Acta Medica et Biologica
Vol. 39, No. 2, 91-99, 1991

Aberrant Keratinization in Skin Neoplasms, with Production of Anti-keratin Monoclonal Antibody (Mo-1)

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Received March 14, 1991

Summary. **Eighteen cases of squamous cell carcinoma (SCC) were examined to reveal its characteristics of differentiation, especially keratinization. Immunohistological procedures were performed with anti-keratin antibodies (Mo-1, K-92, and HKN-5), anti-involucrin, anti-CEA, and anti-epidermal growth factor receptor antibodies. Mo-1 is a monoclonal antibody, produced in the present study, and found to recognize the 58 kd keratin filament of the basal layer of the skin. The results were compared with those of basal cell epithelioma (BCE), Bowen's disease, and seborrheic keratosis.**

In keratinized foci of SCC, Bowen's disease, and seborrheic keratosis, a positive stain of differentiation-associated keratin and involucrin, a negative sign of Mo-1, and a gradually diminished sign of epidermal growth factor receptor were obtained. A membranous stain of CEA and an irregular, "mosaic" pattern of HKN-5 were observed in most of the SCC and BCE, occasionally in Bowen's disease, and rarely in seborrheic keratosis.

Though their keratinized regions were morphologically similar, the expression of intra- and extra-cellular materials of the tumor differed among each other.

INTRODUCTION

Squamous cell carcinoma (SCC) of the skin is a common malignancy arising from keratinocyte. It is characterized by its tendency for keratinization. Histologically, so-called cancer pearls are often observed especially in highly differentiated SCC, revealing the specific nature of this lesion.[1] The number of cancer pearls varies among cases. In poorly differentiated SCC, tiny keratinizing foci can be seen. Seborrheic keratosis, a quite common, benign skin tumor, is characterized by a proliferation of basaloid cells and keratinized cells, and a hyperkeratotic, horny structure. In portions rich in inflammatory cells, horny whorls of various sizes, so-called pseudohorn cysts, are often formed, and the lesion sometimes resembles a malignancy. Bowen's disease is a common type of squamous cell carcinoma *in situ*, which is characterized by the presence of dyskeratotic cells. Basal cell epithelioma (BCE), an intermediate malignancy of the skin, undergoes several forms of differentiation, e.g., sebaceous, cystic or keratotic differentiation.

In this study, the author performed an immunohistological procedure with several antibodies — one of which was newly produced and characterized — to investigate the characteristics of the keratinization in squamous cell carcinoma, and to compare them with those of Bowen's disease, BCE and seborrheic keratosis.

MATERIALS AND METHODS

Specimens

Eighteen cases of squamous cell carcinoma were surgically extirpated, routinely fixed, paraffin-embedded, and stained with hematoxylin-eosin. The degree of keratinization was evaluated by Broders' grading.[1] Ten of them were highly differentiated (grade-1 or 2) carcinomata (seven males and three females, aged between 57 and 87, mean 68.3 years old), while eight of them were poorly differentiated (grade-3 or 4) carcinomata (three males and five females, aged 73-88, mean 80.5 years old). A part of each specimen was snap-frozen in liquid nitrogen for immunohistological procedure. Fifteen cases of seborrheic keratosis (aged 28-80, mean 63.3 years old), as a benign counter-

part, 15 cases of Bowen's disease (aged 43-79, mean 63.3), as a precancerous lesion, and 15 cases of BCE (aged 39-84, mean 64.0), as an intermediate malignancy, were also obtained for comparative studies.

Immunohistological procedures

The frozen materials were cut into 5 μm sections, fixed in aceton, and incubated with the following antibodies for 30 min. HKN-5, an anti-keratin mouse monoclonal antibody, was raised in our laboratory; this antibody has been known to react with the keratin of hair follicles and that of the keratinized portion of several carcinomas.[2,3] Mo-1 is a monoclonal antibody which recognizes the keratin of the basal layer of epidermis. The production and the characterization of this antibody are given below. K-92, another anti-keratin monoclonal antibody, which recognizes differentiation-associated keratin, and anti-carcinoembryonic antigen (CEA) polyclonal antibody were purchased from DAKO Corp. (Santa Barbara, CA). Anti-involucrin from Biomedical Technologies Inc. (Cambridge, MA), and anti-epidermal growth factor receptor (EGFR), from Oncogene Science Inc. (Manhasset, NY) were also obtained. The specificity of each antibody is summarized in Table 1.

Fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG antibody (Cappel Lab., West Chester, PA) was used as the second antibody for HKN-5 and k-92. FITC-labeled anti-rat IgG antibody (Cappel) was used for Mo-1, nand FITC-labeled anti-rabbit IgG antibody (Cappel) for anti-CEA and anti-involucrin antibodies. An alkaline phosphatase anti-alkaline phosphatase (APAAP) method was performed for anti-EGFR.[4] In short, the sections were incubated in anti-mouse immunoglobulins (Cappel) for the second antibody and APAAP complex (Zymed Lab. Inc., San Francisco, CA) for the third reagent. The alkaline phosphatase reaction was obtained in 0.1 M Tris-buffer, pH 8.2, naphthol AS-BI (Sigma Chemical Co., St. Louis, MO), dimethylformamide (Sigma), Fast Blue RR Salt (Sigma), and levamisole (Sigma).

Production of Mo-1, anti-keratin monoclonal antibody

The anti-keratin monoclonal antibody (Mo-1) was produced according to methods by Kohler and Milstein.[5] Three Wistar rats were immunized with EHS sarcoma extract and Freund's complete adjuvant. The extraction buffer was composed of 0.5 M NaCl and 50 mM Tris-acetate buffer, pH 6.0. On Day 14 and 21, a mixture of the extract and Freund's incomplete adjuvant was injected. On Day 24, the spleens were obtained, and suspended in RPMI 1640 medium (Bibco Lab., Grand Island, NY). A mixture of 10^8 cells of the spleen and 10^7 cells of rat myeloma cells (Y3) was made and then 0.005% dimethyl sulfoxide (Sigma) and 50% polyethylene glycol 4000 (Sigma) were added. Washed with the medium, the fused cells were centrifuged, collected, and seeded in three 96-well tissue culture plates. The culture medium contained 10% fetal bovine serum (Gibco), 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterine, 1.6×10^{-5} M thymidine with RPMI 1640 medium. The culture plates were incubated in 5% sodium bicarbonate in the air at 37℃.

Screening of hybridomas was performed on frozen sections with the indirect immunofluorescein technique, as described above. Though the supernatant of a well did not react with EHS sarcoma, it was found to react with the basal cell layer of normal human skin. From its cytoplasmic stain, the hybridoma seemed to produce an auto-antibody against keratin filament.[6] The hybridoma was cloned twice by limiting dilution. The supernatant of the cloned hybridoma

Table 1. Specificity of antibodies

Antibody	Antigen	Tissue specicifity in normal skin
Mo-1	Ck 5	Basal layer
K-92	Ck 10 etc.	Suprabasal layer
HKN-5	Keratins of rapidly keratinizing cells	Negative
Anti-involucrin	Involucrin	Upper spinous layer
Anti-CEA	CEA	Eccrine apparatus
Anti-EGFR	EGFR	Basal layer

Ck: cytokeratin, numbered according to Moll's catalog,[12] CEA: carcinoembryonic antigen, EGFR: epidermal growth factor receptor

