Involvement of transglutaminase 3 (TG3) in hair stiffness changing with age by capture of zinc.

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Abstract

We have reported in 28th IFSCC Congress 2014 that zinc in the cuticle of hair contributes to hair stiffness, and the age-dependent decrease in zinc is one of the reasons for the reduction in hair stiffness. From the results, we speculated that the decrease in zinc in the cuticle with aging may be caused by a decrease in proteins that retain zinc in the cuticle. Then, we extracted proteins from hair cuticle and analyzed to clarify them by mass spectrometry, resulting in identification of transglutaminase 3 (TG3) as a zinc-retaining candidate protein. The binding of TG3 to zinc was confirmed using size fractionation column followed by ICP-MS, and the immunostaining of longitudinal sections of hair root revealed the presence of significant amounts of TG3 in the cuticle. Furthermore, to investigate the decreasing expression level of TG3 depending on the age, hairs from volunteers with various ages were analyzed for the expression of TG3 RNA in the hair roots, demonstrating a trend of decreasing in its expression after the age of 40's. Based on the results, we concluded that the decrease in zinc in hair with age is partly due to the decrease in the expression of TG3 that is possible to retain zinc. In the future, the use of compounds that increase in TG3 expression would expect to be possible inhibitory regulator against the decrease in zinc with age, thereby preventing the decrease of hair firmness.

Keywords: hair stiffness; hair firmness; transglutaminase 3; zinc; aging

Introduction

With aging, hair loses its firmness, making it difficult to create ideal hairstyles. A common method to improve the decrease in stiffness is to form a polymeric dry film on the hair surface, but the surface feels stiff to the touch. We have been investigating the possibility of recovering the decrease in hair stiffness by strengthening the internal structure of the hair, rather than the hair surface. Previously, we reported that the decrease of zinc in the hair by aging correlates with the decrease of hair stiffness, and that the hair stiffness can be restored by supplementing zinc in the hair. In addition, the local structure of zinc in hair was confirmed by X-ray absorption fine structure analysis, and it has been clarified that zinc contributes to hair stiffness by forming a distorted tetrahedral structure with the proteins in the cuticle layer. However, it remains unclear why zinc in the hair decreases with age. We speculated that there are possible zinc-binding proteins in the cuticle layer, which might also decrease depending on age, and tried to identify responsible proteins for binding zinc in the cuticle layer.

After a short treatment of the hair with formic acid, protein was extracted mainly from the surface of the cuticle layer and CMC (cell membrane complex), which was analyzed using a Q-Orbitrap mass spectrometer. Among the list of the extracted proteins, we detected TG3, an enzyme catalyzing a calcium-dependent isopeptide bond formation between glutamine and lysine residues of certain substrate proteins [1]. Specifically in the inner root sheaths and hair shaft, TG3 contributes to cross-linking between trichohyalin and keratin intermediate filaments (KIFs), and also between KIFs, which are necessary to shape the hair and to provide mechanical strength to the hair [2] [3]. TG3 also plays an important role in skin epidermal formation, cross-linking structural proteins in epidermal cells during the late stage of epidermal differentiation to form a strong cornified envelope and enhance skin barrier function [4]. It has been reported that the enzymatic activity of TG3 is inhibited when magnesium binds to the enzyme active area (pocket) instead of calcium [5], although binding to zinc was not investigated. In addition, functions other than the catalytic activity of TG3 in post-keratinization hair had not been reported. We hypothesized that TG3 is a protein that may contribute to hair stiffness by binding to zinc in the hair cuticle layer and attempted various validations.

Materials and Methods

【Mass spectrometry of proteins extracted from hair with formic acid】

We purchased identical Chinese female hair with no history of chemical treatment from Beaulax Co., Ltd. One hundred and fifty hairs were soaked in a 1% SLS solution at 30° C for 10 min, rinsed with water, cut into 15 cm lengths, and coiled onto two plastic picks. After drying in a dryer at 60° C for 1 hour, the picks were removed and the dried coiled hair was enclosed in a 1.5 ml tube. Both cut sides of the hair were placed outside the tube.

