Expression of Hard α-Keratins in Pilomatrixoma, Craniopharyngioma, and Calcifying Odontogenic Cyst

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Abstract

To examine the properties of shadow and ghost cells, 3 kinds of antibodies were raised against human hair proteins and their immunoreactivity was examined in tumors expressing those cells: pilomatrixoma, 14 cases; craniopharyngioma, 17 cases; and calcifying odontogenic cyst (COC), 14 cases. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analyses demonstrated that 2 polyclonal antibodies, PA-HP1 and PA-HP 2, reacted strongly with type I acidic and type II neutral/basic hard α-keratins. The other monoclonal antibody, MA-HP1, reacted with type II neutral/basic hard α-keratins. Immunohistochemical examination revealed that all 3 antibodies reacted only with the hair shaft in sections of normal skin and dermoid cyst. In all pilomatrixoma cases, 3 antibodies reacted with the cytoplasm of transitional and shadow cells but not with that of basophilic cells. Positive reactions were found only in shadow cells of all 13 adamantinomatous craniopharyngiomas. In all COCs, the antibodies reacted only with ghost cells, not with other epithelial components. Immunoreactivity for phosphothreonine, detected in hard α-keratins, also was found in transitional, shadow, and ghost cells. The appearance of shadow or ghost cells might represent differentiation into hair in these 3 kinds of tumors.

In pilomatrixoma, a common benign tumor with hair matrix cell differentiation, tumor nests are composed of basophilic, transitional, and shadow cells, corresponding to the epithelial components of mature hair follicles.1 The appearance of shadow cells in the tumor might represent the endpoint of hard keratinization of matrix cells into hair cortex.1,2

There are 2 other tumors displaying shadow cells or their analogue, ghost cells: craniopharyngioma of the pituitary gland3,4 and calcifying odontogenic cyst (COC) of the jaw.5 Recent studies have documented the similarity of the accumulation and alteration of β-catenin in these 3 types of tumors, suggesting an important role of activation of the Wnt-β-catenin-TCF-Lef (T-cell factor/lymphoid enhancer factor) pathway in the pathogenesis of these tumors.6-10 It has been reported that this activated pathway induces differentiation to hair shafts.8-14 Cribier et al2 demonstrated the expression of messenger RNA for human hair keratin basic 1 in transitional cells by in situ hybridization and suggested that the expression of hard keratins in transitional cells might resemble the maturation process of the hair shaft. Tateyama et al15 demonstrated immunohistochemically the expression of human hard α-keratins in shadow cells of adamantinomatous craniopharyngioma and proposed that this tumor might show hair follicle differentiation. Although there have been no English-language articles on the expression of hard α-keratins in ghost cells, melanin pigmentation as seen in cortex cells of the normal hair shaft occasionally is observed in some COC cases.16,17 These findings suggest that differentiation into hair occurs in COC.

To examine the properties of shadow and ghost cells in pilomatrixoma, craniopharyngioma, and COC, 2 polyclonal antibodies and 1 monoclonal antibody were raised against human hair proteins, and their immunoreactivity was tested on
sections of these 3 types of tumors. In addition, immunoreactivity for phosphothreonine, which has been detected in hard α-keratins, also was examined.

Materials and Methods

Extraction of Hair Proteins

Human hair proteins were extracted according to the method of Nakamura et al.18 (the Shindai method). Human hair was washed with ethanol, and the external lipids were removed by treating with a mixture of chloroform and methanol (2:1, vol/vol) for 24 hours. The delipidized hair (20 mg) was mixed with a solution (5 mL) containing a 25-mmol/L concentration of tris(hydroxymethyl)aminomethane (Tris) hydrochloride, pH 8.5, a 2.6-mol/L concentration of thiourea, a 5-mol/L concentration of urea, and 5% (vol/vol) 2-mercaptoethanol at 50°C for 24 hours. The mixture was filtered and centrifuged at 15,000g for 20 minutes at room temperature. The obtained supernatant was dialyzed against 2 L of distilled water with 5 changes and used as hair protein fraction.

