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Effects of human hair and nail proteins and their films on rat mast cells

Toshihiro Fujii · Shinya Murai · Kousaku Ohkawa · Toshihiro Hirai

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Abstract Human hair and nail are valuable materials for producing individual corresponding biocompatible materials. A rapid and convenient protein extraction method (Shindai method) and novel procedures for preparing their protein films from their extracts have been developed using human hair and nail. The effects of the human hair and nail proteins and their films on histamine release from rat peritoneal mast cells were investigated. Both protein solutions and their films, mainly consisting of keratins and matrix proteins, did not induce histamine release from the mast cells. Scanning electron microscopy (SEM) also showed that the mast cells were only slightly affected by adding the human hair and nail proteins or by incubating on their protein films. The IgE-dependent histamine release was inhibited by the hair and nail proteins and their films. Incubation of the mast cells with the hair and nail proteins prior to the addition of the IgE serum resulted in a high inhibition (50%) of the histamine release, while the inhibition was approximately 10% when the protein solutions were mixed with the mast cells after incubation with the IgE serum. These results suggest that the human hair and nail proteins and their films will be useful materials for antiallergic actions.

T. Fujii (✉) · S. Murai
Department of Kansei Engineering, Faculty of Textile Science and Technology, Shinshu University, Tokida 3-15-1, Ueda 386-8567, Japan
e-mail: fujiit1@shinshu-u.ac.jp

K. Ohkawa
Institute of High Polymer Research, Faculty of Textile Science and Technology, Shinshu University, Tokida 3-15-1, Ueda 386-8567, Japan

T. Hirai
Department of Materials Chemistry, Faculty of Textile Science and Technology, Shinshu University, Tokida 3-15-1, Ueda 386-8567, Japan

1 Introduction

Human hair and nail are valuable materials for producing individual corresponding biocompatible materials. Hair proteins mainly consist of microfibrial α-keratins with molecular masses of 40–65 kDa and matrix proteins with 10–20 kDa based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A rapid and convenient protein extraction method (Shindai method) was developed for quantifying the human hair and nail components [1]. The amount of protein extracted by the Shindai method was 2.5–3.5 times more than those obtained by other conventional methods [1, 2].

Novel procedures were also developed for preparing human hair protein films (Pre-cast, Post-cast, and soft Post-cast methods) [2–4]. These protein films were water-insoluble and mainly consisted of α-keratin. Reinforcement of the human hair protein films with cotton gauze made it possible to apply them on human skin for several days. A patch test using human skin indicated that the protein films made from individual and multiple human hairs did not cause itching, drying smarting, and pain on the contact areas of arms [4]. Yamauchi et al. reported that mouse fibroblast cells adhered, spread and grew on wool keratin-coated dishes as well as collagen- and polyvinyl alcohol-coated ones [5]. Few problems with the cultivation of the fibroblast cells were also confirmed using wool keratin sponge scaffolds [6]. We examined the antibody-producing activity against mouse body hair

proteins, human hair proteins, and human nail proteins using BALB/c mice. The reactivity of sera against the mouse body hair proteins was less than those against the human hair and nail proteins, indicating the presence of an immunological specificity [7]. The protein films prepared from human hair and wool were biodegraded by proteases [8, 9].

Mast cells are widely distributed throughout the epithelia and vascularized tissues and responsible for type I allergic disorders such as dermatitis, allergic rhinitis and fool allergies [10, 11]. Mast cells release histamine and other potent inflammatory mediators in response to various stimuli. The interaction between the IgE-directed antigen and the IgE receptors on the mast cell surfaces causes cross-linking between the IgE receptors and the release granules, the latter of which contain the performed inflammatory mediators and generate inflammatory lipids and cytokines [11, 12]. The stimulation of mast cells is also induced by proteins and peptides [12, 13].

The allergic effects of the hair protein containing α-keratins have not yet been studied. As model experiments, we examined the effects of the human hair and nail proteins and their films on rat peritoneal mast cells.