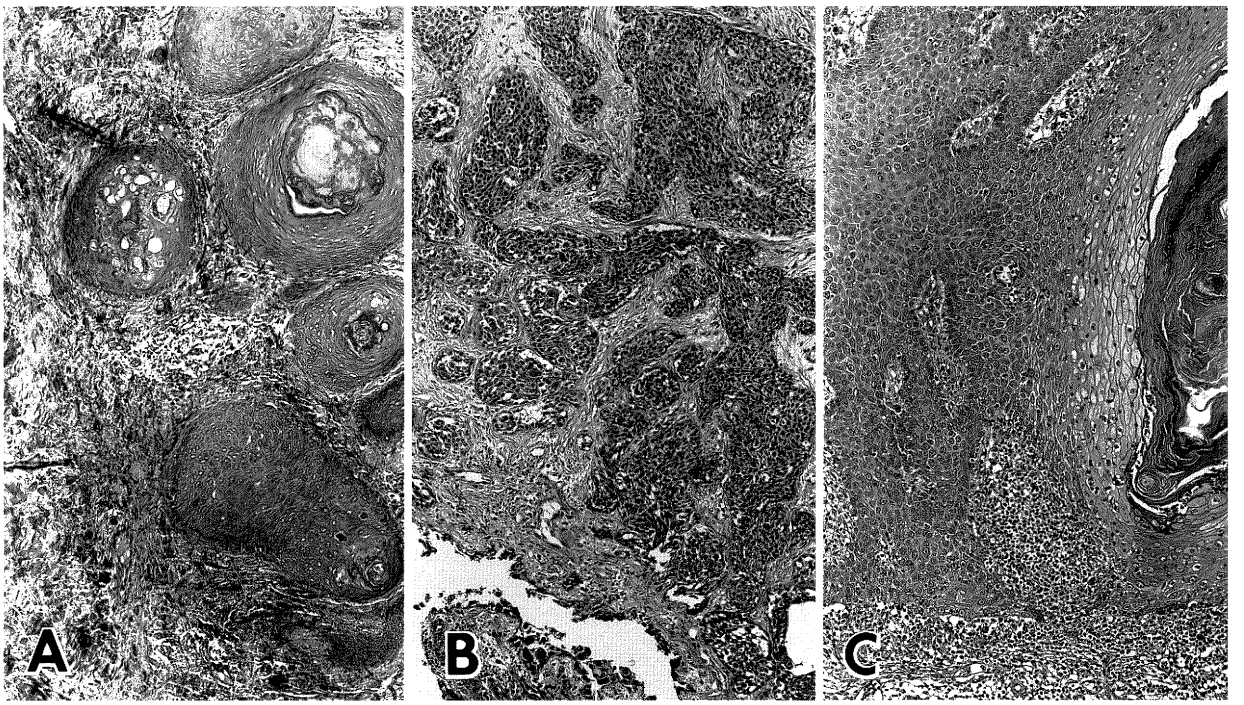

Fig. 1. Light micrographs of different grades of squamous cell carcinomas (SCCs) and seborrheic keratosis. In grade-1 SCC (A), a number of keratinized whorls are formed. The keratinization is less prominent in grade-3 SCC (B). In both cases, numerous atypical cells can be found with mitotic figures. Some cells of the seborrheic keratosis (C) show cellular atypism in the focus of inflammatory cell infiltration. Hematoxylin-eosin stain. ×90

was collected, and preserved in −70℃ for characterization.

Characterization of Mo-1

To determine the specificity of antibody (Mo-1), an immunoblot analysis, according to the methods by Laemmli[7] was performed. Keratin filament was extracted from scalp epidermis and hair follicles by the method of Franke et al.[8] In short, the sample was extracted with several steps of EDTA, Triton X-100, and Tris-HCl buffers. The final residue was dissolved in Tris-HCl buffer containing 5% 2-mercaptoethanol, 0.5% glycerine, 4 M urea, and 1% sodium dodecyl sulfate (SDS, Bio-Rad Lab., Richmond, CA), and then separated in 10% polyacrylamide (Bio-Rad Lab.) gel with 0.1% SDS at pH 8.3. Standard molecular weight markers (Biochemical Products for Life Science, Tokyo) were simultaneously separated. A part of the gel was cut, fixed and stained in Coomassie Blue (Bio-Rad Lab.); the rest of the gel was processed with electrophoretical transfer onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk (Difco Lab., Detroit, MI) in phosphate-buffered saline (PBS), and then incubated overnight with Mo-1. After washing with 0.05% Tween 20-PBS, biotin-cojugated anti-rat IgG antibody (Cappel) for the second antibody, and peroxidase-conjugated Streptavidin (Seikagaku Kogyo Co., Tokyo) for the third reagent were used. The peroxidase reaction was obtained with 3,3-diaminobenzidine (Sigma).

RESULTS

Typical microscopic pictures of SCC and seborrheic keratosis are shown in Fig. 1. Grade-1 SCC (Fig. 1A) formed numerous whorls of keratinized cells, or so-called cancer pearls. In grade-3 SCC (Fig. 1B), some keratinizing foci could be observed, but they were less prominent. Both of them showed marked cellular atypism. Seborrheic keratosis with inflammatory cell infiltration (Fig. 1C) also possessed keratinizing portions of a pseudo-horn cyst.

Immunohistologically, basal-layer keratin, recognized by Mo-1, was rarely stained either in SCC (Fig. 2A), Bowen's disease, or seborrheic keratosis. Most of the cells of BCE were positive (Fig. 2B). K-92 positive cells were scattered among highly and poorly differentiated SCCs (Fig. 2C), Bowen's disease (Fig.