Production of Antibodies

Hard α-keratins of molecular masses of 40 to 65 kd separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were mixed with the Freund complete adjuvant and injected subcutaneously into rabbits during a 1-month period. Serum samples were obtained from the immunized rabbits, and their reactivity with hair protein fractions was confirmed by enzyme-linked immunosorbent assay. Two kinds of IgG fraction (polyclonal antibody against hair proteins 1 and 2 [PA-HP1, PA-HP2, and MA-HP1]) were purified by protein G affinity chromatography.

To obtain a murine monoclonal antibody, a mixture of hair proteins and the Freund complete adjuvant was injected intraperitoneally 4 times into BALB/c mice during a 2-month period. Spleen cells from immunized mice were fused with P3-X-63-Ag8-U1 cells using polyethylene glycol 4,000. Hybridomas were grown and selected in three 96-well tissue culture plates using a medium containing hypoxanthine, aminopterin, and thymidine. After 10 days, the culture supernatants were screened by enzyme-linked immunosorbent assay. Hybridomas producing antibodies that reacted strongly with hair proteins were cloned under limiting dilution conditions and a monoclonal antibody (monoclonal antibody against hair protein 1 [MA-HP1, IgG1, κ]) was obtained.

Collection of Samples

Formalin-fixed, paraffin-embedded tissue samples from 14 cases of pilomatrixoma, 17 cases of craniopharyngioma, and 14 cases of COC were used. Samples were fixed in 10% (vol/vol) buffered formalin and embedded in paraffin wax. Sections of normal skin and dermoid cyst were used as positive control samples. Each section was prepared for immunohistochemical analysis.

Immunohistochemical Analysis

Deparaffinized sections were immersed in absolute methanol containing 0.3% (vol/vol) hydrogen peroxide for 20 minutes at room temperature to block endogenous peroxidase activity. After washing with running water and phosphate-buffered saline (PBS, pH 7.4), the sections were immersed in a 0.01-mol/L concentration of citrate buffer (pH 6.0) and heated in a microwave oven for 15 minutes as described by Shi et al.19 The sections were incubated in 2% (wt/vol) bovine serum albumin in PBS for 15 minutes at room temperature to block nonspecific reactions. Appropriately diluted PA-HP1, PA-HP2 (10 µg/mL), MA-HP1 (supernatant, 1:50), or a monoclonal antibody against phosphothreonine (Affiniti Research Products, Mannhead, England; 1 µg/mL) were applied to each section for 1 hour at room temperature. The tissue sections were washed with PBS and then incubated with biotinylated goat antirabbit IgG (heavy and light chains) antibody (dilution 1:200; Vector Laboratories, Burlingame, CA) or biotinylated horse antimouse IgG (heavy and light chains) antibody (dilution 1:200; Vector Laboratories) for 30 minutes at room temperature. An appropriately diluted streptavidin-peroxidase antibody (1:200; GIBCO-BRL, Grand Island, NY) was applied to the tissue sections for 30 minutes. The sections were washed with PBS, immersed for 10 minutes in 0.05% (wt/vol) 3,3’-diaminobenzidine tetrahydrochloride in a 0.05-mol/L concentration of Tris-hydrochloride buffer (pH 7.6) containing 0.01% (vol/vol) hydrogen peroxide, and then counterstained with Mayer hematoxylin.

Western Blot Analysis

Extracted hair protein was examined by using SDS-PAGE with a 10% (wt/vol) slab gel. Proteins in the gel were stained with 0.25% (wt/vol) Coomassie brilliant blue R-250, 50% (vol/vol) acetic acid, and 50% (vol/vol) ethanol for 1 hour and

Table II

| Pattern of Immunostaining by Three Newly Raised Antibodies (PA-HP1, PA-HP2, and MA-HP1) |
|-----------------------------------|----------------|----------------|
| Tumor                            | Reactivity     | Positive Cells |
| Pilomatrixoma (n = 14)            | 14             | Transitional and shadow cells |
| Cranioopharyngioma (n = 17)       |                | Shadow cells    |
| Adamantinomatous (n = 13)         | 13             |                |
| Papillary (n = 4)                 | 0              |                |
| Calcifying odontogenic cyst (n = 14) | 14         | Ghost cells    |

