with 100 μg/mL PF-S and 100 μg/mL PF-S fractionated using an ODS column. Data are expressed as means ± SD (n = 3). Statistical analyses were performed using the paired t test: $*$, $p < 0.1$.

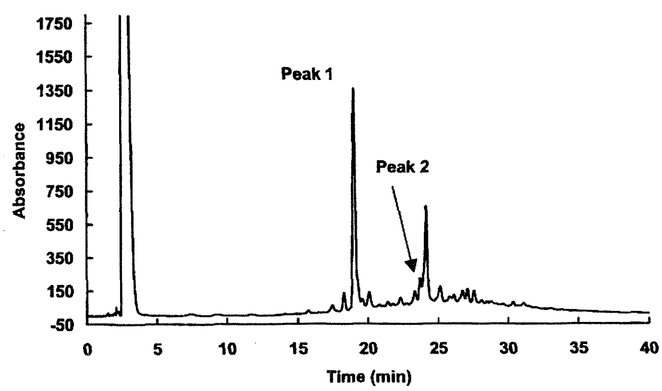

Figure 5. Determination of the concentration of piceatannol and resveratrol in passion fruit seed using chromatographic measurements. Peak 1 represents piceatannol, and peak 2 represents resveratrol. Standard piceatannol and peak 1 showed retention times of 19.01 and 19.13 min and UV (λ_{max}) of 236, 303, and 324 nm and 238, 303, and 325 nm, respectively. Standard resveratrol and peak 2 showed retention times of 23.43 and 23.61 min and UV (λ_{max}) of 237, 306, and 319 nm and 236, 306, and 319 nm, respectively.

to piceatannol, which was present in passion fruit seed in large amounts (**Figure 6A**). The retention time and the UV spectrograph coincided with those of standard piceatannol, with piceatannol and peak 1 showing retention times of 19.01 and 19.13 min and UV (λ_{max}) of 236, 303, and 324 nm and 238, 303, and 325 nm, respectively. The LC/MS data of PF-S also coincided with piceatannol, with the $[M-H]^-$ peak at m/z 243 (piceatannol molecular weight of 244). Piceatannol was not detected in the rind or pulp. Resveratrol (**Figure 6B**) was also determined and is represented in peak 2. The retention time and the UV spectrograph coincided with those of standard resveratrol, with resveratrol and peak 2 showing retention times of 23.43 and 23.61 min and UV (λ_{max}) of 237, 306, and 319 nm and 236, 306, and 319 nm, respectively. The LC/MS data of PF-S also coincided with resveratrol, with the $[M-H]^-$ peak at m/z 227 (resveratrol molecular weight of 228). The amount of piceatannol and resveratrol contained in freeze-dried passion fruit seed was 4.8 and 0.22 mg/g, respectively, which translates to 2.2 and 0.1 mg/g, respectively, in raw passion fruit seed.

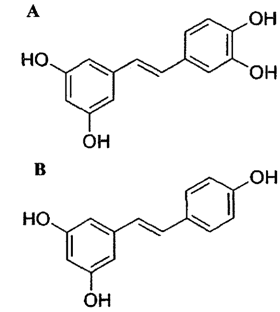

Figure 6. Chemical formulas of (A) piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene) and (B) resveratrol (3,5,4′-trihydroxy-*trans*-stilbene).

