Keratin Subunit Expression in Human Cultured Melanocytes and Mouse Neural Crest Cells Without Formation of Filamentous Structures

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The synthesis of keratin is considered to occur in epithelial and epidermal cells. Previous studies have not reported on keratin synthesis within melanocytes that derive from neural crest cells. Epithelial and neural crest cells originally develop from ectodermal tissue. We previously reported that the expression of keratin is a universal phenomenon seen in cultured melanoma cell lines, as demonstrated by two-dimensional polyacrylamide gel electrophoresis, western blot, and electron microscopy analyses. To further investigate the specificity of keratin function in melanocytic cells, we first examined the presence of keratin proteins in cultured human melanocytes, and unexpectedly found keratin subunits in melanocytes by the above-mentioned procedures. The keratin (K) subunits were composed of K1, K5, K8, K10, K14, K16, and K18, together with vimentin. Neural crest cells, which contain immature embryonic melanocytes developing from ectoderm, already expressed keratins; however, under electron microscopy, the expressed keratin did not form filamentous structures. Although the ATP synthase α-chain, which is expressed universally in cultured epidermal tumor cell lines, was also expressed in cultured melanocytes and neural crest cells, a novel malignant melanoma-related protein (MMRP) was absent in melanocytes and neural crest cells. We concluded that keratin subunits are present in both cells, but do not construct keratin filaments. *Key words: cultured melanocytes/keratin/neural crest cells. Journal of Investigative Dermatology Symposium Proceedings 4:110–115, 1999*

K eratin is a component of intermediate filaments, and the major structural protein of epithelial cells, including keratinocytes. It shows the greatest heterogeneity of all intermediate filament proteins (Osborn and Weber, 1982; Moll *et al*, 1982). In humans, there are more than 20 different keratins (40–67 kDa) encoded by a large multigene family (Fuchs *et al*, 1981). Keratins are differentially expressed as specific pairs of type I (smaller and relatively acidic, 44–63 kDa and pI 4.5–5.5, respectively) and type II (larger and more basic, 53–67 kDa and pI 4.5–7.5, respectively) proteins, both of which are essential for filament formation, and at least one pair of keratins is always expressed in any epithelial cell. Moreover, changes in differentiation (Fuchs and Green, 1980; Woodcock-Mitchel *et al*, 1982) and development (Moll *et al*, 1982; Dale *et al*, 1985) in epithelial cells often coincide with alterations in keratin synthesis, suggesting that the expression of keratins might be finely tailored to suit the particular and varied structural requirements of each epithelial cell.

We have recently demonstrated that keratin expression is a universal phenomenon in thirteen cultured melanoma cell lines and that the ATP synthase α-chain (Kataoka and Biswas, 1991) is also expressed in many cultured epidermal tumor cell lines (Katagata, 1992, 1997;

Katagata and Yoshida, 1993; Katagata *et al*, 1992, 1996; Katagata and Kondo, 1997). The latter, especially, is considered to act as one of the fundamental energy sources in cultured cells. Use of an aqueous solution for preparation of keratin subunits in cultured cell lines, rather than the solution with a high concentration of salt (high-salt solution) that has been employed by many researchers, resulted in the above findings. Originally, this high-salt solution was intended to eliminate subcutaneous fatty tissues; thus, this solution may be too harsh for cultured cells. Malignant melanomas might be an abnormal development of melanocytes or nevus cells that have been affected by various tumorigenic factors. It is considered genetically that there is no keratin expression in melanocytes and neural crest cells (NCC). To begin, we analysed the keratin expression in cultured human melanocytes as a negative control. As several keratin subunits were unexpectedly recognized in cultured melanocytes, we further examined the keratin expression using mouse NCC, which were in an early developmental stage from ectodermal tissues. With the exception of one report (Jimbow and Fitzpatrick, 1975) of 10 nm filaments observed by electron microscope in human melanocytes, information about keratin expression in melanocytes is scarce.

In this report, we describe keratins expressed in cultured melanocytes observed using biochemical, immunologic, and electron microscopic procedures, including assays of the mRNA level. In addition, NCC, embryonic precursor cells of melanocytes, were also analysed for the expression of keratin subunits by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and electron microscopy.

