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Confirmation and an unusual quality of the differentiated keratin peptide (K1) in cultured human squamous cell carcinomas

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Recently K1 keratin peptide (K1, 68 kDa) was found to be present in two kinds of cultured human squamous cell carcinomas (HSCs) using a low-salt aqueous solution, rather than the high-salt solution containing Triton X-100 employed by many researchers up until now. To confirm whether this phenomenon is universal in cultured HSCs we analyzed K1 peptide in four other kinds of HSCs using the same procedures. Moreover, the K1 peptide detected was a little unusual with respect to solubility versus urea concentration. Epidermal K1 peptide is usually solubilized by 6–8 M urea and reductant; however, the K1 peptide in cultured HSCs was about 80–90% extracted by 1–2 M urea in a stepwise extraction procedure. This finding may have important implications regarding evaluation of keratin extracted from normal epidermal and cultured keratinocytes.

K1 keratin peptide; Cultured squamous cell carcinoma

1. INTRODUCTION

Keratins, epithelial intermediate filament proteins, are especially abundant in stratified squamous epithelial tissues, where they comprise the major differentiation-specific products of these cells. In epithelial tissues, keratins are differentially expressed as specific pairs of type I and type II proteins, both of which are essential for filament formation [1–3]. Thus, for example, K5 (type II) and K14 (type I) are co-expressed in basal cells. As basal cells differentiate and move into suprabasal layers, the above two keratin peptides are down-regulated, and K1 (type II) and K10 (type I) peptides are expressed more in the suprabasal cells of the epidermis. Each keratin is encoded by its own gene and is expressed in keratin pairs containing one acidic (type I) and one basic (type II) protein according to specific rules [4–7]. K1/K10 is a characteristic keratin pair of suprabasal keratinocytes and may be essential in constructing and maintaining structural and functional requirements during terminal differentiation [8].

Hereditary epidermolysis bullosa simplex (EBS), a bullous skin disease, is reportedly based on an abnormal and distinctive organization of cytokeratins [9]. The N- and C-terminal rod domains of epidermal keratin have also been shown to be essential to normal keratin filament formation [10,11]. When the C-terminal deletion mutant K14 keratin gene was used in transient transfection in vitro and transgenic mice in vivo, keratin filament formation was markedly altered, with keratin aggregates in basal cells [12], and the mice exhibited abnormalities in their epidermal architecture [13] resembling a group of genetic disorders known as EBS, i.e. all Dowling-Meara EBS point mutations indicated were in the rod domain of either the K5 [14] or K14 [15] protein. In addition, a similar point mutation was recognized in the central portion of K14 in Koebner EBS [16]. They were actually found to be the genetic cause of the disease [15]. Obviously normal keratin peptide is required to form the keratin filament in the basal cells of the epidermis, based on the many recent findings about EBS above.

Although K5, K6, K14, K16, and K17 keratins have been found in cultured human squamous cell carcinomas (SCC) [17], K1 [18] had never been detected [17,19–21]. Thus, it was previously believed that cultured SCC cells characteristically had no keratins larger than 60 kDa. Quite recently, this K1 peptide, though not abundant, was determined to be present in two cultured HSCs when a different buffer was employed in preparing the cells [22].

In this paper, we report the widespread presence of K1 peptide in cultured HSCs.

2. MATERIALS AND METHODS

Dulbecco's medium (DMEM) and fetal calf serum (FCS) were purchased from Dainihon Co., Osaka, Japan. [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Amersham Co. and used for labeling the cDNA probes.

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Abbreviations: 2D-PAGE, two dimensional-polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol; kDa, kilodalton.

2.1. *Cell cultures*

The established cell lines HSCs-2, -4 and A-431 (Cell-No. JCRB 0622, 0624 and 9009, respectively; The Japanese Cancer Research Resources Bank, Tokyo, Japan) and HSC-1 [23] were derived from well-differentiated SCCs of the mouth, tongue, vulva and epidermis, respectively. These cells were serially cultured at plating densities of about 1.5×10^3 cells/cm^2 in DMEM supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (50 μg/ml), kanamycin (50 μg/ml) and hydrocortisone (0.4 μg/ml) at 37°C in a gas phase which was 95% air and 5% CO_2.

