Expression and Distribution of Vimentin and Keratin Filaments in Heterokaryons of Human Fibroblasts and Amnion Epithelial Cells

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ABSTRACT The expression of intermediate filaments of the keratin- and the vimentin-type was studied in heterokaryons of human fibroblasts and amnion epithelial cells by immunofluorescence microscopy. Fibroblasts and their homokaryons showed a fibrillar, vimentin-specific fluorescence throughout the cytoplasm but were negative when stained for keratin. Amnion epithelial cells and their homokaryons, on the other hand, showed a keratin-specific fibrillar staining, and only some of them contained also detectable vimentin. When suspended epithelial cells were fused with adherent fibroblasts, keratin fibrils spread within 3 h into the fibroblasts, intermixing with the vimentin fibrils. 1–3 d after fusion, both vimentin and keratin filaments were expressed as typical fibrillar cytoplasmic arrays, and the distribution of keratin in heterokaryons resembled closely that of vimentin. A typical cell-to-cell arrangement of keratin fibrils, seen in cultures of amnion epithelial cells, could also be found between heterokaryons. Treatment of the cultures with vinblastine sulphate induced coiling of the vimentin filaments in both homo- and heterokaryons, whereas the keratin organization was only slightly affected. Our results show that both vimentin and keratin filaments are incorporated into the cytoskeleton of heterokaryons formed between fibroblasts and epithelial cells, and that they behave in the same way as in their parental cells. Both epithelial and fibroblastic characteristics thus appear to be coexpressed in such heterokaryons.

Regulation of phenotypic expression has been widely studied using cell fusion techniques (cf. reference 23). Cell hybrids have been especially useful in the study of tumorigenicity and the transformed phenotype (21, 23). We earlier investigated (14, 15) the expression of fibronectin in heterokaryons of fibroblasts and normal and malignant epithelial cells. Neither of these epithelial cells produce fibronectin matrix and consequently matrix-associated fibronectin was lost from both types of heterokaryons (14, 15). This indicated that malignancy as such is not obligatory for the loss of fibronectin matrix. The phenotypic changes may rather have been caused by epithelial cell characteristics dominating over the fibroblastic phenotype in such heterokaryons. These results prompted us to study the expression of other fibroblastic and epithelial cell characteristics in heterokaryons.

At least five immunologically and biochemically different types of intermediate filaments can be detected in cells cultured in vitro (2, 8, 16, 32). These include the vimentin-type of filaments found in mesenchymal cells (9), the desmin-type in muscle cells (17), keratin filaments in epithelial cells (10, 27), and neurofilaments and glial filaments (3, 26). A similar distinction in the expression of intermediate filaments has also been found in vivo, and these filaments have thus been considered reliable markers of the differentiated phenotype of cells (5, 25, 29).

Here we show that both the vimentin and the keratin-type of intermediate filaments are coexpressed in heterokaryons of human fibroblasts and normal amnion epithelial cells, indicating that both epithelial and fibroblastic characteristics are coexpressed in such heterokaryons.

MATERIALS AND METHODS

Cell Culture and Cell Fusion

Human embryonic fibroblasts were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. Human amnion epithelial cells were isolated as described by Valle and Penttinen (31) and cultured in RPMI 1640 medium. The media were supplemented with 10% fetal calf serum (Grand Island Biological

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Co. [Gibco], Grand Island, NY) and antibiotics. The cells were mycoplasma-free as tested by DNA staining (24).

LABELING OF CELLS: The cells were labeled with cytoplasmic polystyrene particles before fusion to enable identification of cells and their fusion products as homo- and heterokaryons. The fibroblasts were allowed to ingest large polystyrene particles (diameter 1.23 μm, 25 μl of the suspension supplied by the manufacturer, Polysciences, Inc., Warrington, PA, per 90 mm petri dish) for 24 h. To remove unphagocytized particles the cells were first trypsinized and then centrifugated (400 *g*, 4 × 5 min) in Hanks' balanced salt solution (HBSS). Thereafter the cells were plated on glass cover slips and cultivated for 24 h before cell fusion. Human amnion epithelial cells were labeled with small polystyrene particles (diameter 0.765 μm, 25 μl of the suspension supplied by the manufacturer per 90 mm diameter petri dish) for 24 h. Thereafter the cells were trypsinized, pelleted by centrifugation (400 *g*, 4 × 5 min) and used immediately for fusion experiments. In some experiments, unlabeled epithelial cells were used. In such cases the keratin fluorescence served as the marker for the inclusion of epithelial cells in multinucleated cells.