MATERIALS AND METHODS

Cell culture To establish a culture system of normal human melanocytes, a NHEM-Neo (normal human epidermal melanocytes-neonatal) cell kit was
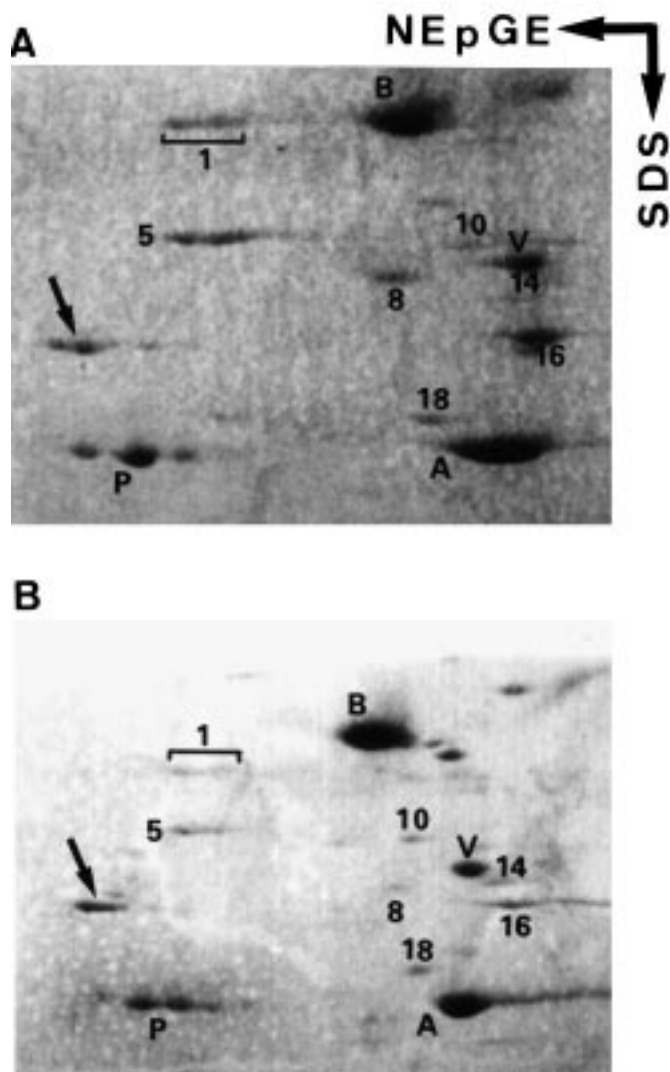
Figure 1. 2D-PAGE of keratin subunits in cultured melanocytes and neural crest cells. (*A*) Cultured melanocytes, reprinted from *FEBS Lett*, 407:25–31, 1997; Y. Katagata and S. Kondo, copyright (1997) with permission from Elsevier Science. (*B*) Neural crest cells. Standard proteins in 2D-PAGE profile: P, 3′-phosphoglycerokinase (pI 7.4, Mr 43 Kd); B, bovine serum albumin (pI 6.35, Mr 68 Kd); A, rabbit α-actin (pI 5.4, Mr 42 Kd). The *arrowhead* is ATP synthase α-chain (Kataoka and Biswas, 1991); V, vimentin. The numbers denote keratin nomenclature according to Moll *et al* (1982). Separation of the proteins in the first dimension was by nonequilibrium pH gradient gel electrophoresis (NEpGE) and in the secondary dimension by 12.5% polyacrylamide gels.

purchased from Biowhittaker (Walkersville, MD). The cells were cultivated in accordance with the manufacturer's protocol using 500 ml of MGM-3 Bulletkit (serum-free medium) and aliquots containing 1 ml bovine pituitary extract (7.5 mg per ml), 0.5 ml hFGF-B (1 μg per ml), 0.5 ml phorbol myristate acetate (10 μg per ml), 0.5 ml insulin (5 mg per ml), 0.5 ml hydrocortisone (0.5 mg per ml), 0.5 ml gentamycin and amphotericin B (50 mg per ml and 50 μg per ml, respectively), and 2.5 ml fetal bovine serum (FBS). In accordance with the manual, the cells were maintained in culture for no longer than 2 wk in each set of experiments to ensure that their phenotype would not change as a result of prolonged *in vitro* passage. Cultured cells were grown as monolayers splitting at a semi-confluent stage in culture dishes (19.6 m², Falcon Plastics, Becton-Dickinson Labware, Franklin Lakes, NJ) at 37°C in a humidified incubator with 5% CO_2. We serially cultured them at plating densities of approximately 2.0×10^4 cells per cm² in serum-free basic liquid melanocyte medium. Cell numbers were determined using a hematocytometer.