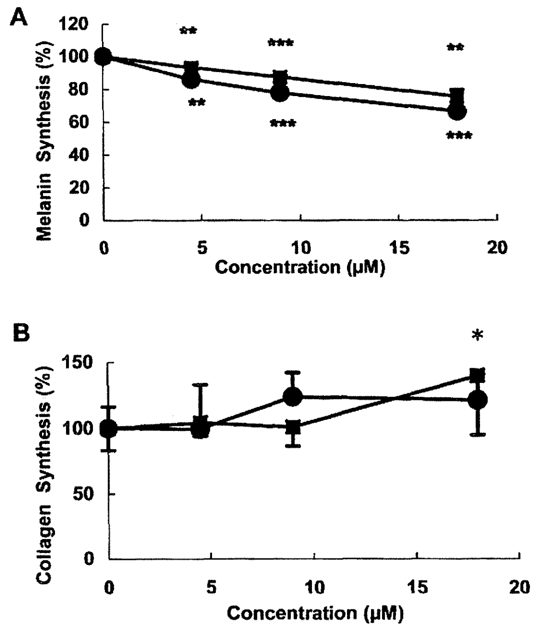

Figure 7. Comparison of the effect of PF-S (●) and piceatannol reagent (■) on (A) melanin synthesis and (B) collagen synthesis. Data are expressed as means ± SD (n = 3). Statistical analyses were performed using the paired t test: $*$, $p < 0.1$; $**$, $p < 0.05$; $***$, $p < 0.01$.

Comparison of the Effects of Piceatannol from PF-S and Piceatannol Reagent in Dermal Cells. To evaluate the contribution of piceatannol to the effects of PF-S on melanin inhibition and collagen synthesis, piceatannol extracted from PF-S and a commercially available piceatannol reagent were prepared to the same concentration, to compare their activity. As shown in **Figure 7A**, piceatannol from PF-S showed a stronger effect in the inhibition of melanin synthesis, although piceatannol reagent also yielded a strong inhibition of melanin synthesis. Both piceatannol samples decreased melanin synthesis significantly, at 4.5 μM. Soluble collagen synthesis was increased after treatment with both piceatannol samples; however, piceatannol reagent showed a significant difference at 18.5 μM (**Figure 7B**).

Comparison of the Effects of Piceatannol and Resveratrol Reagents in Dermal Cells. Piceatannol and resveratrol were examined to compare their effects on dermal cells. As shown in **Figure 8A**, both compounds inhibited melanin synthesis in MNT-1 cells, although piceatannol yielded a higher melanin inhibitory effect compared to resveratrol, because piceatannol led to a significant decrease in melanin synthesis at 5 μM, whereas

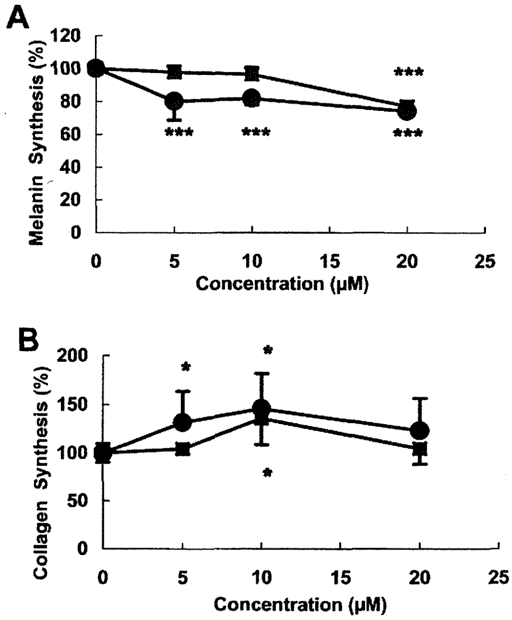

Figure 8. Comparison of the effect of piceatannol (●) and resveratrol (■) reagent on (A) melanin synthesis and (B) collagen synthesis. Data are expressed as means ± SD ($n = 3$). Statistical analyses were performed using the paired t test: ∗, $p < 0.1$; ∗∗, $p < 0.05$; ∗∗∗, $p < 0.01$.

resveratrol led to a significant decrease in melanin synthesis at 20 μM. Moreover, both piceatannol and resveratrol increased soluble collagen in SF-TY cells; piceatannol increased soluble collagen significantly at 5 μM, whereas resveratrol increased soluble collagen significantly at 10 μM (**Figure 8B**).

DISCUSSION

Passion fruit is a tropical fruit that is widely grown in South America and is eaten with the seeds, in its natural state, or converted to juice. The phytochemical compounds of passion fruit juice have been studied (*4–6*), although there are not many on seeds. However, it is reported that passiflin, a novel protein found in passion fruit seed, acts as an antifungal protein (*16*) and that the insoluble fiber from passion fruit seed acts as a potential hypocholesterolemic ingredient (*17*). The oil content in the seed is approximately 23%, of which 72% is linoleic acid (*18*).