CELL FUSION: Cells were fused by the Sendai virus method as described earlier (14, 15). Briefly, fibroblasts cultured on glass cover slips were washed once with NaCl-P buffer (140 mM NaCl, 10 mM sodium phosphate, pH 7.2) and then cooled in 3 ml of HBSS at 4°C for 10 min. Virus adsorption was done at 4°C by adding beta-propiolactone-inactivated (0.03%) (19) Sendai virus (3,000 hemagglutinating units, courtesy of Professor K. Cantell, Central Public Health Laboratory, Helsinki, Finland). After 15 min 0.5×10^6 epithelial cells in 0.5 ml of HBSS were added. The epithelial cells were allowed to adhere to the virus-treated fibroblast monolayer at 4°C for 15 min. Cell fusion was then induced by incubation at 37°C. Finally, the cultures were washed carefully in HBSS and cultivated in medium until fixation.

Indirect Immunofluorescence (IIF) Microscopy

HUMAN AND RABBIT ANTIVIMENTIN ANTIBODIES: Monoclonal human autoantibodies of immunoglobulin M (IgM) class against vimentin have been characterized in detail elsewhere (32) and were used in the present study for double staining experiments. The antibodies reacted specifically with vimentin polypeptide isolated from detergent-resistant cytoskeletons of cultured human fibroblasts (18) as judged by the ELISA method (32). Rabbit antibodies against vimentin were raised against the 58-kdalton polypeptide isolated from the detergent-resistant cytoskeletons of cultured human fibroblasts using the preparative electrophoretic method (32, 33). The antibodies reacted only with the 58-kdalton polypeptide in cultured fibroblasts as shown by the immunoblotting method (30; cf. 32, 33).

RABBIT AND SHEEP ANTIKERATIN ANTIBODIES: Keratin polypeptides were isolated from human plantar callus as described by Sun and Green (28). Antibodies were raised both in rabbits and sheep and were affinity purified on a keratin-Sepharose Cl 4B column as described elsewhere in detail (13, 32, 33). Both antibodies reacted with several polypeptides of human amnion epithelial cells as judged by the immunoblotting method (Fig. 1).

IMMUNOFLUORESCENCE STAINING AND MICROSCOPY: For indirect immunofluorescence staining the cells were fixed in −20°C methanol for 10 min. Thereafter the specimens were first incubated with rabbit antiserum against vimentin or keratin, washed with NaCl-P buffer and then incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit IgG antiserum (N. L. Cappel Laboratories Inc., Cochranville, PA).

For double immunofluorescence staining two methods were used. (*a*) Cells fixed with methanol were incubated first with the human antiserum against vimentin followed by the FITC-conjugated goat anti-human IgM antiserum. The cells were then stained with rabbit antikeratin antibodies followed by tetramethylrhodamine-isothiocyanate (TRITC)-coupled swine anti-rabbit IgG (Dako, Copenhagen, Denmark). (*b*) Alternatively, the cells were first stained with rabbit antiserum against vimentin followed by TRITC-coupled swine anti-rabbit IgG. Thereafter the cells were sequentially exposed to normal rabbit serum, to sheep antikeratin antibodies and then to FITC-coupled rabbit anti-sheep antibodies (Cappel Laboratories Inc.). The specimens were mounted in a mixture of Na-veronal buffer and glycerol (1:1, pH 8.5).

A Zeiss Universal microscope equipped with phase contrast optics and epi-illuminator III RS giving blue excitation light for FITC-fluorescence (HBO 200W lamp, excitation filters BP 455-490, dichroic mirror FT 510 and emission filters LP 520 and KP 560) and green excitation light for TRITC-fluorescence (HBO 200 W lamp, excitation filters BP 510-546, dichroic mirror FT 580, and emission filters LP 590) was used.