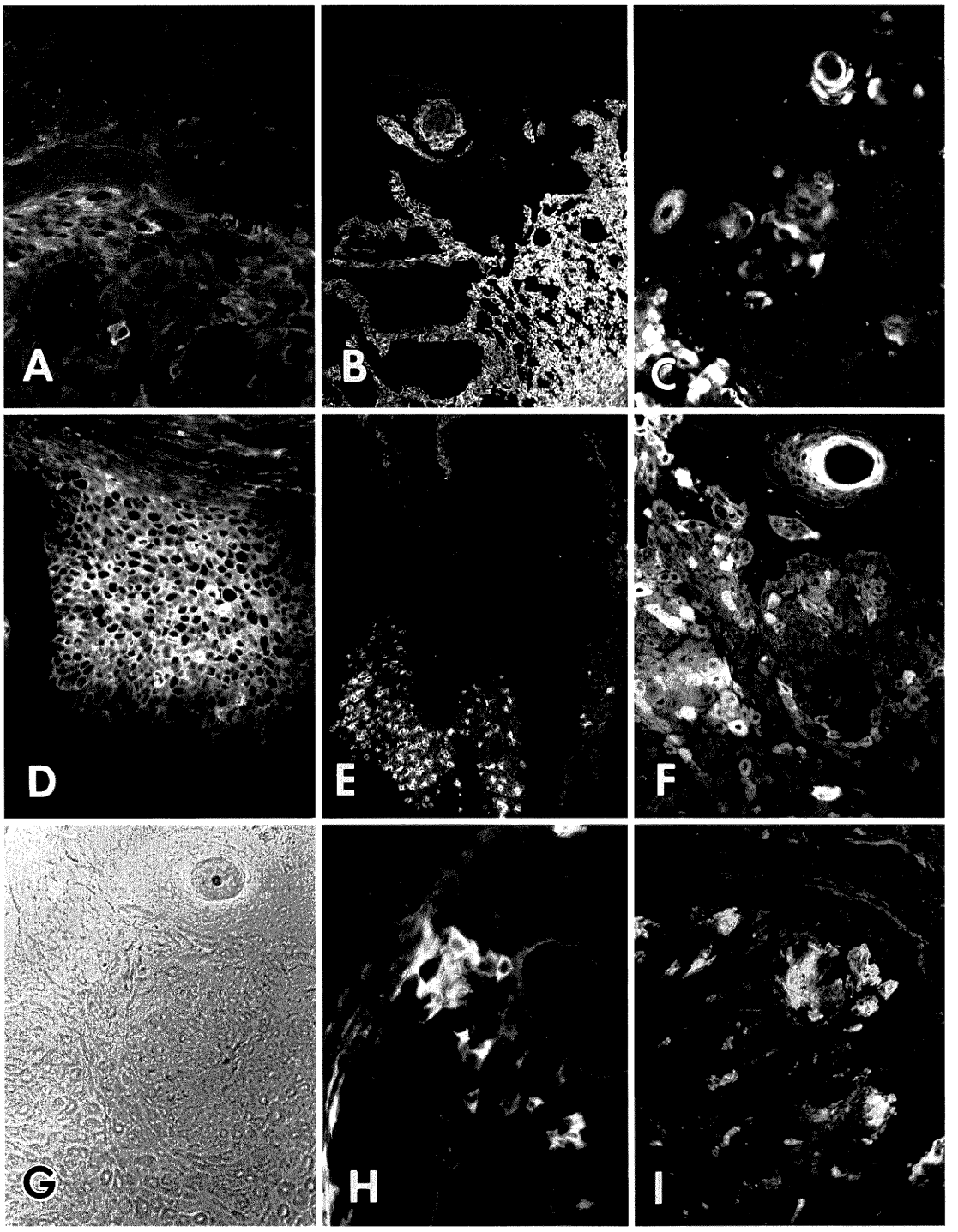

Fig. 2. Immunohistological findings with anti-keratin antibodies. A few Mo-1 positive cells can be found in SCC (A). Most of the cells are positive in BCE (B). K-92 positive, differentiated cells are scattered in SCC (C), in Bowen's disease (D), and in seborrheic keratosis (E). Large foci of HKN-5 positive cells can be seen in grade-1 SCC (F). In grade-3 SCC (H), positive foci are small in number and in size. In both SCCs, the positive cells form mosaic patterns. Some of the positive cells are located in unkeratinized foci. G, phase contrast micrograph of the region identical with F. Some of the BCE cells are positive in keratinized foci (I). FITC stain. A, C, D, F, G, H, I, ×230. B, E, ×150