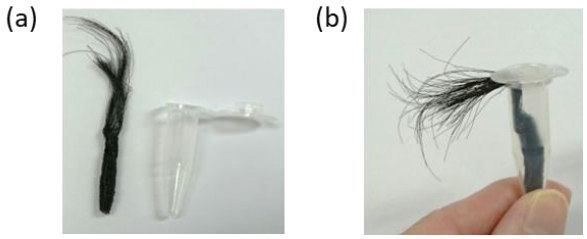

Figure 1 Hair dried in a coiled shape (a) and the cut surface of the hair held outside the container by the tube lid (b).

To obtain an enough amount of protein extracted from the cuticle layer of the hair by the formic acid treatment for short time and for ease of handling, the hair was dried in a coiled shape. To prevent extraction of cortex and medulla proteins from the cut surface of the hair, portion of the hair was kept outside the tube.

The lid of the tube was pierced with a syringe needle, 1 ml of concentrated formic acid was added, and the hair was immersed in the solution. After 5 minutes at room temperature, 100 μl of formic acid-extracted protein solution was harvested with a syringe, placed in another tube with a perforated stopper, and centrifuged at 40° C for 15 min in a centrifugal vacuum concentrator (SpeedVAC, Thermo Fisher Scientific, Goteberg, Sweden) to completely evaporate the formic acid. Then, 1 % SDS and 1 M DTT were added to the dried pellet, vortexed followed by sonication, and heated at 95° C for 3 min. The mixture was applied to a 5-20% gradient gel (e-PAGEL E-T 520L precast gel, ATTO, Tokyo, Japan) and electrophoresed at 100 V, 20 mA. Silver Stain SDS-P (marker A) and Standard Low Rang (marker B, Bio-Rad, CA, USA) were used as markers. Gels were taken out and permeabilized with immobilization solution for 20 min, followed by silver staining using Silver Quest TM Staining Kit (Invitrogen). For analysis by molecular weight range, the gels were divided into 10 equal pieces, desalted, and each was subjected to Q-Orbitrap mass spectrometry (Q-Exactive, Thermo Fisher Scientific).

【Verification of TG3 binding to zinc】

To verify the binding of TG3 to zinc, recombinant hexahistidine-tagged human TG3(His-TG3) (Zedira, Darmstadt, Germany), was mixed with 1 mM zinc sulfate, incubated at 30° C for 1 hour, and then applied to a size fractionation column (Zeba™ Micro Spin Desalting Columns, 40K MWCO, 75 µL) followed by centrifugation at 1500 *g* for 2 min at 4° C. The unbound zinc was removed by this procedure and zinc-bound TG3 eluate was obtained. Using a portion of the eluate, the protein concentration was determined to calculate the mol of TG3. The remaining eluate was diluted with nitric acid aqueous solution and the amount of zinc eluted with TG3 was analyzed by inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7700, Agilent Technologies, CA, USA). Conditions without zinc sulfate added to His-TG3 were used as a negative control, and alkaline phosphatase (ALP) (Alkaline phosphatase calf intestine,TAKARA, Kyoto, Japan), which binds to zinc in the prepared solution, was used as a positive control.