RESULTS

Identification of Cells

The cells were first identified in phase-contrast microscopy by the number of nuclei and by cytoplasmic labeling with polystyrene particles. In these experiments only the fibroblasts were labeled with polystyrene particles. The efficiency of the labeling method was assayed by studying 200 cells. 98% of the fibroblasts contained more than five particles per cell. Keratin fluorescence served as a marker for the presence of epithelial cells in the multinucleated cells. Multinucleated cells with cytoplasmic keratin fluorescence and more than five cytoplasmic polystyrene particles were therefore regarded as heterokaryons. To exclude the possibility that keratin fluorescence could be extinguished in some of the heterokaryons the epithelial cells were labeled with small cytoplasmic particles. 97% of the 200 epithelial cells counted contained more than five particles per cell at the time of fusion. Thus, in this case multinucleated cells with more than five large and five small cytoplasmic polystyrene particles were regarded as heterokaryons. In cocultivation experiments 90–98% of the cells contained particles of only one kind. The rest of the cells were either negative (1–8%) or contained two kinds of particles (1–2%). Only a few loose latex particles were seen to contaminate the cultures.

The frequency of multinucleated cells was low before Sendai virus treatment (3–6% in cocultivation experiments at 1–3 d after plating the cells). This indicates that spontaneous fusion was a rare event under our experimental conditions. These and our previous results (14, 15) show that the identification of heterokaryons was reliable.

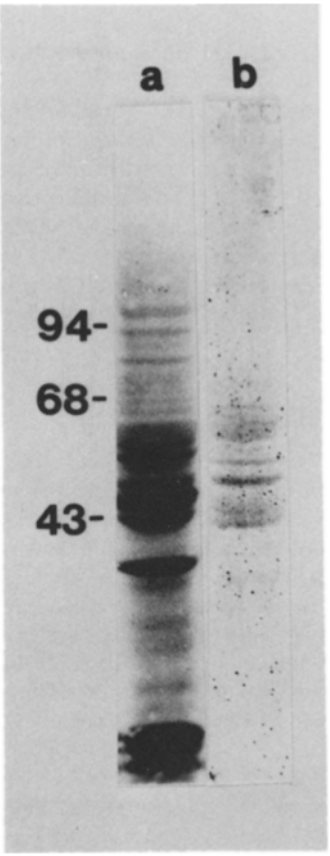

FIGURE 1 Electrophoretically separated polypeptides of human amnion epithelial cells transferred onto a nitrocellulose sheet (lane *a*). In immunoblotting (lane *b*) seven distinct polypeptides can be visualized using rabbit antikeratin antibodies and the immunoperoxidase method (30). Molecular weight markers ($\times 10^3$) are indicated on the left, *43*, ovalbumin; *68*, bovine serum albumin; *94*, phosphorylase b.

Vimentin and Keratin Filaments in Homokaryons of Fibroblasts and Epithelial Cells

Both single, unfused fibroblasts and their homokaryons showed a typical wavy fibrillar fluorescence pattern in indirect immunofluorescence (IIF) with antivimentin antibodies. The filaments extended throughout the entire cytoplasm in both

fused and unfused cells (Fig. 2*a* and *b*). No staining could be observed with antikeratin antibodies in the fibroblasts or their homokaryons (Fig. 2*c*).

Human amnion epithelial cells and their homokaryons showed an extensive, fibrillar cytoplasmic keratin-specific staining in IIF (Fig. 3*a* and *c*). The keratin-positive fibrils of one cell often appeared to be closely related to those of another epithelial cell forming typical cell-to-cell arrangements (Figs. 3 and 4). Interestingly, vimentin-specific fluorescence could be seen only in some (~5%) of the amnion epithelial cells in primary or secondary culture as judged by double IIF (Fig. 5; cf. reference 32).

Vimentin and Keratin Filaments in Heterokaryons

30 min after induction of cell fusion the amnion epithelial cells were seen to adhere to the cultured fibroblasts (Fig. 6*a*). In the fusing cells, vimentin-specific staining was confined to the fibroblastic cell (Fig. 6*b*), whereas the keratin-positive fluorescence localized exclusively in the smaller amnion epithelial cell (Fig. 6*c*). At this stage, the two filament systems were completely separated. 30 min later fused cells could be seen in which the two filament systems were still mostly separated, although located within the same cell (Fig. 7). Keratin fibrils then appeared to invade gradually the cytoplasm of the fibroblastic cell (Fig. 8) and within 24 h to occupy the entire cytoplasmic area of the heterokaryon (Fig. 9).