Polyphenols are scavengers of reactive oxygen species (ROS). Although ROS are a biogenic substance, exposure to UV rays, stress, medication, and many other environmental conditions lead to ROS production to an excessive degree. The total polyphenol content and the antioxidant activity, as assessed using superoxide dismutase (SOD) activity, which is a well-known antioxidant index, were especially high in passion fruit seeds (data not shown). SOD activity is often used as an index of the strength of antioxidant activity. Polyphenols are thought to be natural antioxidants, and polyphenols, such as tea catechins (*19*) and proanthocyanidin (*11*), have been reported as having antioxidant activity in dermal cells. The high antioxidant activity found in PF-S may derive from its high polyphenol content.

Piceatannol is a tetrahydric polyphenol and an analogue form of resveratrol. The content of piceatannol in freeze-dried passion fruit seed was 4.8 mg/g when extracted using 70% acetone, which translates into 2.2 mg/g of raw material. There are many studies on the functions of piceatannol, which include anti-allergic effects (*20*) and inhibition of melanin synthesis (*21*). Piceatannol is found in a limited number of plants, in limited amounts: 138–422 ng/g of dry sample of highbush blueberry (*Vaccinium corymbosum*) and deerberry (*Vaccinium stamineum*) (*22*) and 0.052 μg/g of fresh weight from grapes (*Vitis vinifera* L., cv. Cabernet Sauvignon) (*23*). Because of these reasons, Ku et al. (*24*) reported that piceatannol can be produced in a large amount from the callus of peanut by UV irradiation. Our results showed that passion fruit contains a very high content of piceatannol in a natural state, which is nearly 50 times larger than that found in grapes (described above). Here, we report for the first time that piceatannol is present in passion fruit seed and that this content of piceatannol is much higher than that known for any other plants. Piceatannol was not detected in the rind or pulp of passion fruit. Resveratrol was also detected in passion fruit seeds, at a concentration of 0.22 mg/g of freeze-dried seeds.

Piceatannol is often compared to resveratrol because of their similar chemical structure. Resveratrol is one of the well-known polyphenols contained in grapes and peanuts (*23, 25*) and reportedly contributes to skin photoprotection and antioxidation (*26, 27*), among other functions. Moreover, piceatannol is a derivative of resveratrol and is metabolized from resveratrol via the cytochrome P450 enzyme CYP1B1, which is involved in cancer prevention (*28*). We compared the melanin-inhibitory and collagen-synthesis effects of piceatannol and resveratrol. Both compounds led to the inhibition of melanin synthesis and the production of soluble collagen; however, piceatannol yielded stronger effects in both dermal cell types. The larger number of hydroxyl groups in piceatannol may contribute to its stronger effect compared to resveratrol; in addition, the catechol structure may yield a high antioxidant effect.

To evaluate the contribution of piceatannol to the effects observed for PF-S, we prepared a reagent at the same concentration of the piceatannol contained in PF-S and compared their activity. The data show that piceatannol contributes to the inhibition of melanin synthesis and that the effect of PF-S depended strongly upon the effect of piceatannol. The slightly stronger effect of PF-S may be due to other bioactive compounds (e.g., polyphenols, such as resveratrol or other antioxidant pigments). These *in vitro* results suggest that piceatannol contributes greatly to the inhibitory effect of PF-S on melanin synthesis and to the PF-S-mediated promotion of collagen synthesis.