2 Materials and methods

2.1 Preparation of mast cells

Peritoneal mast cells were obtained from the abdominal cavities of the male rat (Wister; 300–400 g) [13]. Briefly, the rats were anesthetized with ether and injected with 20 mL of mast cell medium (MCM) which was comprised of 144 mM NaCl, 2.7 mM KCl, 4.7 mM Na_2HPO_4, 2 mM KH_2PO_4, 5.6 mM glucose, 10 U mL^{-1} heparine and 1 mg mL^{-1} bovine serum albumin (BSA), pH 7, into the peritoneal cavity, and then the abdomen was massaged for about 5 min. The peritoneal cavity was opened and the fluid was collected using a pipette. The mast cells were purified to at least 95% homogeneity by centrifugation through a 2 mL cushion of 24% metrizamide, and washed twice by centrifugation at 4 °C. The cells were re-suspended in MCM buffer at a density of approximately 5×10^5 mL^{-1} and used for each experiment.

2.2 Preparation of human hair and nail protein and their films

Human hair and nail protein solutions were prepared according to the "Shindai method", which has been previously described by us [1, 2]. Briefly, the human hair and nail were washed with ethanol and incubated with a solution consisting of 2.6 M thiourea, 5 M urea, 5% 2-mercaptoethanol, and 25 mM Tris–HCl (pH 8.5) at 50 °C for 2–4 days. After filtration, the solution was dialyzed against MCM buffer without BSA for 3–4 days at 25 °C. The obtained dialysate was further centrifuged at 15,000× *g* for 20 min at 25 °C, and the supernatant was used as the protein solution.

The human hair and nail protein films were prepared by the Post-cast and soft Post-cast methods [3, 4]. The hair and nail proteins dissolved in the Shindai solution were cast in solution containing 100 mM acetate buffer (pH 4 and 5) (Post-cast method) and 40 mM $MgCl_2$ (soft Post-cast method). After standing for 1 h at room temperature, membrane-like protein films were formed and washed by rinsing with water for 36–48 h and then immersed (replaced) into MCM for 12–15 h.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 13.5% polyacrylamide gel according to the method of Laemmli [14]. Proteins in the gel were stained with 0.1% Coomassie brilliant blue R-250, 10% acetic acid and 40% ethanol for 1–3 h and destained in 10% acetic acid and 40% ethanol. The protein concentrations were determined according to Bradford [15] using BSA as the standard.

2.3 Assay of IgE-independent and -dependent histamine release

The suspended mast cells (2×10^4 per tube) were preincubated with the hair and nail proteins or on the dishes coated with protein films for 5 min at 37 °C. After the addition of 0.1 mM $CaCl_2$, the cell suspension was further incubated for 10 min at 37 °C. The reactions were terminated by adding 1.8 mL of ice–cold MCM buffer containing 1 mM EGTA. The mast cells were stimulated by compound 48/80 (0–0.4 μg mL^{-1}).

The IgE sensitized mast cells were prepared by incubation for 60 min at 37 °C with rat IgE and phosphatidyl serine (50 μg mL^{-1}) and then washed once by centrifugation. The mast cells (0.18 mL) were preincubated with the hair and nail proteins or on the dishes coated with the protein films for 5 min at 37 °C and ovalbumin as an antigen at 50 μg mL^{-1} (0.02 mL) was added to the sensitized cells for stimulation. Incubation was carried out for 10 min at 37 °C, and the reactions were stopped by adding 1.8 mL of ice–cold MCM. After centrifugation, the histamine content of the supernatant (1 mL) was determined using *o*-phthalaldehyde as described by Shore et al. [16]. The total histamine was released by disrupting the cells with boiling. Histamine release from the mast cells was calculated using the

following equation: [(histamine release) − (spontaneous histamine release)]/(total histamine − spontaneous histamine release) × 100.

2.4 Scanning electron microscopy (SEM) observation

Mast cells were prefixed with 2.5% glutaraldehyde and postfixed with 1% OsO_4. The cells were dehydrated in a graded alcohol series and critical point-dried using liquid CO_2. The protein films were sputtered with gold and examined using a scanning electron microscope (Hitachi S-2380N) [17].

3 Results and discussion

3.1 Effect of hair and nail proteins on mast cells

The human hair and nail proteins were prepared by the Shindai method, and their protein components were analyzed by SDS-PAGE (Fig. 1a). The hair protein sample was comprised of the type I (acidic) and type II (neutral/basic) hard α-keratins and matrix proteins. The nail protein sample contains both the hard α-keratins and the soft keratin, the latter of which has a molecular mass of 56 kDa [1, 2].