Since the recombinant TG3 has His-tag (hexahistidine-tag) that is possible to bind zinc, it was necessary to distinguish the bound zinc at either His-tag or TG3 upon detection by ICP-MS. Therefore, EGFP (enhanced green fluorescence protein) and SPR3 (small proline-rich protein 3) were selected as proteins to which does not capture zinc, and hence these His-tagged proteins (His-EGFP and His-SPR3) were prepared in *E. coli* as a recombinant protein. Briefly, *E. coli* BL21 strain harboring the expression vectors pET24d-His-EGFP or pET24d-His-SPR3 were cultured at 37°C. Then, culture with 1 mM IPTG was used for induction of the recombinant proteins for 3 hours. After harvest the *E. coli* by centrifugation at 4° C, 6000 *g*, for 5 min, lysis buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM benzamidine, 1 mM β-mercaptoethanol, 1 mM PMSF) was added, and sonicated for disruption. From the cell lysate, the recombinant protein was purified by metal affinity chromatography (TALON, TAKARA-Clontech) and further subjected to gel filtration purification to obtain highly purified His-EGFP and His-SPR3.

Either His-EGFP or His-SPR3 was mixed with zinc sulfate and passed through a size fractionation column as described in the case of TG3, and the amount of zinc (µg) bound per 1 nmol of His-EGFP or His-SPR3 was calculated from the eluate. Since this amount of zinc was deduced as that bound to the His-tag, the amount of zinc bound to TG3 was determined by subtracting the average amount of zinc bound to His-EGFP and His-SPR3 from the amount of zinc bound to His-TG3.

【Immunostaining of TG3 in the hair cuticle】

Hair was plucked from female Japanese volunteers, and about 1 cm from the hair root was freeze-embedded in Super Cryoembedding Medium (SCEM) (SECTION-LAB Co., Ltd.、Yokohama, Japan). A longitudinal section of the hair root was prepared by cryomicrotome, transferred onto cryofilm (SECTION-LAB Co., Ltd.) and incubated with 1 ng/μl of monoclonal antibody (C2D) against human TG3 followed by reaction with biotinylated secondary antibody (VECTASTAIN ABC mouse IgG kit PK-4002 VECTOR) [6] . Color development was performed using ImmPACT DAB peroxidase substrate kit (SK-4105 VECTOR). Mouse IgG was used for immunoreaction as a negative control.

【qPCR measurement of TG3 expression in the hair root】

To confirm whether the expression level of TG3 in hair root decreases with age, we collected 20 hairs from 49 Japanese volunteers from 20s to 50s (10 to 15 volunteers per age group) after obtaining informed consent. These hairs were immediately stored in liquid nitrogen, and the total RNA in the hair root was extracted with RNeasy ®Mini Kit (Qiagen). For PCR, Hs00162752_m1 (Applied Biosystems, Thermoscientific) was used as the TG3 primer. As endogenous controls, 18S rRNA and GAPDH (glyceroaldehyde-3-phosphate dehydrogenase) were chosen, and the primers Hs02387368_g1 and Hs03929097_g1 were used. Samples were prepared using Premix Ex TaqTM (TAKARA) and expression quantification of each gene was performed by relative quantification method using ViiA™ 7 Real-Time PCR System (Applied Biosystems). Then the expression levels of TG3 were corrected for 18S rRNA and GAPDH expression levels.

Results and Discussion

【Mass spectrometric analysis of proteins extracted from hair】

Hair was immersed in formic acid in order to prepare the protein extract, which enabled to harvest protein mainly from the cuticle surface and CMC. After electrophoresis, the silver-stained gel was cut into 10 pieces by the difference of their molecular mass, as shown in Figure 2.

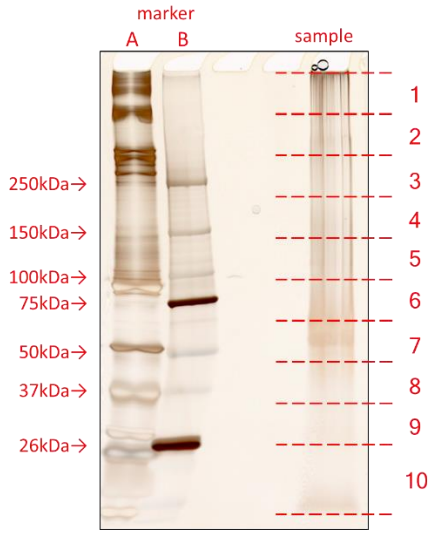

Figure 2. Electrophoresis results of the extracted proteins from hair treated with formic acid. Hair was immersed in formic acid for 5 min, and the resulting extracted protein sample was subjected to SDS-PAGE on a gradient 5%-20% gel followed by silver staining. To run mass spectrometry, the gel was cut into 10 pieces based on molecular mass. The dashed lines indicate the position of the separation.