The culture of embryonic mouse NCC was performed as described by Ito and Takeuchi (1984). The trunk regions, posterior to the forelimb buds, were dissected out from E9.5 mouse embryos by tungsten needles. The trunk tissue was treated with 1% trypsin (Difco, 1:250) in Tyrode's solution containing 10% FBS. The tissues were gently pipetted with a small-pore Pasteur pipette to

separate the neural tubes from other components of the trunk. Neural tubes were explanted individually in 12 well microplates (Falcon) with 1 ml of Eagle, MEM supplemented with 15% FBS, stem cell factor (50 ng per ml), and endothelin-3 (100 nM). After harvesting the NCC at day 15 by treatment with 5 mM EDTA, we subcultured them to analyse keratin subunits. The NCC obtained exhibited the following immunologic characteristics (Kawa and Mizoguchi, unpublished data): anti-c-KIT, strongly positive; anti-tyrosinase-related protein (TRP)-1, anti-TRP-2 and anti-tyrosinase and anti-glial filament acidic protein, positive; anti-neurofilament, negative.

Extraction of keratin and 2D-PAGE When cells reached approximately 80% confluence, the cultured melanocytes and NCC were rinsed twice quickly with calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) and scraped off with a rubber policeman in 1 ml of an aqueous solution [10 mM Tris-HCl (pH 7.4)/10 mM EDTA/phenylmethyl sulfonyl fluoride (PMSF, 10 μg per ml)]. Each cell fraction was sonicated for 15 s using an ultrasonic disrupter (Tomy, UR-20p, Tokyo, Japan) and centrifuged at $18\,000 \times g$ for 10 min. The residue was then washed by centrifugation ($18\,000 \times g$, 10 min) three times to remove aqueous soluble materials. The final residue was dissolved in 50 μl of 10 M urea/10% 2-mercaptoethanol at 37°C for 6 h (Achtstaetter *et al*, 1986). After centrifugation, one-tenth of the supernatant obtained was treated with the same volume of $2 \times$ SDS buffer (Laemmli, 1970) for 3 min at 100°C. The treated solution was then applied to SDS-PAGE (10% polyacrylamide) gels (Laemmli, 1970). Analysis of keratin polypeptides by 2D-PAGE was performed as described (O'Farrell *et al*, 1977) with ampholytes of pH 3.5–10 (LKB, Bromma, Sweden) using the remaining 45 μl of each fraction.

Western blot analyses After SDA-PAGE, proteins on the unstained gels were electroblotted onto polyvinylidene difluoride (PVDF) membranes (0.2 mm, Bio-Rad Laboratories, Hercules, CA) at 2 mA per cm² for 1 h. To block nonspecific protein biding sites on the PVDF membranes, they were incubated with defatted milk for 1 h. Membrane strips were washed in CMF-PBS containing 0.05% Tween 20 and reacted with anti-keratin monoclonal antibodies [34βE12 and 35βH11 from Enzo Diagnostic, NY; DC-10 from DAKO A/S, Glostrup, Denmark; LL002 from Cymbus Bioscience, Hampshire, U.K.; CK-E3 from Sigma, St Louis, MO; LL025 from Ylem, Via Gramsci, Italy; and V9 (anti-vimentin) from Serotec, Oxford, U.K.] at a dilution of 1:50 in CMF-PBS at 37°C for 40 min. The strips were washed in CMF-PBS and incubated with peroxidase-conjugated anti-mouse immunoglobulin rabbit serum (DAKO) at 1:300 at 37°C for 40 min. After washing with CMF-PBS, the strips were stained by the avidin-biotin-complex method (Hsu *et al*, 1981). Staining of standard proteins on the PVDF membrane was performed with 0.04% CBB R-250.