When the mast cells were incubated with the hair and nail protein samples (0–5 mg mL^{-1}), no significant release of histamine was observed in the presence and absence of Ca^{2+} (Fig. 1b), whereas the addition of compound 48/80, a representative stimulator, triggered the histamine release in a concentration-dependent manner (Fig. 1c).

3.2 Morphology of mast cells incubated with the proteins

The surface of the mast cells with diameters of 10–20 μm represents a number of granules (diameters, 0.2–0.5 μm), which contain the pre-formed inflammatory mediators, such as histamine, serotonin, and substance P [12, 13]. Immunological and non-immunological stimuli cause degranulation that releases the mediators from the granules.

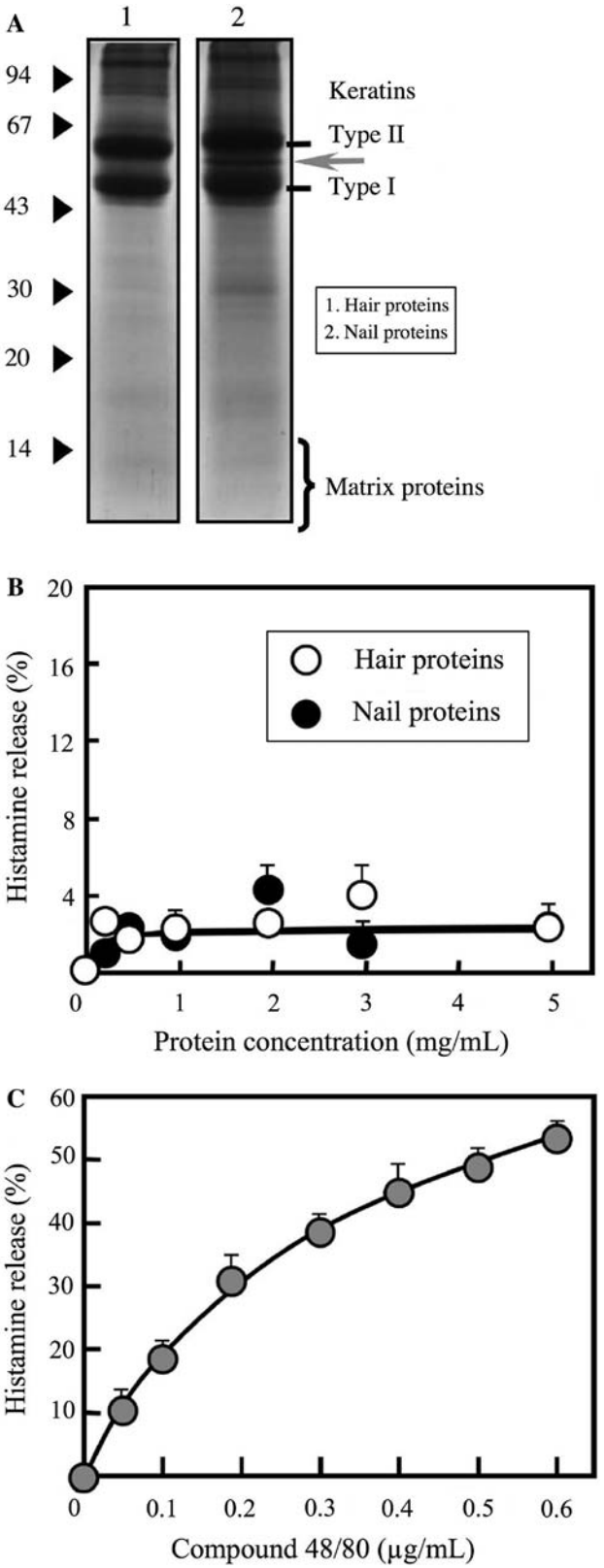

Fig. 1 SDS-PAGE of human hair and nail proteins and histamine release from rat mast cells by compound 48/80, hair proteins, and nail proteins. Proteins were prepared from washed human hair and nail by the Shindai method and subjected to 13.5% SDS-PAGE (**a**). Rat peritoneal mast cells were preincubated for 5 min at 37 °C in the presence of various concentrations of compound 48/80 (**b**) and the hair (◯) and nail proteins (●), as indicated (**c**). The cells were challenged with 0.9 mM $CaCl_2$ for 10 min at 37 °C and the amount of released histamine was measured. Values are expressed as means ± SD for 3–4 experiments