1 d after fusion a fibrillar vimentin- as well as a keratin-specific staining could be seen throughout the cytoplasmic domain of the heterokaryons in IIF (Fig. 9). However, the two filament patterns did not always completely overlap in the heterokaryons (Fig. 10), indicating that the different filaments retained their structural independence also in the fused cells. During 2–3 d of cultivation, heterokaryons continuously expressed both vimentin and keratin-specific staining in IIF (Fig. 11). However, some variation existed in the intensity of staining of the filaments between individual cells: either the keratin or, sometimes, the vimentin pattern appeared to give a more intense fluorescence. Also, some large, multinucleated heterokaryons with only one or two small epithelial cell nuclei were seen. Accordingly, the keratin-specific fluorescence in these heterokaryons appeared weak.

Many heterokaryons formed contact regions with neighboring heterokaryons or epithelial cells. In these contact areas, keratin filaments of one heterokaryon appeared to be continuous or closely related to those of another heterokaryon or to those of neighboring epithelial cells. The filaments at these contact areas showed also the nonfluorescent gaps between cells, typical of desmosome-type junctions. However, these

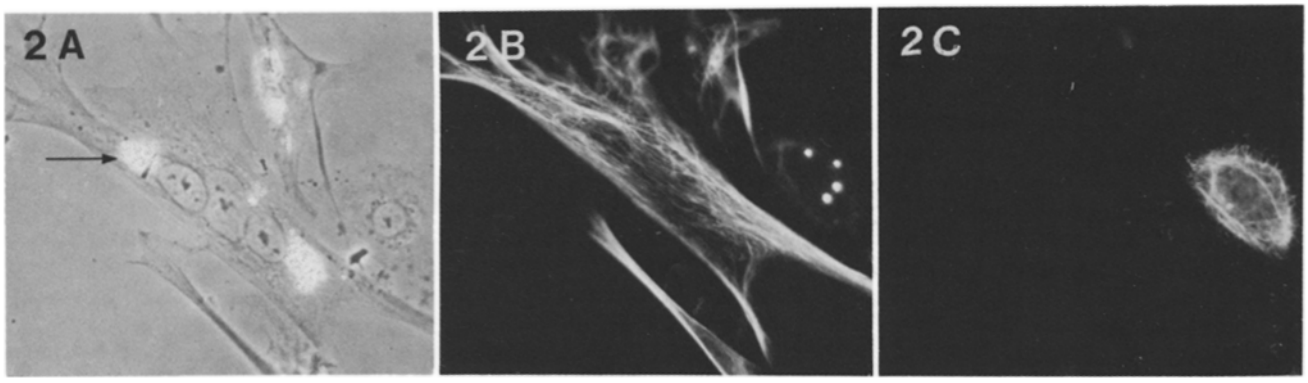

FIGURE 2 (*A*) A fibroblast homokaryon with three nuclei and an unfused epithelial cell double stained for vimentin and keratin 1 d after fusion. Large polystyrene particles are seen in the cytoplasm of the homokaryon in phase-contrast microscopy (arrow). (*B*) After staining for vimentin a strong fibrillar cytoplasmic fluorescence is seen in the homokaryon. (*C*) After staining for keratin the single epithelial cell shows a bright fibrillar fluorescence, whereas the fibroblast homokaryon lacks fluorescence. × 420.