HP, hair protein; MA, monoclonal antibody; PA, polyclonal antibody.
destained in 7% (vol/vol) acetic acid and 50% (vol/vol) methanol. Proteins separated on SDS-PAGE were transblotted onto a nitrocellulose membrane in a solution containing a 48-mmol/L concentration of Tris hydrochloride, a 39-mmol/L concentration of glycine, and 0.4% (wt/vol) SDS in 20% (vol/vol) methanol. The membrane was incubated with 5% (wt/vol) bovine serum albumin in PBS and then with appropriately diluted PA-HP 1, PA-HP2 (1 µg/mL), MA-HP1 (supernatant, 1:20), or a monoclonal antibody against phosphothreonine (0.25 µg/mL) at room temperature for 1 hour. The membrane was washed, and the same procedures that were used for immunohistochemical processing were followed.

The study was performed according to the guidelines of the Meikai University Ethics Committee, Saitama, Japan.

**Results**

SDS-PAGE and Western blot analyses revealed that PA-HP1 reacted strongly with type I acidic and PA-HP2 with type I acidic and type II neutral/basic hard α-keratins. MA-HP1 reacted with type II neutral/basic hard α-keratins. The monoclonal antibody against phosphothreonine reacted with both types of hard α-keratins [Image 1].

**Image 1** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analyses. Lane 1, Type I acidic and type II neutral/basic hard α-keratins stained strongly by Coomassie brilliant blue (CBB). Lane 2, Immunoreactivity of monoclonal antibody against hair protein (MA-HP) 1 with type II neutral/basic hard α-keratins. Lane 3, A strong reaction of polyclonal antibody against hair protein (PA-HP) 1 with type I acidic hard α-keratins. Lane 4, A strong reaction of PA-HP2 with both types of hard α-keratins. Lane 5, A positive reaction for phosphothreonine with type I acidic hard α-keratins.

Immunohistochemical examination revealed that all 3 antibodies, PA-HP1, PA-HP 2, and MA-HP1, produced similar staining patterns. The antibodies reacted only with hair shafts in the sections of normal skin [Image 2A] and dermoid cyst [Image 2B]. Positive reaction products were found in the cytoplasm of transitional and shadow cells but not in that of basophilic cells in all 14 pilomatrixomas (Table 1) [Image 3]. Among 17 cases of craniopharyngioma, 4 were papillary craniopharyngiomas and composed of papillary proliferation...
of squamous epithelium without shadow cells. A positive reaction was found only in shadow cells of the 13 adamantinomatous craniopharyngiomas (Table 1) "Image 4". In all 14 COCs, the antibodies reacted only with ghost cells, not with any other epithelial components (Table 1) "Image 5". In addition, immunoreactivity for phosphothreonine, detected in hard α-keratins, was found in transitional, shadow, and ghost cells in these 3 types of tumors "Image 6", "Image 7", and "Image 8".

**Discussion**

Human hair proteins consist of 2 groups: hard α-keratins forming microfibrous intermediate filaments and nonfilamentous matrix proteins. The hard α-keratins are further divided into 2 families, consisting of type I acidic (40-50 kd) and type II neutral/basic (55-65 kd) members. Although epithelial cytokeratins or soft α-keratins have been well studied, little is known about the hard α-keratins and their associated proteins.

"Image 4" Immunoreactivity of monoclonal antibody against hair protein 1 in adamantinomatous craniopharyngioma. The reaction was positive only in the cytoplasm of shadow cells (original magnification ×50).

"Image 5" Staining pattern of polyclonal antibody against hair protein 1 in calcifying odontogenic cyst showing immunoreactivity only with ghost cells (original magnification ×50).

"Image 6" Immunoreactivity for phosphothreonine in calcifying odontogenic cyst with positive reaction products in the cytoplasm of ghost cells (original magnification ×50).

"Image 7" Immunoreactivity for phosphothreonine in adamantinomatous craniopharyngioma with positive reaction products in the cytoplasm of shadow cells (original magnification ×50).
One reason might be that no antibodies against human hair proteins are available commercially.