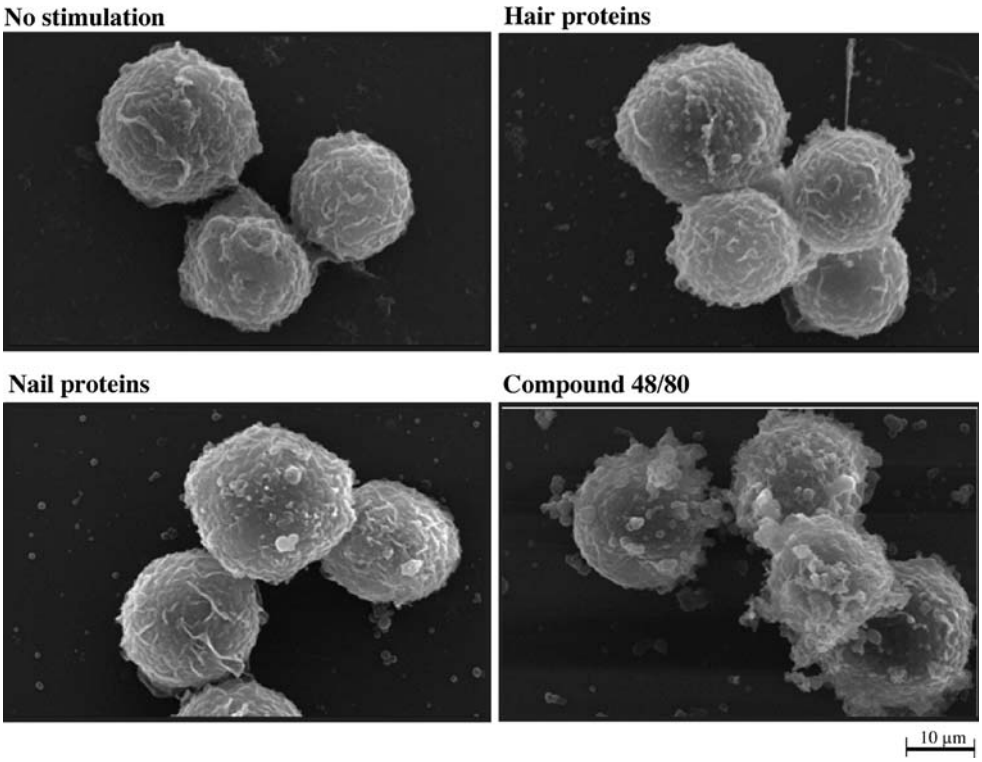

Fig. 2 Morphological observations of mast cells incubated with compound 48/80, hair proteins, and nail proteins. The mast cells were exposed to compound 48/80, the hair proteins, and the nail proteins as shown in Figure 1 and observed by SEM. The concentrations of compound 48/80, the hair proteins, and the nail proteins were 0.3 μg mL^{-1}, 3 mg mL^{-1}, and 3 mg mL^{-1}, respectively. Bars, 10 μm

Figure 2 shows SEM pictures of the mast cells. After stimulation, the appearances of the mast cell surfaces were different from those of the non-stimulated cells whose surfaces were covered with lamellar architectures, which correspond to the granule structures containing the inflammatory mediators. In the presence of compound 48/80 (0.2 μg mL^{-1}), the granule structures were observed on the surfaces of the mast cells. The addition of the hair or nail proteins did not apparently affect the morphology of the mast cells. These results clearly suggest that the human hair and nail proteins had little or no stimulating effect on the peritoneal mast cells.

3.3 Mast cells on hair or nail protein films

In a series of our previous studies, we have developed conventional methods to prepare stable films from the human hair and nail proteins [3, 4]. Figure 3 shows the surface of the hair protein films based on SEM observations. As previously reported, [3, 4] the surface of the protein film prepared by the Post-cast method (acetate buffer pH 4) was rough and covered with a filamentous structure which contains smaller particles having diameters of 0.5–2 μm (Fig. 3a). The surface of the film prepared by the soft Post-cast method was smoother (Fig. 3b) than that by the Post-cast method (Fig. 3c). When the proteins from the hair and nail protein films were extracted again and they were then analyzed by SDS-PAGE, the enriched protein components were found as the hard keratin for the hair protein films and as the hard plus soft keratins for the nail protein films (data not shown).