Each gel piece was analyzed with a Q-Orbitrap mass spectrometer, and a total of 136 proteins were detected in 10 cleaved gels. Since the amount of zinc in hair is approximately 140 μg per 1 g of hair [7], we considered that in order to retain a large amounts of zinc in hair, there must be also a possible amounts of proteins that capture zinc. We selected proteins with a maximum detection score of 500 or higher in total of the excised gels, resulting in limited to 16 proteins as shown in Table 1.

Table1. Detection of proteins in each gel with scores higher than 500 .

Symbol	Description	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6	Gel 7	Gel 8	Gel 9	Gel 10
KRT1	Keratin, type II cytoskeletal 1	8,296	6,064	2,406	2,665	2,373	7,969	4,335	2,129	2,606	1,403
KRT9	Keratin, type I cytoskeletal 9	7,373	4,976	2,143	2,077	1,473	8,705	3,841	1,550	1,850	694
KRT10	Keratin, type I cytoskeletal 10	8,123	1,951	1,080	1,577	1,539	1,483	4,297	1,431	1,407	588
KRT2	Keratin, type II cytoskeletal 2 epidermal	3,248	1,126	968	1,204	1,055	1,975	1,550	677	876	443
KRT16	Keratin, type I cytoskeletal 16	1,045	860	884	1,038	1,145	1,064	3,129	1,346	1,076	380
KRT14	Keratin, type I cytoskeletal 14	1,128	864	750	952	949	1,042	3,063	1,510	1,042	467
KRT6B	Keratin, type II cytoskeletal 6B	1,462	581	691	729	756	1,030	2,165	954	348	319
KRT6A	Keratin, type II cytoskeletal 6A	1,328	566	601	682	669	858	1,886	881	337	319
KRT6C	Keratin, type II cytoskeletal 6C	1,405	511	480	562	525	895	2,005	822	337	319
KRT5	Keratin, type II cytoskeletal 5	1,187	413	330	467	552	830	1,667	573	418	186
KRT17	Keratin, type I cytoskeletal 17	420	235	262	283	387	529	1,074	984	333	204
KRT13	Keratin, type I cytoskeletal 13	1,202	266	264	326	438	390	571	343	380	201
FABP5	Fatty acid-binding protein, epidermal	137	0	0	0	210	62	39	117	306	1,212
DSP	Desmoplakin	830	109	0	0	0	59	0	0	469	51
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	143	0	0	0	39	0	73	527	244	406
TGM3	Protein-glutamine gamma-glutamyltransferase E	69	0	0	0	0	0	102	0	51	698

Of the 16 proteins detected in large quantities, most were keratin cytoskeletons, which are proteins related to keratin fibers, while others were fatty acid-binding protein, epidermal, desmoplakin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and transglutaminase, protein-glutamine gamma-glutamyltransferase E (TG3) were detected. Fatty acid-binding protein, epidermal is a protein that specifically binds C18 fatty acids, and desmoplakin is a major macromolecular protein of desmosomes and is involved in intercellular adhesion. GAPDH is implicated in glycolysis, which is constantly expressed in many cells. It is predictable that these proteins could be detected in large amounts in the cuticle layer. However, even if TG3 is the enzyme that forms an isopeptide bond between glutamine and lysine residues, the fact that its detection as zinc-bound protein in larger amounts was unexpected. TG3 is synthesized as a precursor form, followed by cleavage via responsible protease for activation, and then TG3 has enzymatic activity by binding to calcium, a divalent metal [8]. From the results, we suspected that TG3 also binds to zinc, which is also a divalent metal, Ca and Mg, but there have been no reports that TG3 binds to zinc. Therefore, in order to confirm whether TG3 binds to zinc, we tried verification using recombinant TG3 (His-TG3).