Northern blot analysis Total RNA, prepared by the method of Chomczynski and Sacchi (1987) was separated on a denaturing formaldehyde-agarose (1%) gel, transferred to a nylon membrane (Magnagraph, Micron Separations, Westbord, MA), fixed to the membrane, and hybridized with ^{32}P-labeled probes. Prehybridization (2 h) and hybridization (overnight) were performed at 42°C in 45% formamide/5 mM Tris-HCl (pH 7.4)/100 mM NaCl/ $10 \times$ Denhardt's solution/0.1% sodium pyrophosphate/100 μg per ml salmon sperm DNA/1% SDS. The DNA probes used for hybridization were human K5 DNA (Galup and Darmon, 1988) and K14 (Vassar *et al*, 1989) probes provided by Dr K. Nomura (Department of Dermatology, Hirosaki University School of Medicine, Hirosaki, Japan). These DNA probes were isolated from vector DNA by appropriate restriction endonuclease digestion prior to random-priming in the presence of [α-^{32}P]dCTP. Labeled probes were then denatured by boiling and added directly to the prehybridization solution. The hybridized membranes were washed at various stringencies ($1 \times$ SSC, 1% SDS to $0.1 \times$ SSC, 0.1% SDS) at room temperature and exposed to Kodak X-Omat AR film in the presence of a Du Pont cronex intensifying screen at –80°C.

Immunostaining of melanocytes Harvested melanocytes were frozen immediately and embedded in OCT compound (Miles, Elkhart, IN) at –80°C. They were serially sliced at 4 μm thickness by a cryostat (Tissue-Tek II, Miles) at –20°C. Each slice was fixed in cold acetone at 4°C for 10 min and then exposed to 0.3% H_2O_2 for 5 min at 4°C to inhibit endogenous peroxidase. The slices were then exposed to primary antibodies to keratins [34βE12, LL002, V9, DE-K10, CAM 5.2 (Becton-Dickinson, San Jose, CA) and PKK3 (Labsystems Helsinki, Finland)] and CMF-PBS as a negative control at 4°C for 12 h. After washing the above slices with CMF-PBS three times, each slice was incubated with anti-mouse goat immunoglobulin labeled with fluorescein isothiocyanate (FITC) at 37°C for 1 h, followed by extensive washing with CMF-PBS. Controls for specificity of the antibodies were routinely carried out. Photomicrographs were taken with Nikon ECLIPSE E600 photomicroscope (Nikon, Tokyo, Japan).
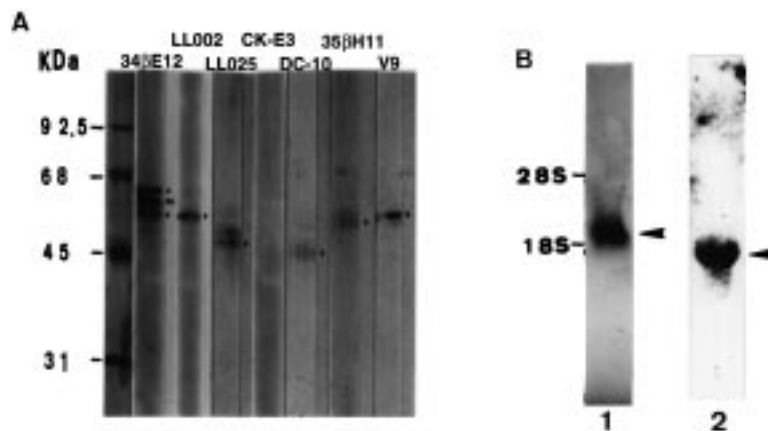
Figure 2. Western blot and northern blot analyses of cultured melanocytes. (*A*) Western blot analysis of keratin subunits; 92.5, phosphorylase A; 68, bovine serum albumin; 45, ovalbumin; 31, carbonic anhydrase, kDa ($\times 10^3$). Left lane is standard proteins stained with 0.04% CBB R-250 after transfer. 34βE12 (against K1, K5, K10, and K14), LL002 (against K14), LL025 (against K16), CK-E3 (against K17), DC-10 (against K18), 35βH11 (against K8), V9 (against vimentin). (*B*) Northern blot analysis: total RNA (10 μg) prepared from the cultured melanocytes was subjected to northern blot analysis, then blotted onto a nylon membrane. The membrane was hybridized with ^{32}P-labeled K5 or K14 keratin fragments as described in *Materials and Methods*. The *arrowheads* indicate the positions of both mRNA: 2.4 Kb (*lane 1*) and 1.7 Kb (*lane 2*), respectively. The positions of ribosomal RNA, 28S (4.7 Kb) and 18S (1.9 Kb), are indicated at the left.

