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Immunoelectron Microscopy of Keratin Filaments in Cultured Malignant Melanomas and Squamous Cell Carcinomas

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ABSTRACT

Heretofore, epithelial cells have been considered to be the only source of keratin (K) polypeptides that assemble into 10-nm filaments to form an extensive cytoplasmic network in conjunction with nuclear and cytoplasmic membranes. However, K was recently found to be expressed also in cultured non-epithelial normal and tumor cells: melanocyte, fibroblast, endothelial cell, malignant melanoma, fibrosarcoma, and angiosarcoma. Nevertheless, electron microscopy was incapable of detecting the K filaments (Katagata et al., *J Dermatol Sci*, 30, 1-9, 2002, see ref. 11). That is, K may present as subunits in each of the cultured cells named above, not as a filament formation. We used squamous cell carcinoma observed with immunoelectron microscopy, a more precise and conclusive technique, to further confirm whether or not K filament is formed in those cultured cells. HaCaT, an immortalized keratinocyte cell line used as a positive control, yielded elegant immunoelectron microscopic images. Considerable K filament formations existed in malignant melanoma using anti-K or anti-vimentin antibodies, as revealed by the presence of linear immune gold particles on high electron density substances. In the case of squamous cell carcinoma, the gold particles were fewer than those of malignant melanoma. By contrast, no K filaments were detected in the other non-epithelial normal and tumor cell lines: fibroblast, endothelial cell, fibrosarcoma and angiosarcoma. These results suggest that the formation of K filaments in malignant melanoma (and slight presence in squamous cell carcinoma) is a particular and cell-dependent characterization.

Key words: tumor cells, keratins, filament formation, immunoelectron microscopy

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INTRODUCTION

Polypeptide subunits of 10 nm intermediate filaments in epithelial cells are classifiable into two large multigene families: types I and II keratin (K). They form cage-like structures around the nucleus and extend from the perinuclear region to the cell periphery. Each K is encoded by its own gene and is expressed in K pairs containing one acidic (type I: K9-K20, 44-60 kDa) and one basic (type II: K1-K8, 48-67 kDa) protein according to specific rules¹. For example, simple epithelia express K8/K18 with variable levels of K19 and K20, whereas stratified epithelia express K5/K14 in basal cells and K1/K10 in suprabasal cells. The K filaments, which are considered to be particularly important for epithelial resilience against mechanical stress, form a stable network for specific cell contact of the desmosome type. Regarding the physiological function of K, in addition to its important role in helping cells cope with stress^{2,3}, it is hypothesized that filament reorganization plays an integral role. However, skin abnormalities associated with mutations of specific epidermal Ks have provided strong evidence of a mechanical function of the intermediate filaments in the epidermis⁴. Furthermore, K-attenuated tumor necrosis factor was shown recently to induce cytotoxicity⁵ and to have an association with Fas-mediated apoptosis⁶. Discovery of such new K functions might constitute a breakthrough in keratinocyte biology.

A series of biochemical and immunological studies of the relationship between K and epithelial or non-epithelial tumor cells has demonstrated that K also appears in cultured tumor cells^{7-11, 16, 17}. The K expressed in

respective cultured cells are present as K subunits, not K filaments. Data were analyzed using SDS-PAGE. Electron microscopy showed no filament formation.

We have demonstrated that K expression is a universal phenomenon in cultured SCC and MM using two-dimensional polyacrylamide gel electrophoresis, Western blot analysis and measurement of the mRNA expression level⁷⁻¹⁰. Cultured fibroblasts (SF-TY), endothelial cells (HMVEC-dAd), fibrosarcomas (HT-1080) and angiosarcomas (ISO-HAS), which were derived from mesenchymal cells and their sarcomas reportedly express several K subunits¹¹. Nevertheless, K filament formation was unclear in electron microscopy data of the above cultured cells. The present study employed immunoelectron microscopy to clarify whether or not K filaments are expressed in SCC, MM, HT-1080, and ISO-HAS. In addition, HaCaT, an immortalized keratinocyte cell line, was used as a positive control. Although cultured SCCs¹³ and MMs⁹ have been analyzed by electron microscopy, immunoelectron microscopy of two kinds of cultured cells was undertaken to further elucidate K filament formation.