【Verification of TG3 binding to zinc】

The total amounts of zinc bound to His-TG3, His-EGFP, His-SPR3, and ALP after elution from the size fractionation column were measured, and then calculated as the quantity of zinc (μg) bound per 1 nmol of each protein, shown in Figure 3. The average value of zinc bound to His-EGFP and His-SPR3 indicated that the amount of zinc bound to the His tag portion was 0.3 μg per 1 nmol of protein. Hence, 1.83 μg of zinc appeared to bind the TG3 portion.

Since the amount of zinc bound to ALP, which was used as a positive control, was about 0.89 μg per 1 nmol, TG3 was able to bind about twice as much zinc as ALP, demonstrating that it has a high affinity for zinc.

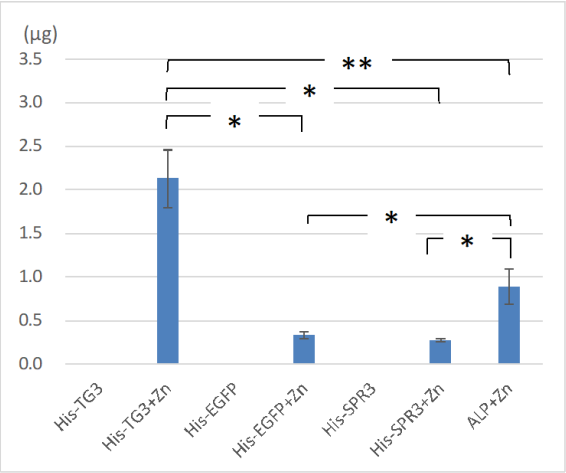

Figure 3. Amounts of zinc (μg) bound to 1 nmol of protein.

The amount of zinc bound to each protein in the eluate from the size fractionation column was measured, and the results calculated as the amount of zinc (μg) bound per 1 nmol of each protein. * $P<0.05$, ** $P<0.01$

【Immunostaining of TG3 in the hair cuticle】

Although TG3 is contained in the hair, the precise location has been remained unclear. Previously, some reports indicate that it is expressed in the cuticle layer and cortex [9], while others indicate that it is expressed in the cortex and medulla [10]. In both cases, detection by fluorescent antibody staining was performed in the state of follicle sections in which inner and outer root sheaths were present, so it was difficult to confirm that TG3 was clearly expressed in the cuticle layer. Therefore, to confirm whether TG3 was present in the cuticle layer clearly, we prepared longitudinal sections of plucked hair root and performed immunostaining using TG3 monoclonal antibody (C2D). In parallel, reaction with purified normal mouse IgG was used as a negative control. As a result, it was confirmed clearly that TG3 was present in large amounts in the hair bulb area and also in large amounts in the cuticle layer (Figure 4).

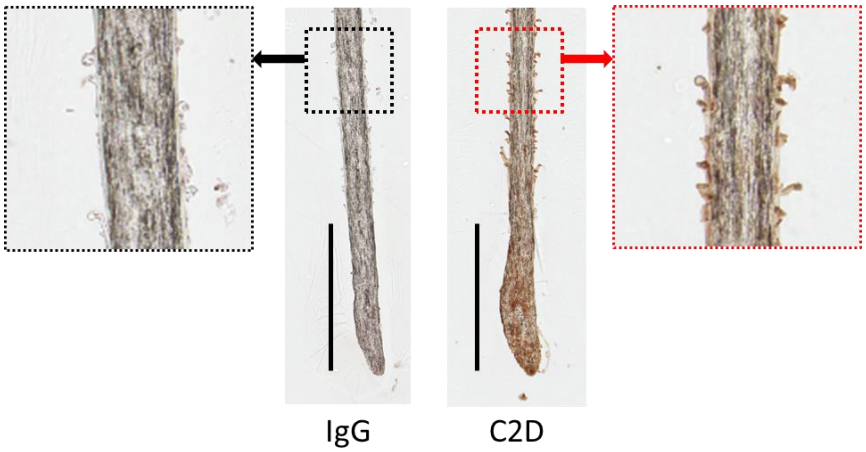

Figure 4: TG3 antibody staining of hair root.