Electron microscopy   Cultured melanocytes and NCC were prepared separately for electron microscopic study after fixation with 2.5% glutaraldehyde and 2% osmium tetroxide for 30 min in 2 mM cacodylate buffer at pH 7.4. The gathered cells were dehydrated by passages through a graded ethanol series and substituted with QY-2. Then, the samples were fixed in Quetol 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under an electron microscope.

RESULTS

Confirmation of keratin subunits in the cultured melanocytes and NCC   The cultured melanocytes obtained showed the typical dendritic cell shapes in MGM-3 medium (data not shown). Keratin subunits were prepared from cultured melanocytes at about 80% confluence. We used an aqueous solution for keratin subunit preparation, rather than using the high-salt solution [1.5 M KCl/0.5% Triton X-100/140 mM NaCl/10 mM Tris-HCl (pH 7.4)/1 mM EDTA/0.4 mM PMSF] that has been employed in previous studies of cultured cells by many researchers. The extracts were subjected to SDS-PAGE, and the resultant profiles indicated the presence of several bands of keratin subunits with Mr sizes between 40 and 67 kDa (Moll *et al*, 1982) (data not shown). To identify the keratin subunits present in the melanocytes, we performed 2D-PAGE (Laemmli, 1970). The results confirmed the presence of seven keratin (K) subunits (K1, K5, K10, K14, K8, K16, and K18) and vimentin, which were compared with the relative mobilities of three standard proteins (**Fig 1*A***). In addition, we detected the ATP synthase α-chain at the same position (Mr, ca. 48 kDa, pI 8.2) as shown in **Fig 1** (*arrow*). The protein has been recognized in cultured squamous cell carcinoma (SCC) (Katagata, 1992, 1997; Katagata *et al*, 1992; Katagata and Yoshida, 1993) and melanoma cell lines (Katagata *et al*, 1996; Katagata and Kondo, 1997); however, a novel protein, malignant melanoma related protein (MMRP; Mr, ca. 80 kDa, pI 7.8) that has been confirmed in established melanoma cell lines (Byers *et al*, 1991; Katagata and Kondo, 1997), was not detected in our melanocyte samples. On the other hand, NCC revealed the same seven keratin subunits and ATP synthase α-chain on 2D-PAGE (**Fig 1*B***).

Almost all anti-keratin monoclonal antibodies employed reacted reasonably well to the keratin subunits in the cultured melanocytes (**Fig 2*A***). As the Mr and pI of K16 and K17 are similar, we employed CK-E3 (monoclonal antibody against K17) to distinguish them and confirmed that the melanocytes did not express K17. Vimentin expression was confirmed with V9 anti-vimentin monoclonal antibody. These results were highly reproducible when the experiments were repeated several times. To investigate keratin expression by another method, we analysed the total RNA by northern blots using radiolabeled probes of K5 and K14 subunits. Both mRNA were found at correct sizes [2.4 kilo bases (Kb) and 1.7 Kb, respectively] (**Fig 2*B***).

Furthermore, for confirmation of keratin(s) and vimentin in the melanocytes, we carried out an immunohistologic study using several keratin and vimentin antibodies and detection with an FITC-conjugated secondary antibody. In the frozen sections of melanocytes, PKK3 (K18), CAM 5.2 (K8, K18), and V9 (vimentin) antibodies strongly reacted within the cytoplasm of the cells (**Fig 3*A, B, G***). The intracellular fluorescence intensity of the other keratin antibodies employed, 34βE12 (K1, K5, K10, and K14), LL002 (K14), and DE-K10 (K10), was weak compared with that of the above antibodies. Intracellular granular fluorescence was observed in the 34βE12 specimen under high magnification (× 1000) (**Fig 3*H***). We therefore concluded that the intracellular expression of keratins and vimentin was confirmed in cultured human melanocytes.