The mast cells were exposed to the dishes coated with the hair and nail protein films and incubated for 0–90 min at 37 °C, then the release of histamine and the morphological changes of the mast cell surfaces were examined (Fig. 4). Time-dependent continuous releases of histamine (approximately 4%) from the mast cells were found in the hair and nail protein films as well as the control polystyrene dish (Fig. 4a). These results indicate the spontaneous degranulation occurred during the incubation periods. SEM observations also revealed no significant morphological change in the mast cells, which were attached on the films after incubation for 60 min (Fig. 4b). This result strongly suggests that the hair and nail protein films, like their protein solutions (Figs. 1 and 2), had little stimulating effect on the mast cells.

3.4 IgE-dependent histamine release

The release of the pre-formed inflammatory mediators is generally triggered by a cross-linking between the IgE

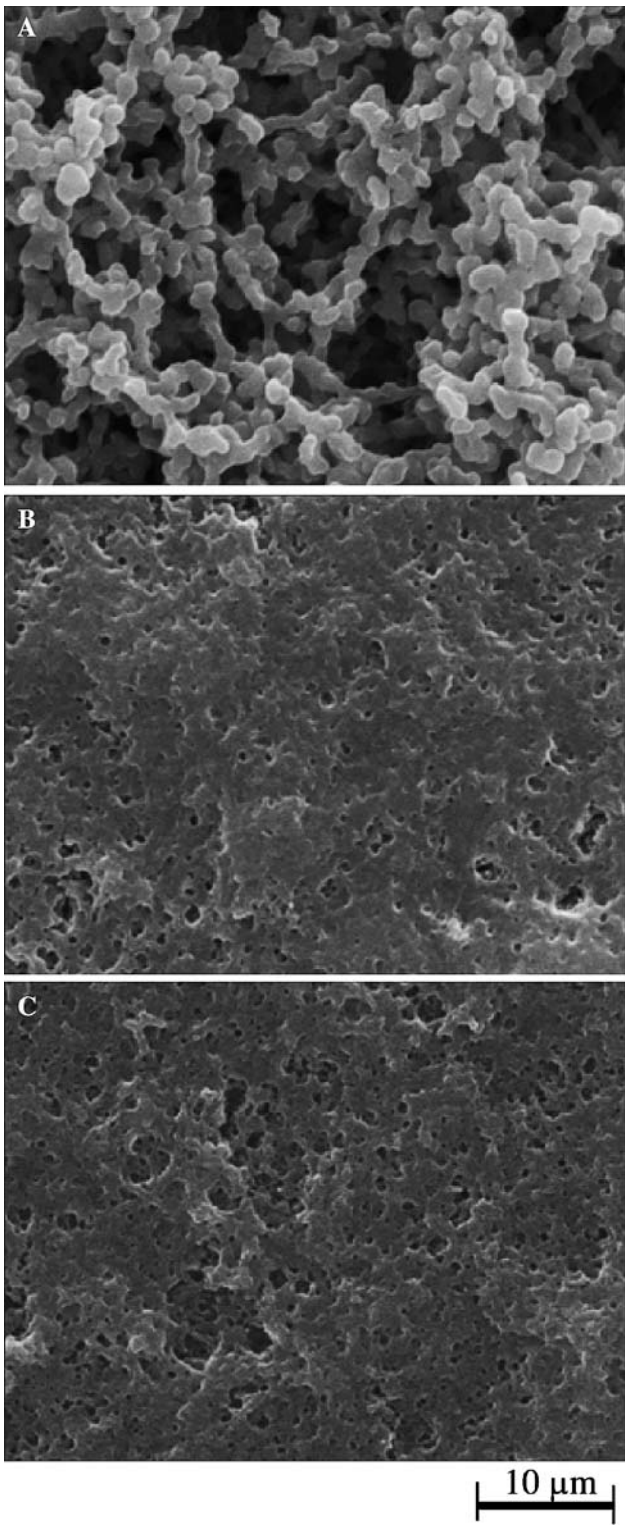

Fig. 3 Morphological observation of hair protein films. The hair protein films were prepared by the Post-cast method (**a**, 100 mM acetate buffer, pH 4 and **b**, 100 mM acetate buffer, pH 5) and soft Post-cast method (**c**, 40 mM $MgCl_2$) and observed by SEM. Bars, 10 μm