We found that piceatannol was the major polyphenol contained in passion fruit seed and that its melanin-synthesis inhibitory activity was higher compared to the other parts of the passion fruit, although the rind and pulp also contain polyphenols (*4, 29*) and other pigments, such as carotenoids (*5*). Studies on the mechanism of the biogenesis of melanin and the inhibition of melanogenesis have progressed actively. Cytokines, such as endothelin-1 (ET-1) and the α-melanocyte stimulating hormone (α-MSH), induce melanogenesis; conversely, suppression of these cytokines inhibits melanogenesis (*30,31*). Moreover, tyrosinase is the main enzyme that contributes to melanogenesis. Tyrosinase catalyzes several steps of the melanin pigment biosynthetic pathway, via oxidation. There are reports on the inhibitory action of polyphenols from plant seeds on tyrosinase, e.g., those of safflower seeds (*10*), and also of grape-seed-mediated inhibition of melanogenesis in guinea pig (*11*). In addition, gnetol (2,3′,5′,6-tetrahydroxy-*trans*-stilbene), which is another tetrahydroxyl stilbene compound isolated from gnetum, with a structure similar to that of piceatannol, has a strong inhibitory effect on tyrosinase and suppresses melanin biosynthesis in melanoma cells (*32*). Luteolin, the polyphenol contained in the rind of passion fruit (*29*), inhibits melanin synthesis in B16 melanoma cells (*33*); however, our results did not show inhibitory effects for rind extracts. Yokozawa et al. (*21*) compared the melanin inhibitory activities of piceatannol and resveratrol in melanoma cells and concluded that the melanin inhibitory activity of piceatannol was higher than that of resveratrol because of the higher antioxidative properties of piceatannol. Similar results were obtained in the present study. Piceatannol contains an additional hydroxyl residue, which provides a stronger reduction effect. The high content of polyphenols in PF-S may inhibit tyrosinase activity, and its high antioxidative activity may scavenge ROS; however, further studies are needed to identify the precise mechanism underlying these effects.

Collagen represents $^1/_3$ of the total protein content in the body and plays many important roles, including cell−cell adhesion, cell proliferation, and cell differentiation. Collagen is produced in fibroblast cells, and approximately 70% of the dermis consists of collagen. The functional properties of the skin depend upon the quality and condition of the collagen present in the dermis. Some food components promote collagen synthesis effectively in the skin. Other substances act as co-enzymes of prolyl hydroxylase and lysyl hydroxylase, which are the key enzymes in collagen synthesis, and some induce transforming growth factor β1 (TGF-β1), which stimulates the accumulation of type-I procollagen mRNA in human fibroblast cells (*12*). Inhibition of the degradation of collagen via inhibition of matrix metalloproteinases (MMPs) is another effective approach to the maintenance of collagen in the dermis. Piceatannol is an inhibitor of the JAK1/STAT-1 pathway, which induces the expression of the *MMP-1* gene in cultured human dermal fibroblasts (*34*). Moreover, catechins, well-known polyphenols, also inhibits UV-induced expression of MMPs in mouse skin (*13*). These reports led us to speculate that the PF-S-induced increase in the levels of collagen may be the result of the inhibition of MMPs or of their activity by the polyphenols present in PF-S; however, additional experiments are required to elucidate these issues.

In conclusion, we found that piceatannol was contained in passion fruit seeds at very high levels and that it exerted positive effects on cultured dermal cells: inhibition of melanogenesis and synthesis of collagen. The synthesis of melanin and the degradation of collagen are accompanied by the production of ROS, and ROS scavengers are effective for these damages. We postulate the possibility from our *in vitro* data that oral and topical application of PF-S may contribute to a decrease in the skin damage, which leads to skin melanogenesis, wrinkles, and other skin abnormalities. However, additional *in vitro* and *in vivo* studies are needed for human application. Although passion fruit is usually eaten with the seeds in its natural state, the seeds are discarded when it is processed to tropical juice. Extraction of piceatannol from seeds could be an efficient use of the seeds wasted during the production of passion fruit juice.

ABBREVIATIONS USED

PF-R, passion fruit rind extract; PF-P, passion fruit pulp extract; PF-S, passion fruit seed extract; PVPP, polyvinylpolypyrrolidone; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SOD, superoxide dismutase; HPLC, high-performance liquid chromatography; ODS, octa decyl silyl; LC/MS, liquid chromatography/mass spectrometry; PDA, photodiode array; ROS, reactive oxygen species; ET-1, endothelin-1; α-MSH, α-melanocyte stimulating hormone; TGF-β1, transforming growth factor β1; MMP, matrix metalloproteinase.

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