Hair was plucked from a Japanese volunteer female, immediately freeze-embedded in SCEM, and cryomicrotomed for longitudinal section, which was subjected to immunostaining using TG3 antibody. The scale bar means 300 μm.

【qPCR measurement of TG3 expression in the hair bulb】

Finally, to confirm whether decreases in the TG3 expression depends on age, we examined TG3 expression in hair root of Japanese volunteers in their 20s to 50s (10 to 15 volunteers in each age group). Two types of endogenous controls, 18S rRNA and GAPDH, were used. As a result, we found that TG3 expression had tendency to decrease with age, even when corrected for both endogenous control, and that the decrease was particularly apparent after the age of 40s, as was the case with zinc. [7]

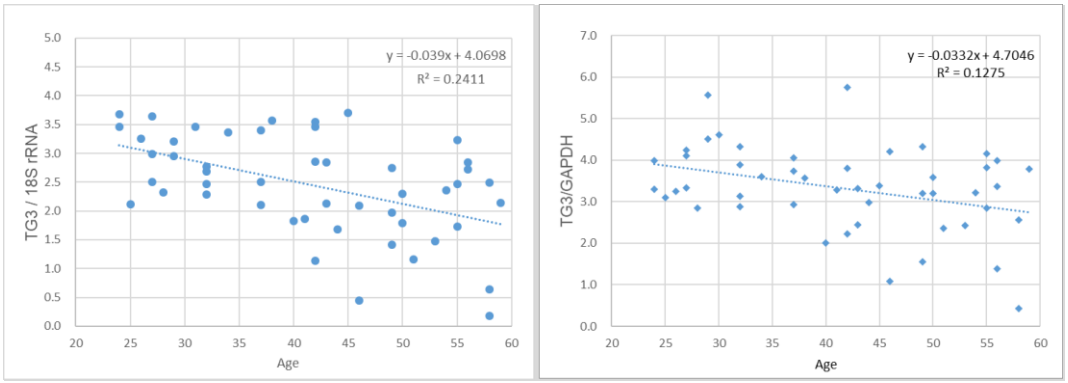

Figure 5. TG3 mRNA expression level in the hair at various aged group (endogenous control: 18S rRNA, GAPDH)

Hair was plucked from Japanese volunteers, and total RNA was immediately extracted from the hair root. TG3 expression levels were measured by qPCR. GAPDH and 18S rRNA were used as endogenous controls, and the results were corrected for their relative ratio.

Conclusion

By a series of experiments, the decrease in zinc in the hair and also its firmness with age appeared to be partly due to the decrease in the expression of TG3. Beside of the protein cross-linking activity this enzyme was identified as zinc binding protein in the cuticle layer. Depending on age, it becomes more difficult to retain zinc in the cuticle layer. Consistent with this change, the expression levels of TG3 gradually decreased, newly analyzed by our qPCR analysis. TG3 has been known as an enzyme that provides hair stiffness by cross-linking structural proteins with strong isopeptide bonds, but here we showed a novel role of this enzyme that also contributes to hair stiffness by retaining zinc in the cuticle layer of post-keratinization hair.

In the future, application of compounds that enhance TG3 expression to the scalp is strongly expected to keep hair firmness, by preventing age-dependent decrease in TG3 and its captured zinc in the hair.

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Conflict of Interest Statement

The authors declare that they have no conflicts of interest with contents of this research article.

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