From an electron microscopic study, the keratin filaments in NCC and the cultured melanocytes were found to be extremely thin and immature (**Fig 4**). Only trace amounts of the filament-like substance were recognized. They were not present at perinuclear areas or near the plasma membrane, but were near the centre of the cytoplasm (**Fig 4*B***, *arrowhead*). These photographs indicated that keratin filaments were probably not formed in cultured melanocytes, compared with keratin filaments of cultured melanoma cells (**Fig 4*C***, *arrow*).

DISCUSSION

As melanocytes derive developmentally from NCC (Holbrook *et al*, 1989), not from epithelial cells, we had not expected to observe keratin expression in cultured human melanocytes. In fact, no previous studies appear to have found keratin expression in melanocytes. In order to prepare keratin subunits from malignant neoplasms (Viac *et al*, 1982; Moll *et al*, 1984), a high-salt solution (1.5 M KCl/140 mM NaCl/1% Triton X-100, etc.) has commonly been employed. This solution was originally used to remove large amounts of subcutaneous fat tissues found in native tissues or tumors (Franke *et al*, 1981; Schweizer and Winter, 1983); however, despite the substitution of *in vitro* experimental systems (cultured cells) for *in vivo* ones (malignant neoplasms), many researchers have adopted the same high-salt solution to prepare keratin subunits in cultured SCC (Rubin *et al*, 1989; Sacks *et al*, 1989) and malignant melanomas (MM) (Trejdosiewicz *et al*, 1986; Hendrix *et al*, 1992). It was considered that the employed procedure was too harsh for cultured cells, because we confirmed expression of the K1 subunit in six kinds of cultured SCC (Katagata *et al*, 1992; Katagata, 1992; Katagata and Yoshida, 1993) and found keratin subunits to be universally expressed in cultured MM (Katagata *et al*, 1996; Katagata and Kondo, 1997), using an aqueous solution instead of the high-salt solution. Thus, in this study, we employed an aqueous solution as a buffer to prepare keratin subunits from cultured human melanocytes. In other words, this experiment was carried out as a negative control, and cells were not expected to present keratin(s) during our studies on the relationship of epithelial tumor invasion to keratin expression.