receptors and the polyvalent allergens on the mast cell surfaces. We prepared a serum containing anti-ovalbumin IgE from a rat and constructed an experimental system for IgE-dependent exocytosis. The mast cells were preincubated with the serum and phosphatidyl serine vesicles for 60 min at 37 °C in order to prepare the IgE-sensitized mast cells. When ovalbumin as an antigen was added to the IgE-sensitized mast cells, the extent of the histamine release increased with the increasing concentration of the applied ovalbumin (Fig. 5a). The maximum histamine release reached approximately 20–25% of the total histamine content.

The effects of the hair and nail proteins on the IgE-dependent histamine release were subsequently examined using two procedures. First, when the mast cells were preincubated with the hair and nail proteins for 5 min at 37 °C before incubation with the IgE serum and then stimulated by the addition of ovalbumin, the histamine release was inhibited up to approximately 50% in the presence of the hair and nail proteins above 0.5 mg mL^{-1} (Fig. 5b). Next, when the hair and nail proteins were added to the mast cell solutions after incubation with the IgE serum and then stimulated by ovalbumin, approximately 10% of the histamine release was inhibited by over 0.5 mg mL^{-1} of the hair and nail proteins (Fig. 5c). The extent of the inhibition was higher when the mast cells were preincubated with the hair and nail proteins prior to the addition of IgE.

We examined the effects of the hair and nail protein films on the IgE-dependent histamine release from the mast cells (Fig. 6). The IgE-sensitized mast cells were preincubated for 60 min at 37 °C on the dishes coated with the hair and nail protein films as shown in Fig. 4. After incubation, ovalbumin was added to the dishes and further incubated for 10 min at 37 °C. Similar to the previous results, addition of ovalbumin stimulates the IgE-sensitized mast cell for the histamine release. The extent of the histamine release from the mast cells attached on the protein films was approximately 60% compared to that from the mast cells on the control dishes. No significant difference was found between the hair and nail protein films. These results indicated that the hair and nail proteins and their films suppressed the IgE-induced histamine release from the mast cells, and the extent of inhibition was dependent on the order of addition of the protein, IgE, and mast cells.

The antiallergic effects towards rat mast cells in vitro were analyzed as part of our continuing research of the human hair proteins and their films having biocompatibility. In this study, unlike compound 48/80, the human hair and nail proteins and their films did not directly stimulate the mast cells. The mast cells were stimulated by proteins and peptides including protamine, salmine, clupenine, mast cell degranulating peptide, melitin, and mastparan, and substance P [13, 18, 19]. Most of these proteins and peptides are basic, and three or more basic