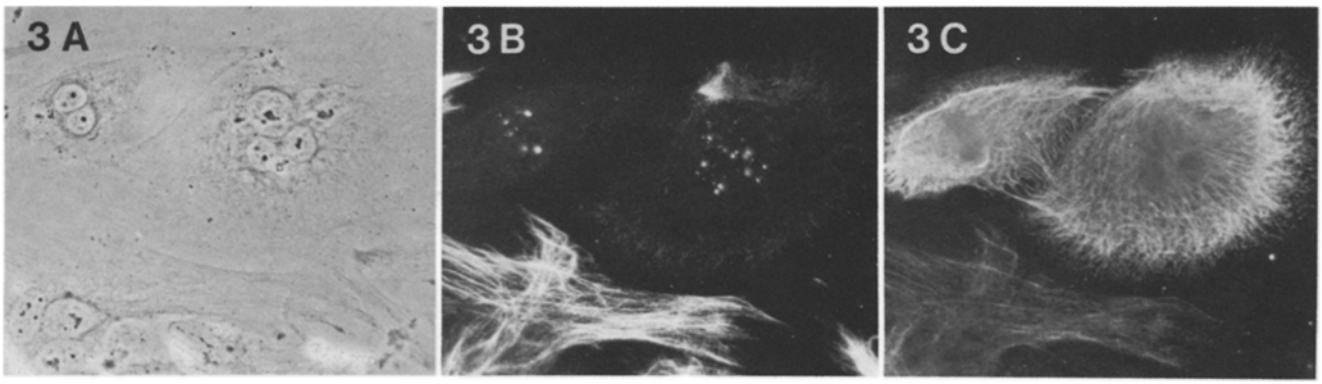

FIGURE 3 (*A*) Two epithelial cell homokaryons (above) and a heterokaryon (below) 1 d after fusion seen in phase-contrast microscopy. (*B*) After staining for vimentin no fibrillar fluorescence is seen in the epithelial cell homokaryons. The heterokaryon shows a strong fibrillar vimentin staining. (*C*) After staining for keratin the epithelial cell homokaryons show a fibrillar keratin-specific fluorescence with typical cell-to-cell connections. The heterokaryon also shows fibrillar keratin-specific fluorescence. × 420.

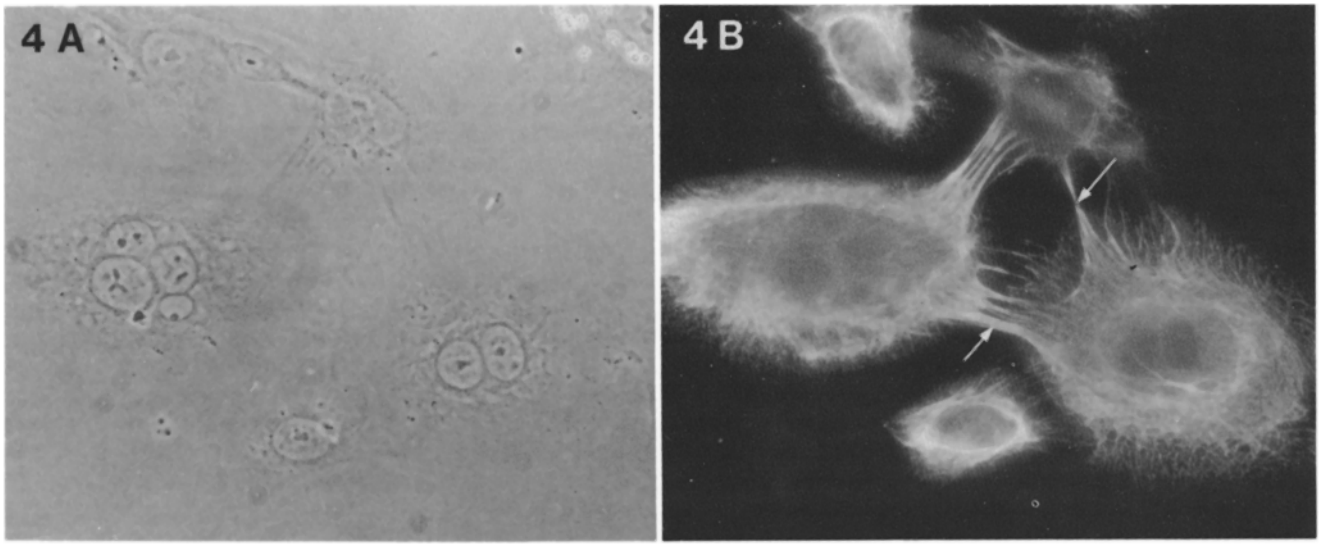

FIGURE 4 (*A*) A binucleated and a trinucleated epithelial cell homokaryon and three unfused epithelial cells in phase-contrast microscopy. (*B*) After staining for keratin a strong cytoplasmic fibrillar fluorescence is seen in all of the cells. Cell-to-cell connections are brightly stained and in the connections a dark unstained area is seen (arrows) corresponding to the location of desmosomes. × 540.