2.2. *Extraction of keratin polypeptides*

After four days, 4 plates of cultured cells were quickly rinsed twice with PBS and scraped with a rubber policeman using 1 ml of 10 mM Tris-HCl (pH 7.4)/10 mM EDTA/phenylmethylsulfonyl fluoride (PMSF; 0.3 mg/ml) (Buffer A). The cells obtained were divided into two fractions. One fraction was washed, sonicated (15 s × 3 times) and centrifuged (10,000 × *g*, 10 min) with 1 ml of Buffer A. The other fraction was treated in a similar manner with 1.5 M KCl/0.5% Triton X-100/140 mM NaCl/10 mM Tris-HCl (pH 7.4)/1 mM EDTA/PMSF (0.1 mg/ml) (Buffer B). After repeating the above procedure 3 times, both the resulting final residues were treated with 50 μl of 10 M urea/5% 2-ME at 37°C for 6 h [24].

2.3. *One- and two-dimensional polyacrylamide gel electrophoresis (PAGE)*

SDS-PAGE was performed according to the method described by Laemmli [25]. Analysis of keratin peptides by two-dimensional PAGE was performed as described by O'Farrel [26] using pH 3.5–10 ampholines (LKB, Bromma, Sweden).

2.4. *Immunoblot analyses*

After electrophoresis, the unstained gels were transferred electrically to nitrocellulose membranes at 100 mA for 16 h, and the membranes were reacted with keratin monoclonal antibody (34βB4 [27–29]; ENZO Diagnostics, NY, USA, RKSE60 [30,31], SANBIO B.V., The Netherlands, reacting with K1 and K10 peptides, respectively) using an indirect immunologic technique [32].

2.5. *Northern blot analysis*

The K1 probe [21] was prepared at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP. A labeled K10 probe was prepared according to the method described in a random primer preparation kit (Takara Shuzou Co., Osaka, Japan) using [α-^{32}P]dCTP and Klenow fragment. Total RNA was prepared from HSCs-1, -2, -4 and A-431 cells by the guanidine isothiocyanate procedure [33]. Poly(A)$^+$RNA, isolated on an oligo(dT) cellulose column [34], was subjected to electrophoresis on 1% agarose/formaldehyde gel and transferred to a nitrocellulose membrane [35]. The RNA blot was hybridized with the above ^{32}P-labeled probes prepared from the 5′ end by the T4 polynucleotide kinase reaction and random primer preparation method, respectively.

2.6 *Urea stepwise-extraction of keratin peptides*

After washing the residue with Buffer A, the residue obtained was treated with 50 μl of 1 M urea/5% 2-ME for 3 h at 37°C. Then the supernatant was removed by centrifugation at 10,000 × *g*, for 10 min at 4°C. The resulting residue was further treated with 50 μl of 2 M urea/5% 2-ME under the same conditions and subsequently extracted with 50 μl of 6 M urea/5% 2-ME for 3 h at 37°C. Each extract obtained in three steps was treated with 2 × SDS sample solution [25] at 100°C for 3 min and then subjected to SDS-PAGE containing 10% acrylamide. The K1 keratins extracted at the three urea concentrations were estimated by LHB 222, Ultro Scan XL, enhanced laser densitometry (LKB, Bromma, Sweden).