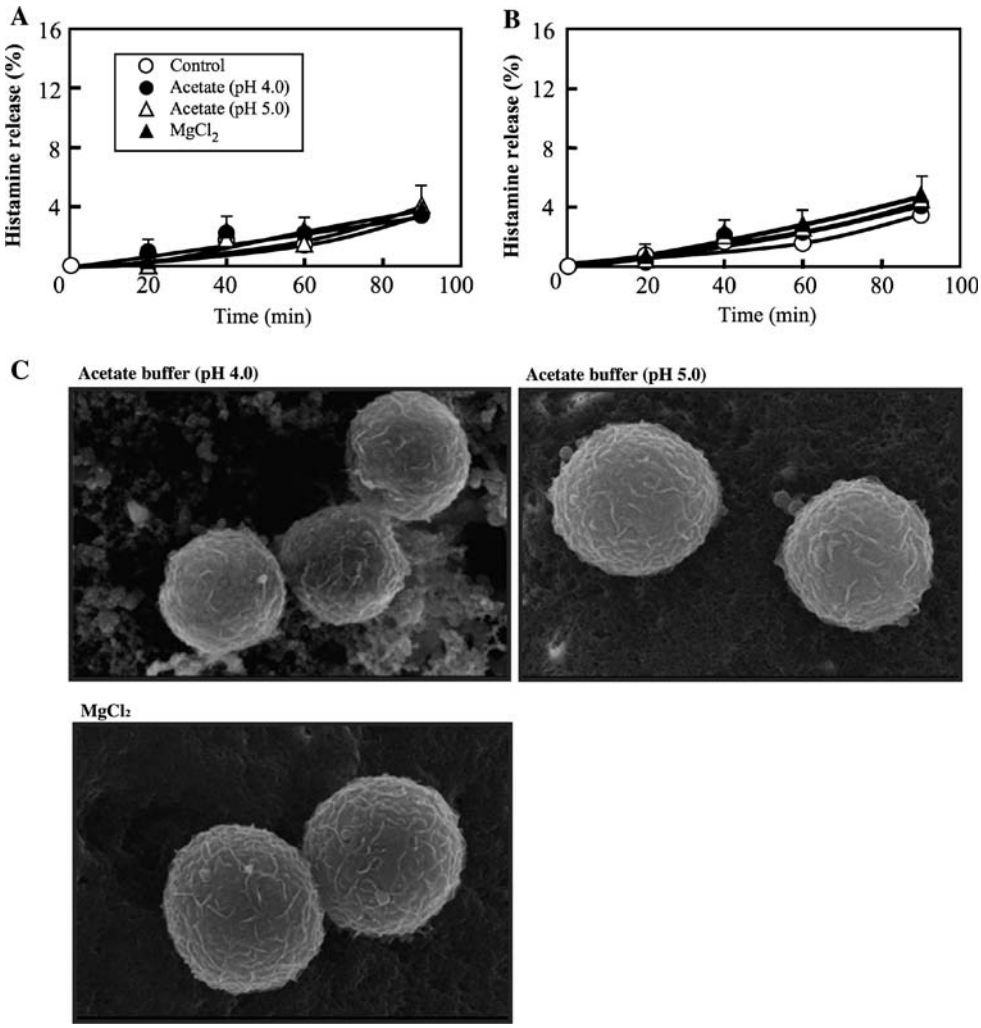

Fig. 4 Histamine release from rat mast cells exposed to hair and nail protein films and morphological observations. The mast cells were incubated for 5 min at 37 °C on the hair (**a**) and nail (**b**) protein films as shown in Fig. 3: ○, Control (dish); ●, acetate buffer (pH 4); Δ, acetate buffer (pH 5); ▲, 40 mM $MgCl_2$. The cells were challenged with 0.9 mM $CaCl_2$ for 10 min at 37 °C, and the amount of released histamine was measured. Values are expressed as means ± SD for 3–4 experiments. The mast cells after incubation with the protein films for 60 min were observed by SEM (**c**)

amino acids are repetitively arranged in their primary structures. Hard keratins occurring in hairs and nails are classified into two subfamilies, which consist of at least 4–9 distinct type I acidic members (40–50 kDa) and 4–6 type II neutral/basic (55–65 kDa) members [20–22]. Among the amino acid sequences of the keratins, the trilysyl (KKK) sequences were located in the type II keratin Hb3 (249–252), while the other repeating sequences found in the keratin do not contain the KKK or triarginyl (RRR) sequence [22, 23]. Tertiary structures of the intermediate filament proteins are generally constructed from three domains, i.e., head, rod, and tail. The KKK sequence of Hb3 was located in the rod domain, which is essential for the assembly of proteins into filaments. This indicates that the KKK sequence that localizes inside the Hb3 molecule cannot directly contact the mast cell surfaces, so that the hair and nail proteins, enriched in keratin, do not stimulate the histamine release from the mast cells.

4 Conclusion

In the present study, we confirmed the inhibitory effects of the human hair and nail proteins and their films on the IgE receptor-stimulated histamine release from mast cells. These findings indicate that the human hair and nail proteins and their films actually interact with IgE, because the inhibition became more remarkable when the mast cells were preincubated with the hair and nail proteins prior to the addition of IgE than that when the cells were preincubated with IgE prior to the addition of the proteins. Human hair and nail are easily collected at an individual level and the extraction by the Shindai method recovers the hair and nail proteins in a high yield. The extracted proteins are fabricated into films, [3–5] fibers, [24] and microspheres [25] from their aqueous solutions. Thus, these products will become some of the self-originating materials with promising biocompatibilities. The potential application of the present materials encompasses health care,