MATERIALS AND METHODS

Cell lines and the cultured methods

We employed the following cultured cell lines: 1) SCC: A-431¹² and HSC-5¹³; and 2) MM: MM-LH, MM-RU and MM-AN. The MM cell lines were established from melanomas that had metastasized respectively to one lung and two lymph nodes¹⁴. The above three cultured melanoma cell lines were gifts from Dr. H. R. Byers (Department of Pathology, Boston University School of Medicine, Boston, MA,

USA). The SCC and MM were cultured in special media under conditions described in our previous paper¹⁰. As normal keratinocytes, we employed the HaCaT cell line¹⁵-a gift from Dr. N. E. Fusenig (Division of Differentiation and Carcinogenesis *In Vitro*, Institute of Biochemistry, German Cancer Research Center, Heidelberg, Germany). Cultured human SF-TY, human HT-1080¹⁴ and human ISO-HAS¹⁵ cell lines were supplied by the Japanese Cancer Research Resources Bank, Tokyo. A human HMVEC-dAd (Biowhittaker Inc., Walkersville, MD, USA) cell kit was used to culture human endothelial cells. Cells were cultured in accordance with the manufacturer's protocol. We maintained the cells in culture for no longer than 10 d in each set of experiments to ensure that their metastatic phenotype would not change as a result of prolonged passage *in vitro*. The cells were cultured continuously as a monolayer in plastic dishes (78.5 cm²; Falcon Plastics, Becton Dickinson Discovery Labware, Franklin Lakes, NJ, USA) at 37 °C in a humidified incubator with 5% CO₂ in air. We serially cultured all the cell lines at plating densities of approximately 1.5 × 10³ cells/cm² in minimum essential medium (MEM, for MM cell lines) and Dulbecco's MEM (for HaCaT and SCC cell lines) supplemented with 10% fetal calf serum (Gibco Corp., Grand Island, NY, USA), penicillin (100 units/ml), streptomycin (50 µg/ml), kanamycin (50 µg/ml), and hydrocortisone (0.4 µg/ml). Cell numbers were determined using a hemacytometer.

Immunoelectron microscopy

Cells were fixed with a mixture of 2% paraformaldehyde and 0.025% glutaraldehyde for 1 h at room temperature. After the cells were rinsed with 0.1 M phosphate buffer (pH

7.2) containing 0.1 M lysine and 0.15 M NaCl and phosphate-buffered saline, they were centrifugated. Then they were immersed in 20% polyvinylpyrrolidone solution for preparation of cryoultramicrotomy sections. Sections were stained with the following anti-mouse antibodies for human K and vimentin: anti-vimentin antibody (1:50 dilution), anti-K antibodies, LP34 (K5, K6 and K18: 1:50 dilution), and 34 E12 (K1, K5, K10 and K14: 1:25 dilution), or normal mouse immunoglobulin solution (1:1 dilution) for four nights at 4 °C. They were then stained with 15 nm gold-conjugated anti-mouse IgG (1:10) overnight at 4 °C. After immunoreaction, the sections were fixed with 1% glutaraldehyde solution for 10 min at room temperature and stained with 1% uranium acetate solution for 10 min at room temperature. Anti-vimentin (V9, 1:100 dilution, Boehringer Mannheim Biochemica, Mannheim, Germany), LP34 and 34 E12 (DakoCytomation Co. Ltd., Glostrup, Denmark) and 15 nm gold-conjugated anti-mouse IgG (Amersham Pharmacia Biotech Inc., Uppsala, Sweden) were used.

RESULTS

We previously examined K filament formation in various types of cultured MMs and SCCs using electron microscopy, not immunoelectron microscopy. Five cultured MMs expressed K filament-like substances⁹. However, cultured SCCs showed extremely small and fine filaments-like substance¹³, respectively derived from non-epithelial and epithelial tumors. Moreover, the K filament-like substances expressed in cultured melanocytes closely resembled those of previously cultured SCCs¹³. After obtaining the unexpected data of

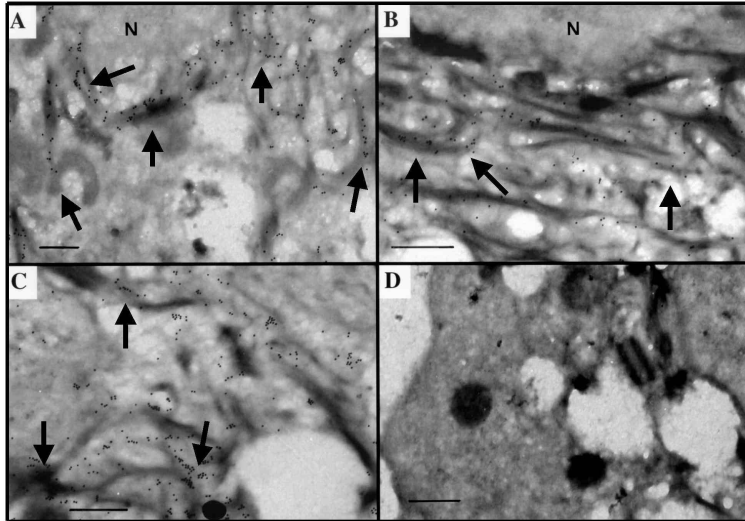


Fig. 1.