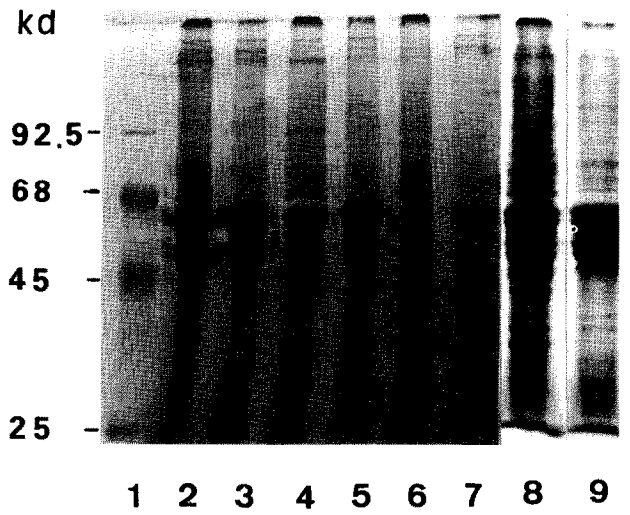

Fig. 1. SDS-PAGE of the keratin peptides extracted from cultured human HSCs-1, -2, -4, and A-431. 0.1% Coomassie brilliant blue (CBB) R-250 staining after electrophoresis of the extracts. (1) Standard proteins, 92.5; phosphorylase A, 68; bovine serum albumin, 45; ovalbumin, 25; chymotrypsinogen A. (2) Extracts of HSC-1 treated with Buffer A. (3) Extracts of HSC-1 treated with Buffer B. (4) Extracts of HSC-2 treated with Buffer A. (5) Extracts of HSC-2 treated with Buffer B. (6) Extracts of HSC-4 treated with Buffer A. (7) Extracts of HSC-4 treated with Buffer B. (8) Extracts of A-431 treated with Buffer A. (9) Extracts of A-431 treated with Buffer B. Numbers on left indicate positions of molecular mass standards (kDa). ➡ = 68 kDa keratin peptide.

3. RESULTS AND DISCUSSION

Up until now many investigators have used high salt/Triton X-100 (Buffer B) as the washing buffer to extract keratin peptides from cultured SCC cells. The above solution was used originally to remove fatty substances and the many hydrophilic substances contained in the experimental materials (tumors or living tissues). Hence, this specific solution can also be used to extract keratin from cultured cells. However, there is generally less extractable material in cultured cells than in living tissue or tumors. Thus, to treat cultured cells with such