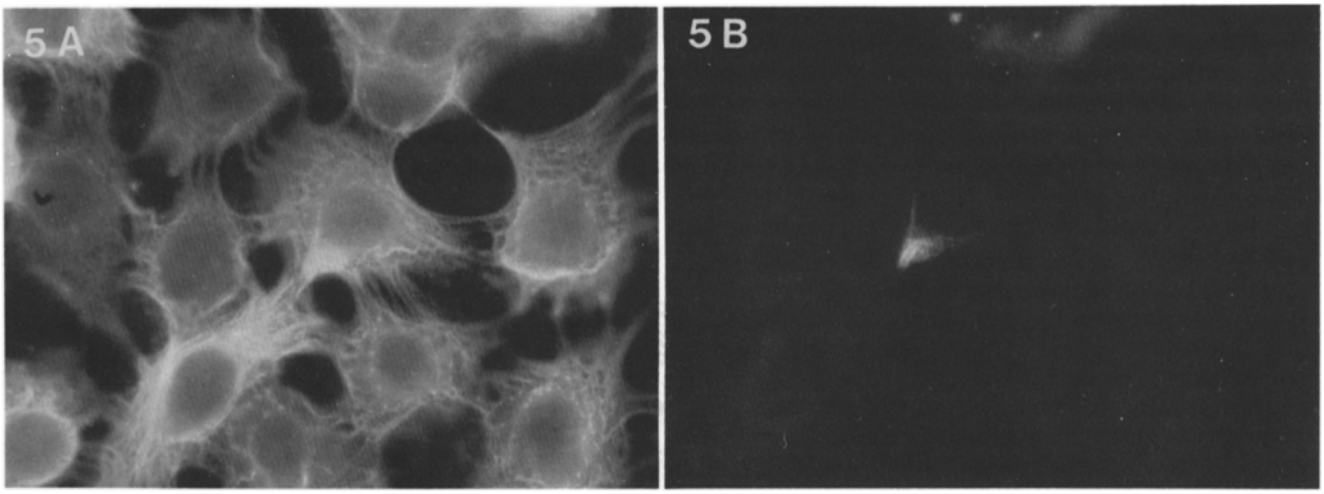

FIGURE 5 (*A*) Human amnion epithelial cells stained for keratin. All of the cells show an extensive fibrillar, cytoplasmic fluorescence. (*B*) The same field after staining for vimentin. Only some of the cells show detectable vimentin. × 540.

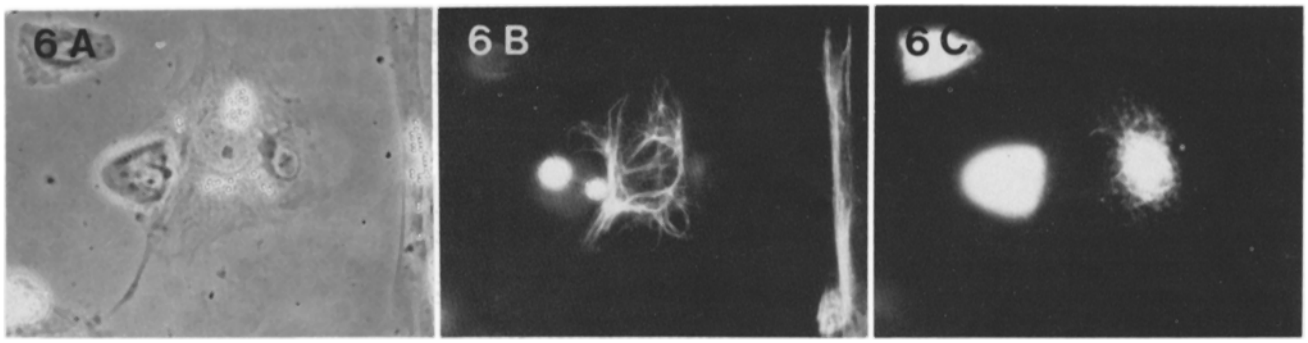

FIGURE 6 (*A*) Two single epithelial cells attached to a fibroblast (center) 30 min after induction of fusion. Phase-contrast microscopy. (*B*) After staining for vimentin the fibroblast is brightly stained, whereas the epithelial cells lack fluorescence or show only spotty staining. (*C*) After staining for keratin the epithelial cells are brightly stained and the area of keratin-specific staining is separated from that of the vimentin-specific staining. Note the lack of keratin-specific fluorescence in the fibroblast (center). × 420.