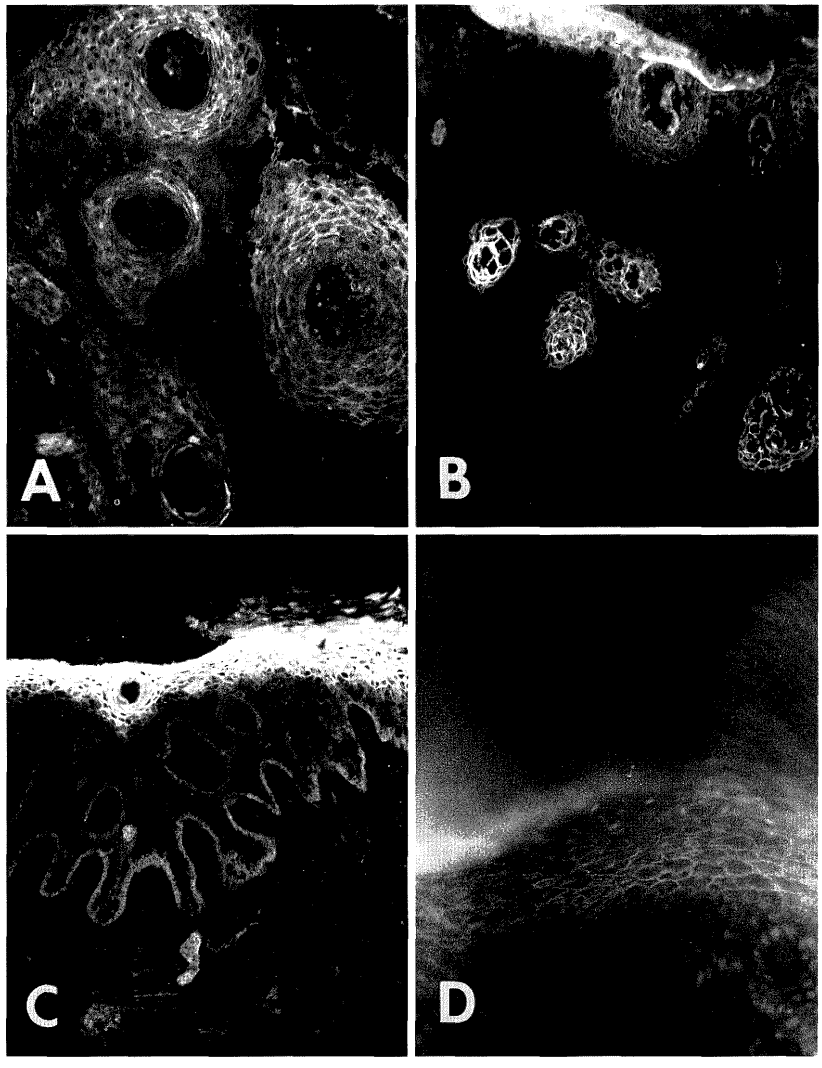

Fig. 3. Immunohistological findings with the anti-involucrin antibody. Membranous staining is seen around the keratinized foci in SCC (A), BCE (B), Bowen's disease (C), and seborrheic keratosis (D). FITC stain. A, D, ×250. B, C, ×160

2D), and seborrheic keratosis (Fig. 2E). The numbers of the positive cells did not vary among the tumors. Either case of BCE was negative.

The keratinized cells of SCCs were stained with HKN-5 in a mosaic pattern. The whorls of positive cells were large and numerous in highly differentiated SCC (Fig. 2F, G), and small in size and number in poorly differentiated SCC (Fig. 2H). Moreover, some unkeratinized foci were stained in both groups of SCCs. Keratinized foci of BCE were stained in the same pattern as SCC (Fig. 2I). Only one case of seborrheic keratosis disclosed a positive reaction in a keratinized portion. Two cases of Bowen's disease revealed the mosaic stain in the upper layers of the tumors.