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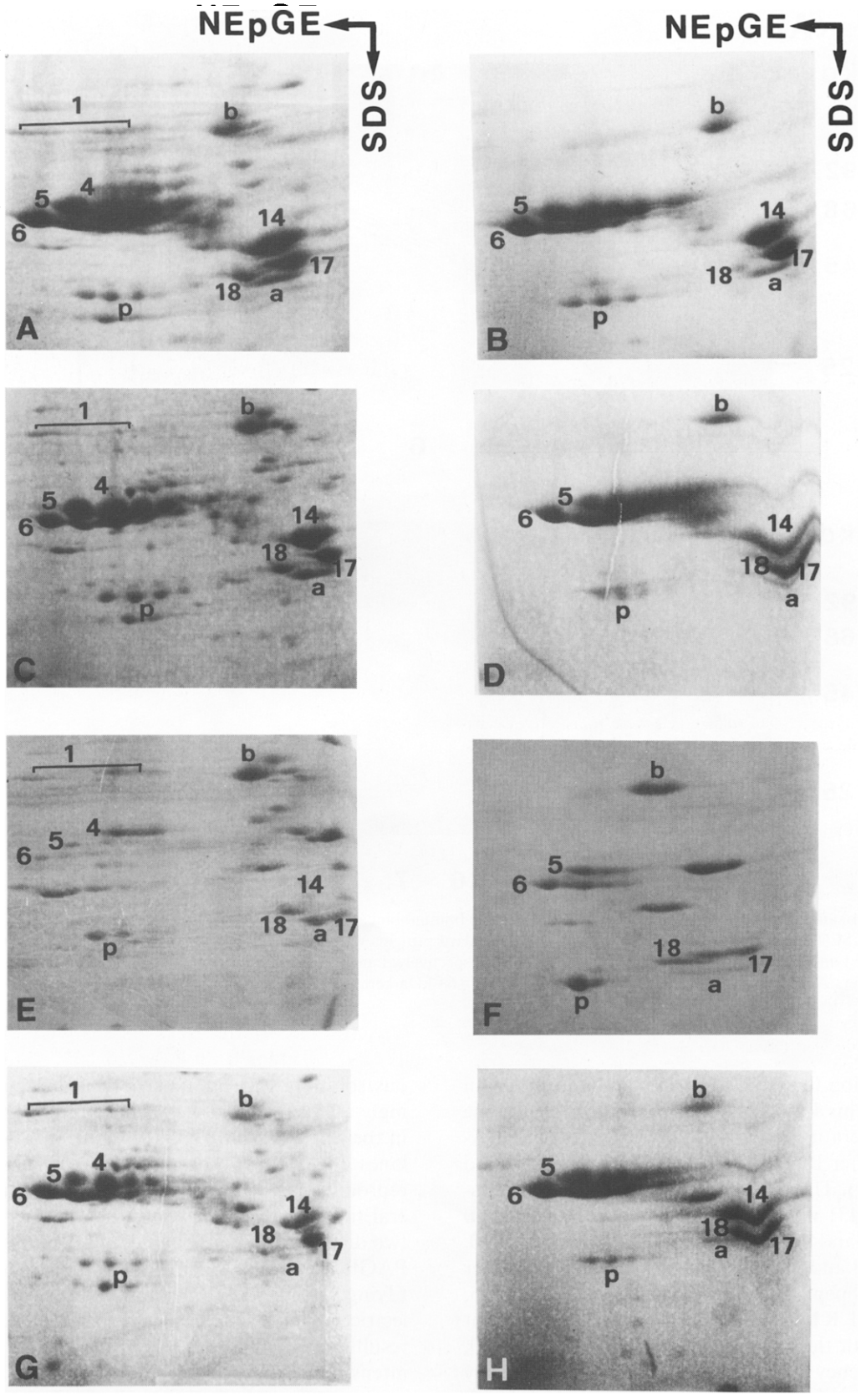
Fig. 2. 2D-PAGE of keratin peptides extracted from cultured human HSCs-1, -2, -4, and A-431 (CBB staining). (A) Extracts of cultured HSC-1 treated with Buffer A. (B) Extracts of cultured HSC-1 treated with Buffer B. (C) Extracts of cultured HSC-2 treated with Buffer A. (D) Extracts of cultured HSC-2 treated with Buffer B. (E) Extracts of cultured HSC-4 treated with Buffer A. (F) Extracts of cultured HSC-4 treated with Buffer B. (G) Extracts of cultured A-431 treated