Immunoelectron microscope images of HaCaT cells stained with anti-K and anti-vimentin antibodies. As a secondary antibody, 15-nm gold-conjugated anti-mouse IgG was used. Linearly aligned immune gold-conjugated anti-mouse IgG particles appear in many places (arrows). A) LP34 (K5, K6 and K18, 1:50 dilution). B) 34 E12 (K1, K5, K10 and K14, 1:25 dilution). C) V9 (anti-vimentin antibody, 1:50 dilution). D) Negative control (normal mouse immunoglobulin). The negative control shows no immunolabels. Scale bar: 0.5 μm with $\times 10,000$ magnification. N, nucleus.

K filament formation and the origin of K⁹, we carried out immunoelectron microscopy to confirm whether or not K filaments are formed in cultured MMs and SCC. Immunoelectron microscopy was carried out using 15-nm gold-conjugated anti-mouse IgG to further clarify whether or not K formed filaments.

Figure 1 shows that HaCaT (a positive control and immortalized keratinocytes cell line) was clearly indicated as present in the K filaments by the two anti-K monoclonal antibodies (LP34 and 34 E12) and the anti-vimentin antibody (V9). The gold particles aligned on filament-like substances (Figs. 1A-1C), showing reactivity to employed K-antibodies and expressed Ks. In addition, the negative control gave a reasonable result (Fig. 1D). These results were estimated to express K filaments and were employed for comparison

with the following results as a standard set of data. Results of K filaments in cultured MMs did not indicate similar levels to those of HaCaT (Figs. 2A-2C). However, some linearly aligned dots were apparent in the results (MMs-LH and -RU, as shown respectively in Figs. 2A and 2B). In MMs used here, scarce gold particles were particularly more numerous in MM-AN (Fig. 2C), than in HaCaT (Fig. 2D). These results among identical tumor cell lines (MMs) indicated that each cultured cell possessed a different degree of K filament formation. Moreover, Fig 3 shows that, using the anti-vimentin antibody (V9), immunoelectron microscopy yielded extremely detailed images. In other words, immunoreaction with V9 was stronger than that of anti-K antibody immunoreaction (Fig. 3D), as shown in (Figs. 3A-3C). These facts imply that vimentin

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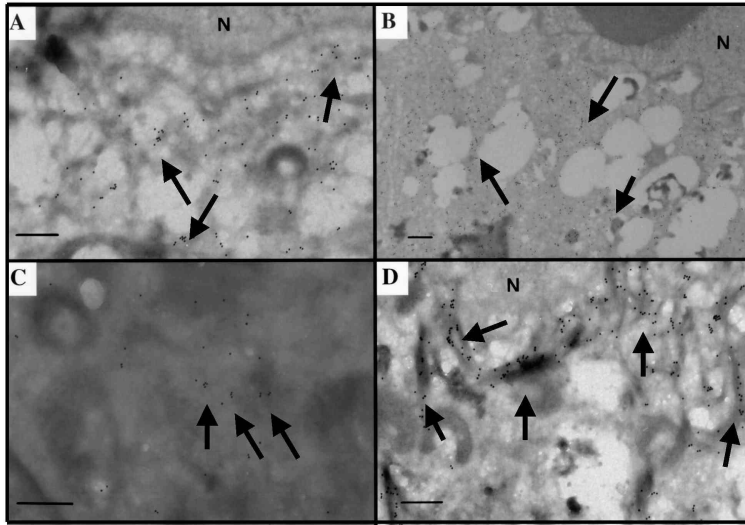


Fig. 2. Immunoelectron microscopy of cultured MM and HaCaT cells stained with anti-K antibody (LP34). Immune gold-conjugated anti-mouse IgG particles are visible and appear in a slightly linear fashion (arrows). A) Cultured MM-LH. B) Cultured MM-RU. C) Cultured MM-AN. D) HaCaT (positive control, Fig. 1A). Scale bar: 0.5 μm with $\times 10,000$ magnification. N, nucleus.