Involucrin, a marginal band protein of keratinocytes,[10] was exhibited at the peripheries of tumor cells in the keratinized regions of SCC (Fig. 3A), BCE (Fig. 3B). In Bowen's disease (Fig. 3C) and seborrheic keratosis (Fig. 3D), the upper layers were positive as seen in normal skin, but the numbers of positive layers were increased.

A positive reaction with anti-CEA was obtained around the cancer pearls of SCCs in a membranous pattern (Fig. 4A). Keratinized foci of BCE and the upper layers of Bowen's disease were occasionally

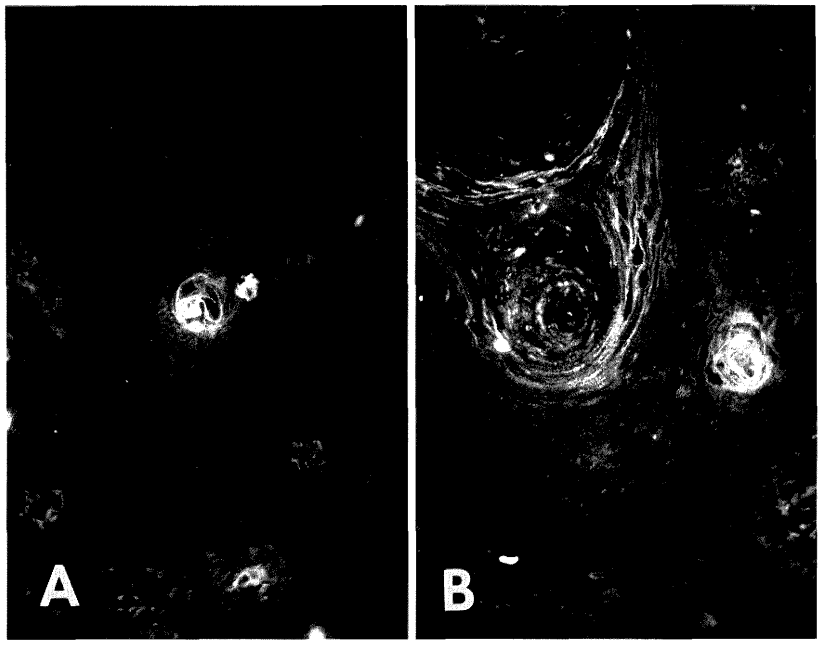

Fig. 4. Immunohistological findings with the anti-CEA antibody. Keratinized foci of SCC (A) and seborrheic keratosis (B) are membranously stained. FITC stain. ×250

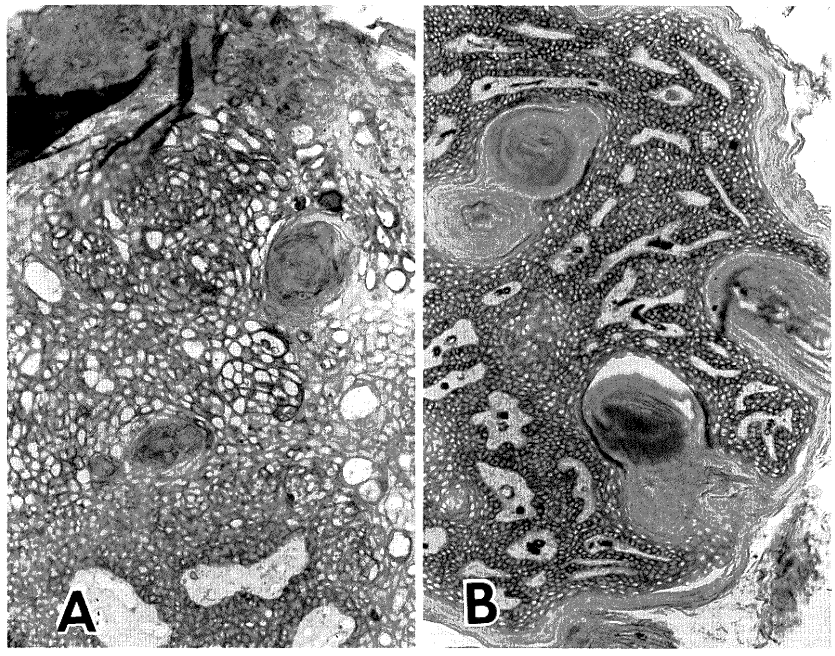

Fig. 5. Immunohistological findings with the anti-EGFR antibody. Cell membranes of all the tumor cells are stained positively in SCC (A), and seborrheic keratosis (B). The staining is less intense at the center of the keratinized portion. APAAP stain. A, ×250. B, ×100