with Buffer A. (H) Extracts of cultured A-431 treated with Buffer B. Standard proteins were used: p = 3′-phosphoglycerokinase (p*I* 7.4, M_r 43 kDa; b = bovine serum albumin (p*I* 6.35, M_r 68 kDa); a = rabbit α-actin (p*I* 5.4, M_r 42 kDa). The numbers denoted keratin nomenclature according to the Moll catalogue [1]. The first-dimensional separation was done by non-equilibrium pH gradient gel electrophoresis (NEpGE), and the second-dimensional separation by SDS-PAGE.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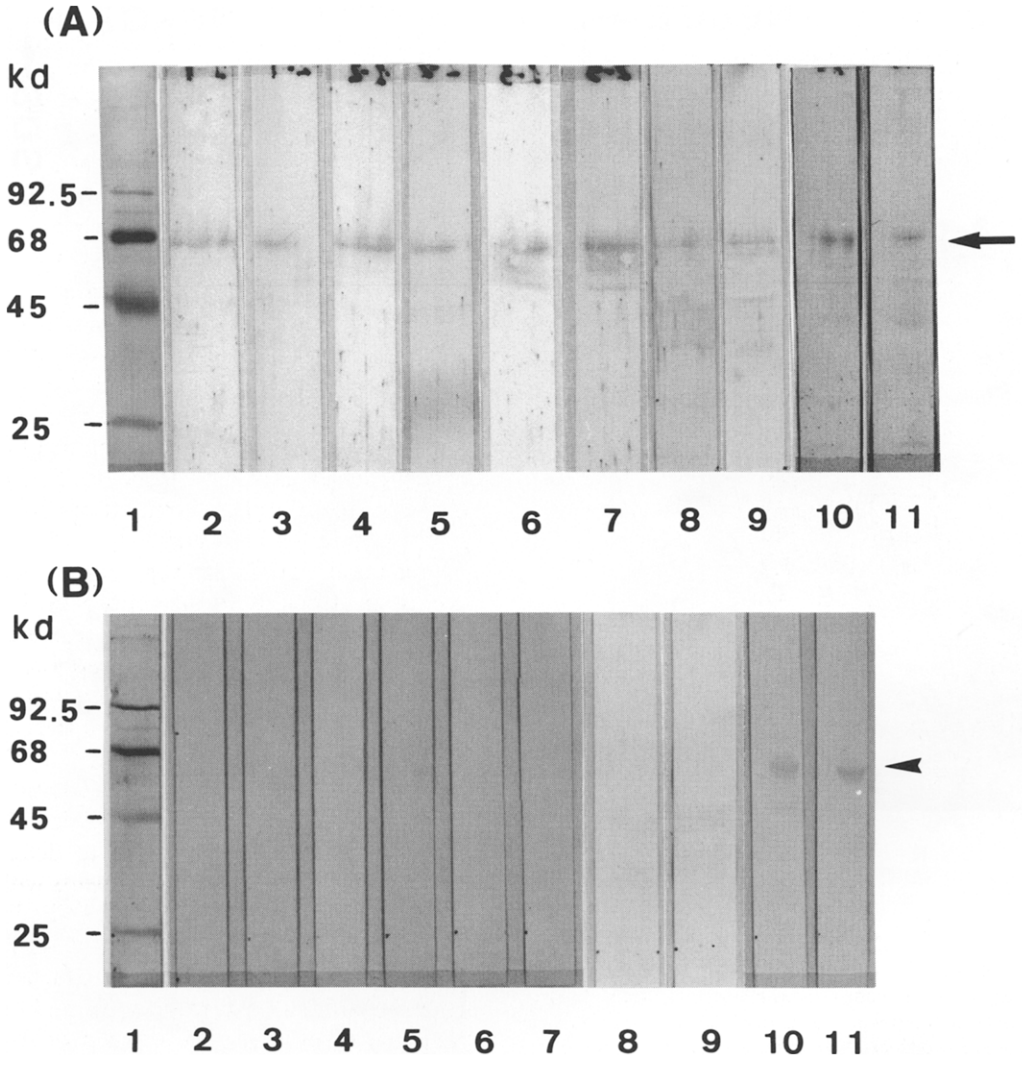