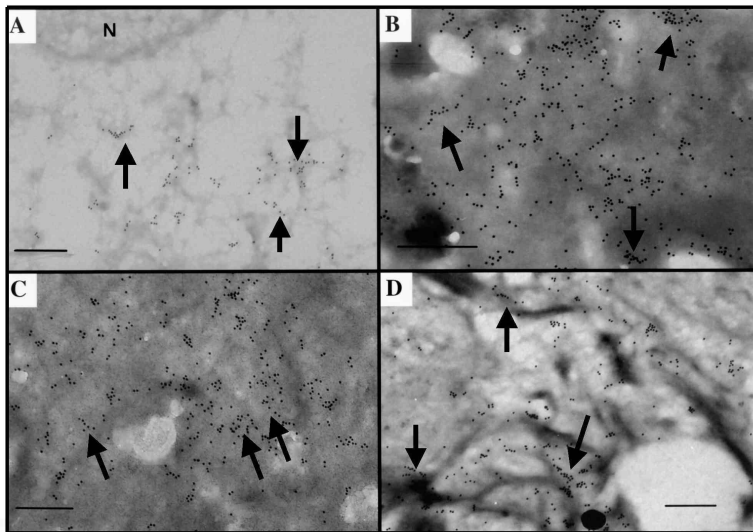


Fig. 3. Immunoelectron microscopy of cultured MM and HaCaT cells stained with anti-vimentin antibody (V9). Immune gold-conjugated anti-mouse IgG particles are seen in some places (arrows). A) Cultured MM-LH. B) Cultured MM-RU. C) Cultured MM-AN. D) HaCaT (positive control, Fig. 1C). Scale bar: 0.5 μm with $\times 10,000$ magnification. N, nucleus.

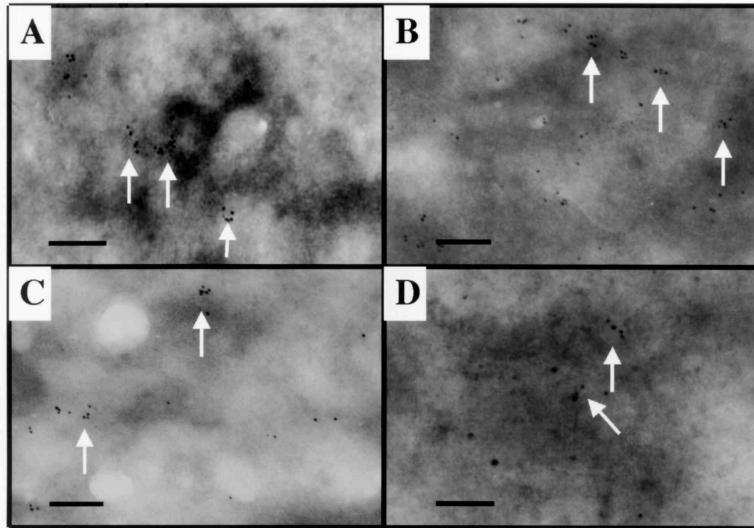


Fig. 4. Immunoelectron microscopy of cultured SCCs (HSC-5 and A-431) stained with anti-K antibody (LP34) and anti-vimentin antibody (V9). Immune gold-conjugated anti-mouse IgG particles are few. However, they appear in a linear alignment (arrows). A) Cultured HSC-5/LP34 anti-K antibody. B) Cultured HSC-5/V9 anti-vimentin antibody. Scale bar: 0.5 μ m with \times 10,000 magnification.

expression is better than that of K in cultured cells in accordance with vimentin contents, as mentioned by many researchers so far. In contrast with the immunoreactive HaCaT and SCCs (HSC-5 and A-431), the latter showed linearly aligned dots on immunoreaction with anti-K antibody (Figs. 4A and 4C) and with V9 (Figs. 4B and 4D), showing their immature filaments. Immunoelectron microscopic investigation of cultured cell lines (SF-TY, HMVEC-dAd, HT-1080 and ISO-HAS) other than the two mentioned above revealed no K filament formation (data not shown).

DISCUSSION

After checking results of immunoreaction with anti-K antibodies of the above cultured cells using phase-contrast microscopy, K filament formation in the selected cultured

cells was confirmed using immunoelectron microscopy. Non-K filament formation and the origins of the four cultured cells might be related to occurrence of the phenomena described in the above section. Moreover, we infer that these results might reflect K and vimentin expressions because the respective expression levels in cultured MMs were approximately one-tenth and one-third of those in HaCaT cells, as described in a previous paper¹⁷⁾. We also infer that the degree of K filament formation account of HaCaT (++) , MM (+) , SCC (\pm) and other cultured cells (SF-TY, HMVEC-dAd, HT-1080 and ISO-HAS) used in this study. These are relevant considering the extremely small amounts of K in each cultured cell employed. They are considered to be characteristic phenomena in these experiments¹¹⁾.

This is the first immunoelectron-microscopic

detection of K filaments in keratinocytes (HaCaT). In addition, the degree of K filament formation in MM was greater than in SCC. These results were unexpected in light of each cell's origin. Lower expression of K10 in SCC than in MM, which has been shown by our previous study⁷⁾, possibly reflects the low degree of K filament formation in SCC.

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