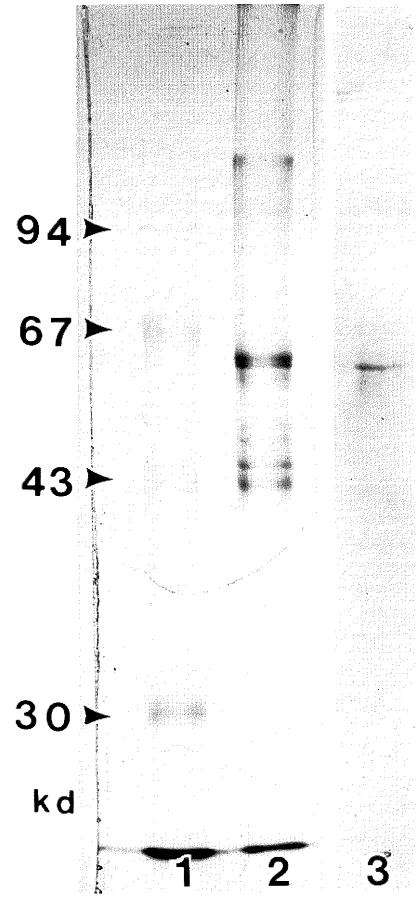

Fig. 6. SDS-polyacrylamide gel electrophoresis of keratins and immunoblot analysis of Mo-1. Sample keratins, extracted from scalp epidermis and hair follicles, are separated in several bands with 10% polyacrylamide gel, and are stained with Coomasssie Blue (Lane 2). Rat monoclonal antibody Mo-1 is blotted on the transfer membrane; 58 kd band is obtained in peroxidase reaction with diaminobenzidine (Lane 3). Lane 1, molecular weight markers.

Table 2. Immunoreactivities of the tumors examined

Antibody	SCC	BCE	Bowen's disease	Seborrheic keratosis
Mo-1	2/18, S	14/15, M	2/15, S	3/15, S
k-92	7/18, S	0/15, —	13/15, S	9/15, S
HKN-5	18/18, K*	13/15, K	2/15, U	1/15, K
Anti-involucrin	18/18, K	13/15, K	15/15, U	15/15, K
Anti-CEA	18/18, K	10/15, K	4/15, KU	1/15, K
Anti-EGFR	18/18, A	15/15, A	15/15, A	15/15, A

The figures indicate: the number of tumors with positive cells / the number of tumors investigated
A All the tumor cells were immunostained.
K Cells in keratinized foci were immunostained.
M Most of the tumor cells were immunostained.
S Positive cells were scattered in tumor nests.
U Cells in upper spinous layers were stained.
* Cells out of keratinized foci were occasionally immunostained.
— Positive cells could not be recognized.

stained. Only one case of seborrheic keratosis presented the same membranous pattern (Fig. 4B).

EGFR existed in the cell membranes of all the tumor cells in every tumor (Fig. 5). In the keratinized foci, the staining lost its intensity toward the center of the whorl. The reactivity of tumors is summarized in Table 2.

Immunoblot analysis of Mo-1 revealed the monospecific reactivity with 58kD keratin of basal layer (Ck5, according to Moll's catalog,[12] Fig. 6).

DISCUSSION

To investigate the differentiation of skin tumors, several markers, e.g., keratins and involucrin[3,9-11] have often been used. Keratin is a type of intermediate filament of 10 nm in diameter, and is divided into 19 types according to its molecular weight and isoelectric point. The keratin expression is defined by histological findings.[12-14] The antibodies K-92 and Mo-1 recognize a differentiation-associated keratin and a basal-layer keratin of Ck5. The antibody monospecific to Ck5 has rarely been reported. With these antibodies, positive cells were demonstrated to be scattered in tumor lesions of SCC, Bowen's disease and seborrheic keratosis. The keratinization in these pathological states is suspected to show altered keratin expressions. In BCE, most of the cells were Mo-1(+), K-92(−). This reaction looks proper when one recalls the morphological resemblance to basal layer cells of the epidermis.