Fig. 3. Immunoblot analyses with keratin monoclonal antibodies. (A) Immunoblot analyses with 34βB4 against K1 keratin peptide. (B) Immunoblot analyses with RKSE60 against K10 keratin peptide. Lane 1 = standard proteins which stained with 0.04% CBB R-250 after transfer. Lanes 2–9 designated in Fig. 1 and each strip transferred from Fig. 1 samples, respectively. Lanes 10 and 11 were transferred from extracts of normal epidermis with Buffers A and B, respectively, as positive controls. ➡ = 68 kDa keratin (K1) peptide; ◀ = 56.5 kDa keratin (K10) peptide.

a strong solution to extract keratin may remove certain unstable keratins along with affinity substances with the buffer. The keratin patterns of HSCs-1, -2, -4, and A-431 using Buffer B are shown (Fig. 1, lanes 3, 5, 7 and 9, respectively). The major bands from cultured HSCs-1, −2, and A-431 were 58 kDa, 56.5 kDa, 52.5 kDa, 50 kDa, 45 kDa and 40 kDa (corresponding to K5, K10, K8, K14, K18 and K19, respectively) [1]. On the other hand, keratin peptides from HSC-4 consisted of K5, K8, K18, and K19. These findings were in excellent agreement with those in many earlier reports [17–20], and this tendency in keratin expression patterns may have two types in human cultured HSCs, combined with results of previous paper [22]. Moreover, no large keratin peptides (60–68 kDa) were present in these cultured cells described thus far. When Buffer A, a low-salt aqueous solution, was used as the washing buffer, surprisingly a 68 kDa band was detected, though not intense, in the extracts from HSCs-1, -2, -4, and A-431 (Fig. 1, lanes 2, 4, 6 en 8). Moreover, these results were highly reproducible when the experiments were repeated several times. To further verify the keratins extracted by two different buffers, we analyzed these extracts by 2D-PAGE, because 2D-PAGE systems are superior in identifying keratin peptides with similar mobilities and isoelectric points. As we expected to some extent from the results in Fig. 1, K1 peptide was present, though not intense, and appropriately detected only when Buffer A was used (Fig. 2A, C, E, and G). On the other hand, this peptide was not present when treated with Buffer B (Fig. 2B, D, F, and H). Though very little K1 peptide

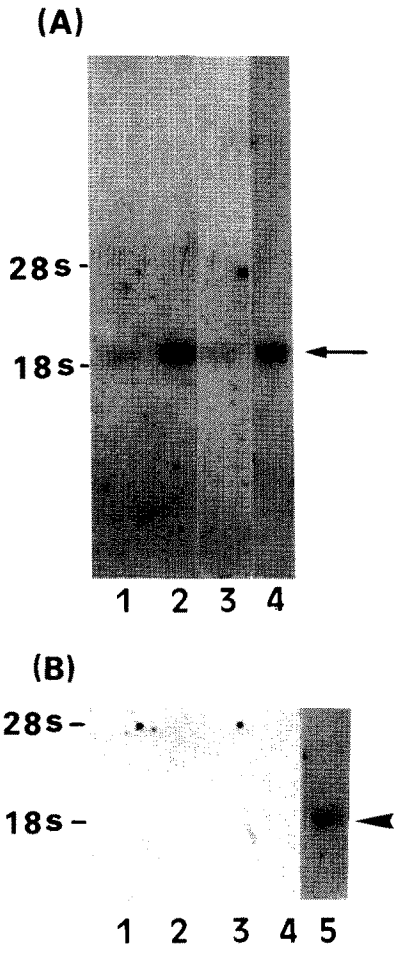

Fig.4. Northern blot hybridization of HSC mRNAs. Poly(A)$^+$ RNAs (2 μg each) prepared from human cultured HSCs-1, -2, -4 and A-431 total RNA were electrophoresed on 1% agarose/0.66 M formaldehyde gel, then blotted onto a nitrocellulose membrane. 1 = HSC-1; 2 = HSC-2; 3 = HSC-4; 4 = A-431; 5 = normal epidermis as a positive control. The membrane was hybridized with a ^{32}P-labeled K1 and K10 probes as described in Materials and Methods. Ribosomal 28 S (4.7 kb) and 18 S (1.9 kb) RNAs were used as size markers. RNA blots (A) and (B) were analyzed with K1 and K10 probes, respectively. ➡ = K1 mRNA (2.5 kb); ◀ = K10 mRNA (2.3 kb).

is involved, these findings suggest its presence in the above HSCs.

To immunologically verify whether the 68 kDa band was K1 peptide [21], we transferred it from the unstained gel to nitrocellulose membranes after electrophoresis and reacted it with the monoclonal antibody 34βB4 [27–29]. The 68 kDa band was detected in extracts and control fractions treated with Buffer A (Fig. 3A, lanes 2, 4, 6, and 8). Moreover, the same band was also observed faintly in extracts treated with Buffer B after treatment with the same antibody (Fig. 3A, lanes 3, 5, 7, and 9). These data suggest that K1 peptide may be removed by Buffer B or remain in only very small amounts, and so many investigators may have overlooked its presence in cultured SCC when staining with CBB alone so far. In addition, K10 peptide, a pair keratin of K1 peptide, also investigated using keratin monoclonal antibody RKSE60. As shown in Fig. 3B, no detection of it was present in these cultured cells, except normal epidermis (Fig. 3B, lanes 10 and 11).