To investigate whether keratin subunits were present in human cultured melanocytes, we performed 2D-PAGE. This system is effective
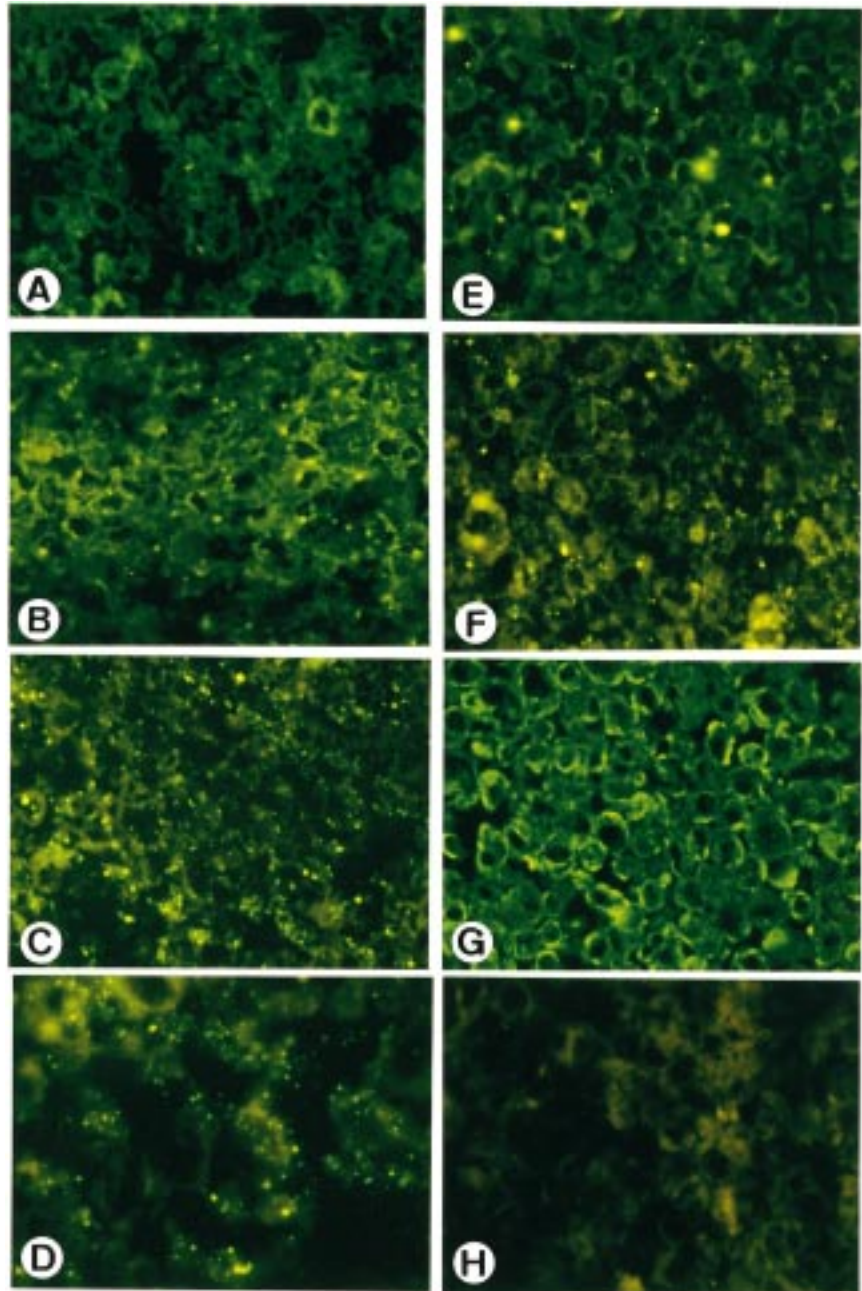
Figure 3. Immunofluorescence microscopy of cultured melanocytes using several monoclonal antibodies for keratins and vimentin. (×400.) (*A*) PKK3 (K18); (*B*) CAM 5.2 (K8, K18); (*C*) 34βE12 (K1, K5, K10, and K14); (*D*) 34βE12 (×1000); (*E*) LL002 (K14); (*F*) DE-K10 (K10); (*G*) V9 (vimentin); (*H*) negative control.
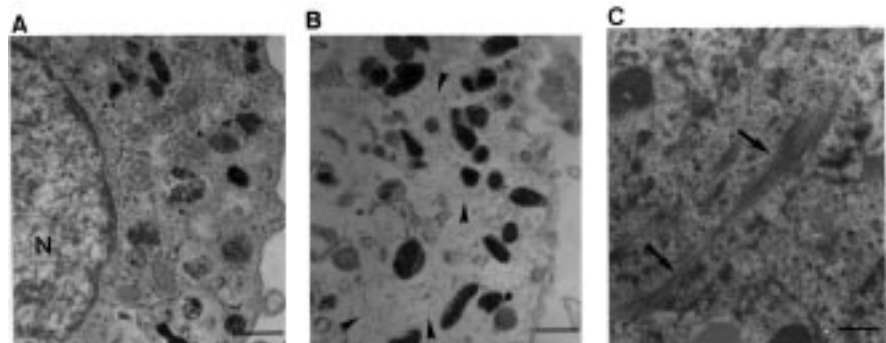

Figure 4. Electron micrographs of cultured melanocytes and neural crest cells. (*A*) Neural crest cells, (*B*) cultured melanocytes, (*C*) cultured melanoma. Reprinted from *FEBS Lett*, 407:25–31, 1997; Y. Katagata and S. Kondo, copyright (1997) with permission from Elsevier Science. Magnification ×20 000. *Scale bar*: 0.5 μm; N, nucleus.