In the present study, 2 rabbit polyclonal antibodies (PA-HP1 and PA-HP2) and 1 murine monoclonal antibody (MA-HP1) were raised against human hair proteins. SDS-PAGE and Western blot analyses revealed that PA-HP1 reacted strongly with type I acidic and PA-HP2 with type I acidic and type II neutral/basic hard α-keratins. MA-HP1 reacted with type II neutral/basic hard α-keratins. Indeed, all 3 antibodies reacted immunohistochemically with hair shafts but not with any other epithelial components in the sections of normal skin and dermoid cyst.

Pilomatrixoma, a benign tumor of the skin, shows differentiation into hair. The tumor nests are composed of basophilic, transitional, and shadow cells, corresponding to the matrix and cortex cells of mature hair follicles. In the present study, all 3 antibodies reacted with the cytoplasm of transitional and shadow cells. These findings seem to correspond to those of Cribier et al., who demonstrated the expression of messenger RNA for human hair keratin in transitional cells by in situ hybridization.

Craniopharyngioma is a benign epithelial tumor of the sellar region, presumably derived from the Rathke pouch. Two entities, adamantinomatous and papillary craniopharyngiomas, can be distinguished. Tateyama et al. demonstrated the expression of human hard α-keratins immunohistochemically in shadow cells of adamantinomatous craniopharyngioma and proposed that the tumor showed differentiation into hair follicles. In the present study, 13 adamantinomatous and 4 papillary craniopharyngiomas were studied. In all 13 adamantinomatous craniopharyngiomas, all 3 antibodies reacted only with the cytoplasm of shadow cells, not with that of any other epithelial components.

COC is an uncommon odontogenic lesion. Because the lesion originally was recognized as a specific odontogenic lesion by Gorlin et al. in 1962, there has been confusion and disagreement about the terminology and classification because of its diversity. The classifications of COC, proposed by Praetorius et al., Buchner, Hong et al., Toida, and Li and Yu categorized the lesion variously as a cyst, a neoplasm, or a combined lesion. The essential feature of COC is the appearance of ghost cells, which are analogues of shadow cells. Furthermore, melanin pigmentation as seen in the cortex cells of normal hair shafts occasionally is observed in some COC cases. These findings suggest that differentiation into hair occurs in the lesion. In the present study, all 3 antibodies reacted only with the cytoplasm of ghost cells, not with that of any other epithelial components in all 14 COCs.

Widely different tumors of the skin, pituitary gland, and jaw showing a common histopathologic characteristic—the appearance of shadow or ghost cells—were examined in the present study. A significant similarity in the immunohistochemical staining for hard α-keratins was found except for a positive reaction in transitional cells in pilomatrixomas. Remarkable similarities in the accumulation and alteration of β-catenin in the pilomatrixoma, adamantinomatous craniopharyngioma, and COC have been described. The Wnt-β-catenin-TCF-Lef activated pathway has an important role in terminal hair shaft differentiation. On the other hand, once the cell has undergone complete keratinization to produce anucleated shadow cells, there is no β-catenin expression.

Hair develops as an invagination from the surface epithelium of the skin. The anterior portion of the pituitary gland, the adenohypophysis, develops as an invagination from the primitive oral epithelium, the Rathke pouch. The enamel organ develops from an epithelial dental lamina derived from the epithelium of the primitive oral cavity. In each case, there is an interaction between these developing epithelial components and adjacent neuroectodermal mesenchymes (ie, the dental papilla, the infundibulum of the floor of the third ventricle, and the dental papilla). Thus, hair formation, formation of the adenohypophysis, and tooth formation show significantly similar embryologic features, and it is not surprising that some tumors derived from these structures might show similar histopathologic findings, namely the appearance of shadow cells or their analogue, ghost cells. It has been suggested that these 3 tumors share the same pathogenetic mechanism of tumorigenesis, which probably is related to the unique pattern of keratinization and shadow cell formation. In addition to the reactivity of these 3 newly raised...
antibodies, the positive reaction for phosphothreonine, detected in hard $\alpha$-keratins, also was found in transitional, shadow, and ghost cells in the tumors. These findings suggest that the appearance of shadow or ghost cells might represent differentiation into hair in the 3 types of tumors.

References