As reported to involve K1 mRNA in HSCs-3 and -5 in our previous paper [22], to investigate whether the mRNA of K1 was present in HSCs-1, -2, -4, and A-431 cells, we analyzed their mRNAs by Northern blots using a radiolabeled probe specific for K1 peptide. The specific K1 probe hybridized to a single RNA band of about 2.5 kilobases (kb), which was consistent with the size of K1 mRNA [21], as shown in Fig. 4A. However, the mRNA content of the various HSC cells was not constant, even though equal amounts of poly(A)$^+$ RNA were employed in the experiments. This may be related to the degree of cell differentiation and K1 peptide expression in individual cells.

In general, K1/K10 forms keratin filaments and are expressed especially in the suprabasal keratinocytes of the epidermis [4–8]; however, K1/K10 expression was less than that of other keratin peptides in HSCs-1, -2, -4 and A-431 (Figs. 1 and 2). These findings may be due to exposure of the cultured cells to the strong buffer, or may be due to the development of epidermal tumors rather than normal epidermis. Though not in large amounts, K1 peptide was present in HSCs-1 to -5 or A-431 and, we also attempted to determine whether K10 peptide formed keratin filaments with K1 in HSCs. Immunological reactions using RKSE60 keratin monoclonal antibody and preparation of K10 mRNAs were performed using the same methods described above. The results are shown in Figs. 3B and 4B and no K10 peptide was found in HSCs-1 to -5 or A-431. Thus, K1 peptide may not form keratin filaments with K10 peptide, or if K1 peptide is formed with other type I keratin peptides, the filaments formed may be unstable and incomplete. As it is said that type II keratin induces type I keratin [36], the K1 peptide itself may be unstable or be a point mutation in the case of epidermolytic hyperkeratosis as reported quite recently [37–39]. Hence, we investigated whether K1 peptide in HSCs retains its original solubility properties in urea. As described in Materials and Methods, each extract obained by urea stepwise-extraction was subjected to SDS-PAGE. As shown in Fig. 5, intact epidermal K1 peptide is extracted by 6–8 M urea/2-ME [40]; about 80–90% of this K1 peptide was extracted in the 1–2 M urea/reductant fraction according to the data in Fig. 5. Since the so-called K1 peptide was extracted from the epidermis with 6 M reductant, as shown in Fig. 5, we may reasonably conclude that the K1 peptide extracted had changed considerably. Finally, we performed the above experiments 3–4 times each and obtained the very reproducible results described in this paper. Based on our findings in this study, the following appears to be true. (1) While K1 was not expressed abundantly, its presence was con-