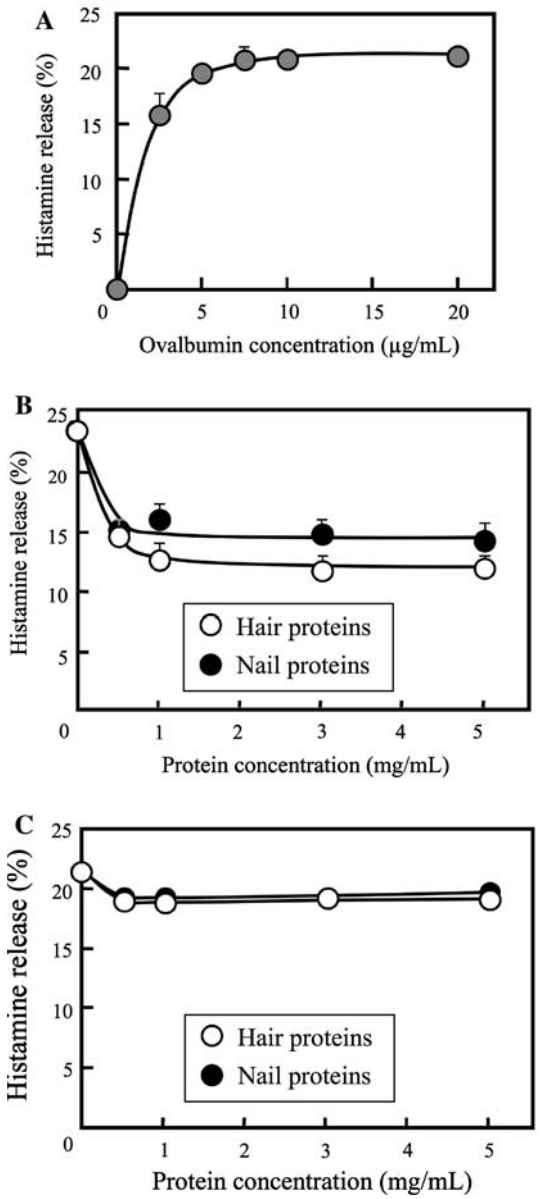

Fig. 5 Effects of the hair and nail proteins on the IgE-dependent histamine release from mast cells. The mast cells were incubated with IgE serum for 60 min at 37 °C and then stimulated for 10 min at 37 °C with various concentrations of ovalbumin as indicated (**a**). The histamine release was measured when the obalbumin was added after preincubation of the mast cells with hair (◯) and nail proteins (●) (**b**) and when the hair and nail proteins were added after preincubation of the mast cells with IgE serum (**c**). Values are expressed as means ± SD for 3–4 experiments

cosmetic, and medical uses, such as wound dressing films or drug releasing films.

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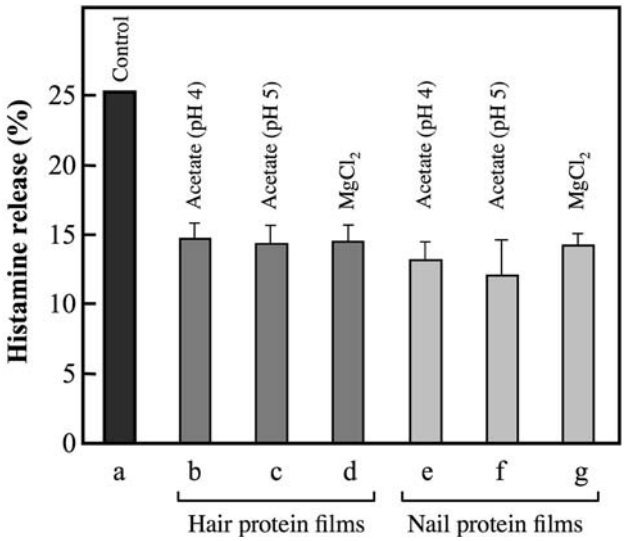

Fig. 6 Effects of hair and nail protein films on IgE-dependent histamine release from mast cells. The mast cells on the control dish (a), hair protein films (b–d), and nail protein films (e–g) were incubated with IgE serum for 60 min at 37 °C and stimulated for 10 min at 37 °C with 10 μg mL^{-1} ovalbumin. The dishes were coated with protein films; Post-cast method (b and e, 100 mM acetate buffer, pH 4), Post-cast method (c and f, 100 mM acetate buffer, pH 5), and soft Post-cast method (d and g, 40 mM $MgCl_2$). Values are expressed as means ± SD for 3–4 experiments

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