HKN-5 reacts with a basic 59kD keratin peptide (Ck4) and an acidic 59Kd peptide.[15] This acidic antigen is thought to be a hard keratin peptide. Most of the keratinizing whorls and less keratinized foci of SCC reacted to this antibody, as did the keratinized foci of BCE. Only rarely did seborrheic keratosis and Bowen's disease stain positively. Keratinized foci of an epidermoid carcinoma cell line A-431 were also stained with HKN-5 (unpublished data). HKN-5 seems to have widely recognized the keratinizing cells in SCC. Because the A-431 cell line did not express Ck4,[12] HKN-5 seemed to react with the hard keratin specifically expressed in SCC. Despite the anti-hard keratin antibodies reported recently, this characteristic reactivity has not been reported so far. Therefore, HKN-5 could be used as a sensitive marker of the keratinization of SCC.

Involucrin is a precursor of envelope proteins in cornified cells of the epidermis.[10] In all the tumors, a positive sign was obtained in the membranous pattern. As could be predicted from the morphology, the squamoid foci of SCC and BCE, and the upper layers of Bowen's disease and seborrheic keratosis revealed positive reactions. The numbers of positive layers were larger in the latter two lesions than in normal skin. Involucrin could be immunostained in a membranous pattern with unfixed specimens, and in a cytoplasmic pattern with formalin-fixed specimens, as several papers have reported.[10,16] Because of its solubility, involucrin might be washed out from the cytoplasm in the course of PBS-washing in an unfixed state. An alteration in the cytoplasmic stain in tumors and a change in intensity of the stain from cell to cell were reported by Murphy et al. under fixed

conditions.[17] Moreover, a minor change in antigenicity could occur during the fixation. Therefore, the author took the safer way for the antigen, and the results suggested that the change of expression of involucrin could occur gradually, but not abruptly in each of the tumor cell.

The membranous pattern could be obtained also with anti-CEA polyclonal antibody in SCC, Bowen's disease and BCE, and rarely in seborrheic keratosis. The function of this substance is obscure. In 1989, Benchimol et al.[18] reported that anti-CEA polyclonal antibody, against a purified CEA from human colonic carcinoma, impaired cell-to-cell adhesion of a colon carcinoma cell line *in vitro*. Therefore, CEA has been presumed to serve as an intercellular adhesion molecule. The author recently made an adhesion assay on a SCC cell line and observed that anti-CEA polyclonal antibody, which was used in the present study, could also dissociate the SCC cells *in vitro* (unpublished data). CEA and closely related family members,[18] which react with the antibody, may form another group of intercellular adhesion molecules existing in the keratinizing cell membrane of SCC.

An early observation reported that EGFR expression in seborrheic keratosis was restricted only in a special subject, such as in a patient with neoplastic lesions.[19] Recently, Nazmi et al.[20] reported that EGFR was expressed in all the specimens of both seborrheic keratosis and SCC. The present observation also indicates that every tumor cell of arsenical keratosis (submitted for publification), SCC, BCE, Bowen's disease and seborrheic keratosis (the present study), is positively stained with the anti-EGFR antibody. This discrepancy could be caused by the differences in sensitivity among investigating methods. Therefore, the highly sensitive APAAP technique was chosen for the present study. The intensity of the stain was the same among the tumors, and was decreased in the keratinized foci of each lesion. EGFR owns the tyrosine kinase activity within its intracellular domain, and is related to the proliferation of tumor cells.[21-23] In keratinized foci, the tumor cells are thought to be differentiated. Thus, they are thought to have lost the ability to proliferate. The decreased EGFR stainability in the keratinized foci might be understandable in this context.

Through the present study, the efficacy of the antibodies against keratins, involucrin, CEA and EGFR was discussed, for research of differentiation, especially keratinization in the neoplasms of the skin. In comparison with seborrheic keratosis, the particular differences of staining were obtained with the HKN-5 and anti-CEA antibodies. It is suggested that the immunoreaction for HKN-5 may reveal intracellular alterations, and that this reaction may be useful in detecting early signs of abnormal keratinization in malignant tumors. The expression of CEA also seems to indicate altered intercellular conditions. Further investigations should be made to clarify the significance and regulation of its expression.

Acknowledgements. The author would like to express his sincere gratitude to the following individuals: Prof. Y. Sato, Associate Prof. M. Ito, Drs. T. Kazama, T. Tazawa, K. Ito, and K. Katsuumi for their support, and Mr. T. Imai for his technical assistance.

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