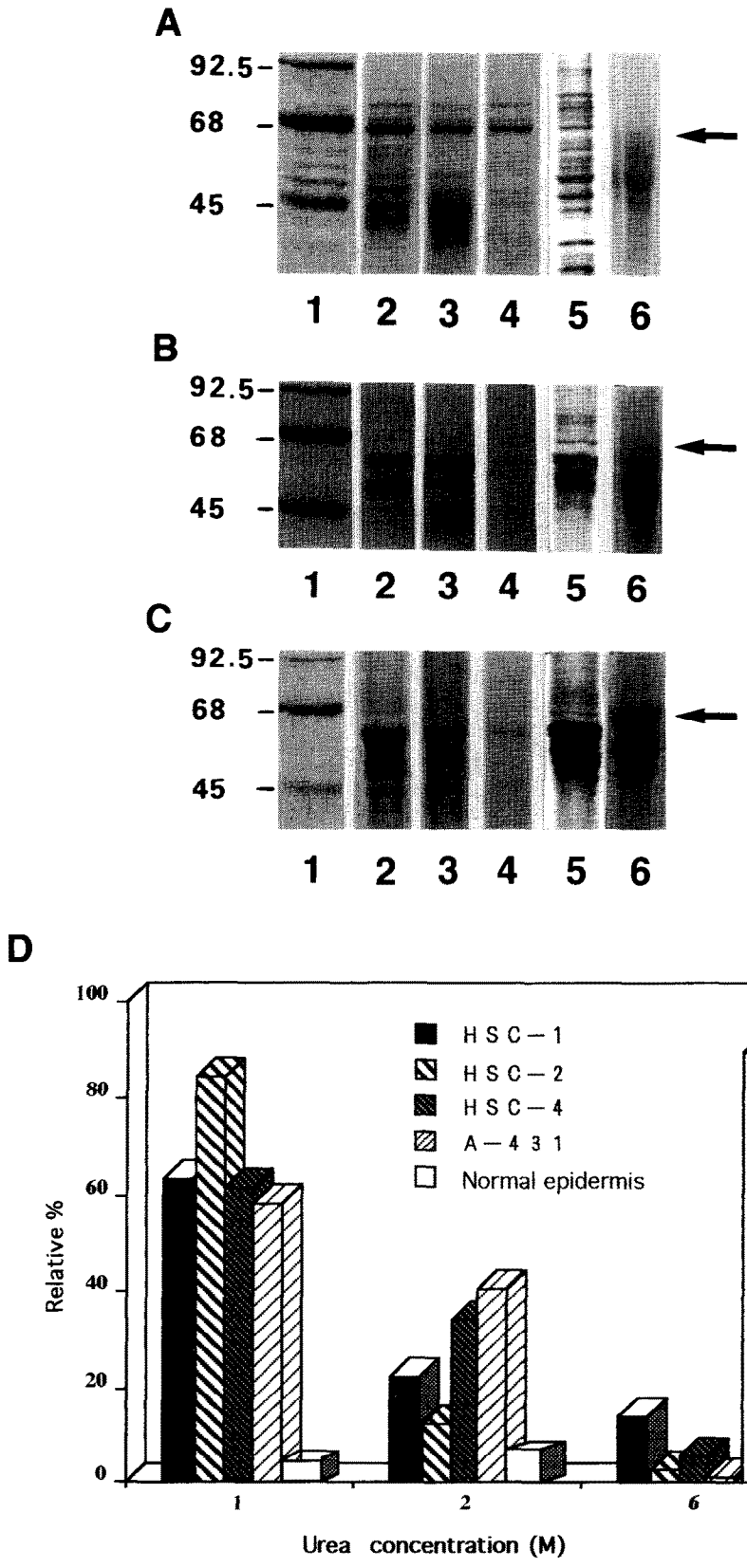

Fig. 5. SDS-gel electrophoresis water-insoluble HSC keratin peptides sequentially extracted with increasing concentrations of urea, stained with CBB R-250. About 1% (v/v) of proteins sequentially extracted with 10 mM Tris-HCl (pH 7.4)/5% 2-ME, containing 1 M urea (A), 2 M urea (B) and 6 M urea (C) were analyzed on 10% acrylamide gels. 1 = standard proteins; 2 = HSC-1; 3 = HSC-2; 4 = HSC-4; 5 = A-431; 6 = normal epidermis. (D) Quantitative analyses of K1 peptide extracted from four HSCs and normal epidermis by sequential urea concentrations.

firmed in HSCs extracts treated with Buffer A. Based on these findings and the results of the previous study [22], K1 keratin peptide may be widely present in HSCs. (2) K10 peptide was not found in HSCs, i.e. K1 may form keratin filaments with other type I keratins, but not K10 peptide. (3) The K1 peptide may be altered with respect to its urea solubility and become unstable or developed affinity when exposed to Buffer B. Many investigators may therefore have overlooked K1. It was discovered after the chance use of an aqueous buffer solution.

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