and can easily differentiate the 21 kinds of keratin subunits (Moll *et al*, 1982; Katagata, 1997), all of which have a similar Mr (40–67 kDa) and pI (4.9–7.8). As shown in **Fig 1**(*A*), seven distinct keratin subunits (K1, K5, K8, K10, K14, K16, K18) and vimentin were deduced and identified. K8, K18, and K13 are known to be related to tumor

invasion and to change in epithelial–mesenchymal interactions (Markey *et al*, 1991; Caulin *et al*, 1993; Chu *et al*, 1993). K13 was not present in our melanocyte samples. By contrast, K8 and K18 were present in minimal amounts. Our findings from this experiment clearly indicated that keratin subunits were expressed in cultured human melanocytes,

not only in keratinocytes. Furthermore, almost the same keratin subunits were identified in mouse NCC, embryonic precursor cells, during the melanocytes development (**Fig 1B**). The expression of the ATP synthesis α-chain may be common in all of the cultured cells, because the expression of this polypeptide has been noted in six cultured SCC lines (Katagata *et al*, 1992; Katagata, 1992; Katagata and Yoshida, 1993) and thirteen cultured MM (Katagata *et al*, 1996; Katagata and Kondo, 1997), as well as in cultured melanocytes and NCC in this study (**Fig 1A, B**, arrow). It is likely that this component is a resident protein, playing the same fundamental role *in vitro* as an energy source. MMRP, which is a novel malignant melanoma-related protein expressed in five particular melanoma cell lines (Byers *et al*, 1991; Katagata and Kondo, 1997), was not expressed in either cell type. From the above results, we estimated that melanoma tumorigenesis might be related to the presence of K8 and K18 subunits, including effects of many other factors in melanocytes. All of these findings were dependent on our use of an aqueous solution instead of a high-salt solution as the preparation solution for our cultured melanocytes. Therefore, other investigators may have overlooked some keratin subunit(s) and the ATP synthase α-chain, due to the use of a high-salt solution.

To further confirm the presence of keratin subunits in cultured melanocytes, we performed western blot analysis using several anti-keratin and vimentin monoclonal antibodies. As shown in **Fig 2(A)**, the results indicated co-expression of vimentin and keratin in the melanocytes, which is in agreement with the 2D-PAGE profiles (**Fig 1A**). The results of northern blot analysis (**Fig 2B**) further confirmed the expression of keratin subunits in the melanocytes. The co-expression of these molecules may be necessary to maintain the internal cell structure in melanocyte development. In general, there are no desmosomes on the outer aspect of the plasmalemma, but condensation of some granular materials of the cytoplasm can be observed on the inner leaflet of the plasmalemma facing the basal lamina. Keratinocytes do not form hemidesmosomes against melanocytes, although they are dotted by these structures on the surface adjacent to the basal lamina.

Proving keratin expression in melanocytes by procedures other than those described above, would serve to confirm our results. Therefore, we attempted to confirm keratin expression with several antibodies and an FITC-conjugated secondary antibody for the slices of frozen melanocytes. As shown in **Fig 3**, all antibodies were positive, indicating some degree of difference in fluorescent strengths. In particular, 34βE12 (K1, K5, K10, and K14) showed FITC-labeled granules in intracellular regions of melanocytes when at 1000× magnification (**Fig 3D**). These results were in good agreement with those of our other experiments presented in this paper.

As electron microscopy showed only very thin keratin molecules (**Fig 4A, B**), filaments might not have been formed. On the other hand, we confirmed that keratin formed complete filaments in cultured melanoma cells (**Fig 4C**, Katagata and Kondo, 1997). The filament formation may start to alter during the processing from melanocytes to melanoma cells by some factor(s). With the exception of one report (Jimbow and Fitzpatrick, 1975) of 10 nm filaments in human melanocytes, there is no biochemical evidence that keratin and vimentin filaments are present in cultured melanocytes. The discrepancy between the findings of other researchers and ours may be due to the differences that exist between native human melanocytes and cultured melanocytes. The evidence of keratin subunits may also be a result of a change caused by moving native tissues to cultured cell conditions, in which some factor(s) switched in. Our results suggest that keratin subunits exist in cultured melanocytes and NCC and may play some unique roles that are different from those in cultured melanoma cell lines (Katagata *et al*, 1996; Katagata and Kondo, 1997).

In conclusion, keratins are expressed in melanocytes and NCC, not only in keratinocytes. This is the first evidence of keratin expression in the above cells; however, the expressed keratin did not form filamentous structures. As we have previously demonstrated the expression of keratin filament structures in several melanoma cell lines (Katagata and Kondo, 1997), keratin filament formation may be one sign of transition from melanocytes to melanoma cells caused by many

risk factors. We speculate that the intracellular keratin filaments may play particular role(s) during the development of melanocytes.

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