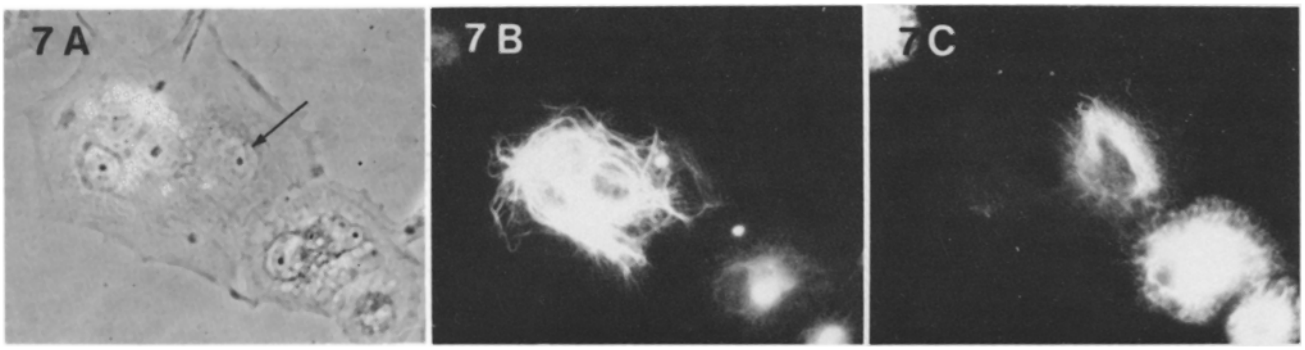

FIGURE 7 (*A*) 1 h after fusion an epithelial cell (arrow) is seen to fuse with three fibroblasts. After double staining for vimentin (*B*) and keratin (*C*) the staining patterns are still mostly separated at this point. × 420.

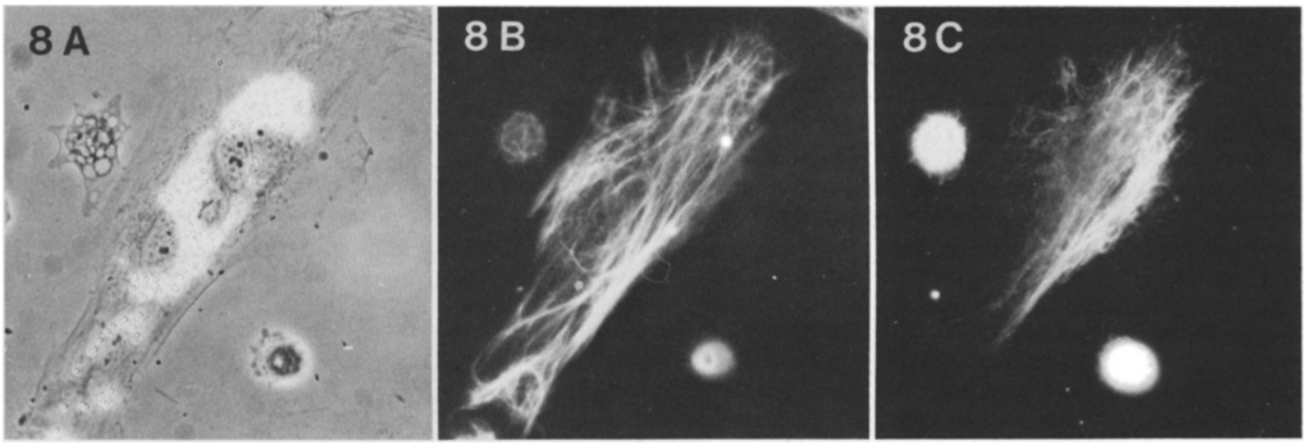

FIGURE 8 (*A*) A multinucleated heterokaryon 1 h after fusion. (*B*) The vimentin filaments have spread over the entire cytoplasmic area of the heterokaryon. (*C*) The keratin filaments occupy a larger area of the cytoplasm of the heterokaryon than in Fig. 7. × 420.

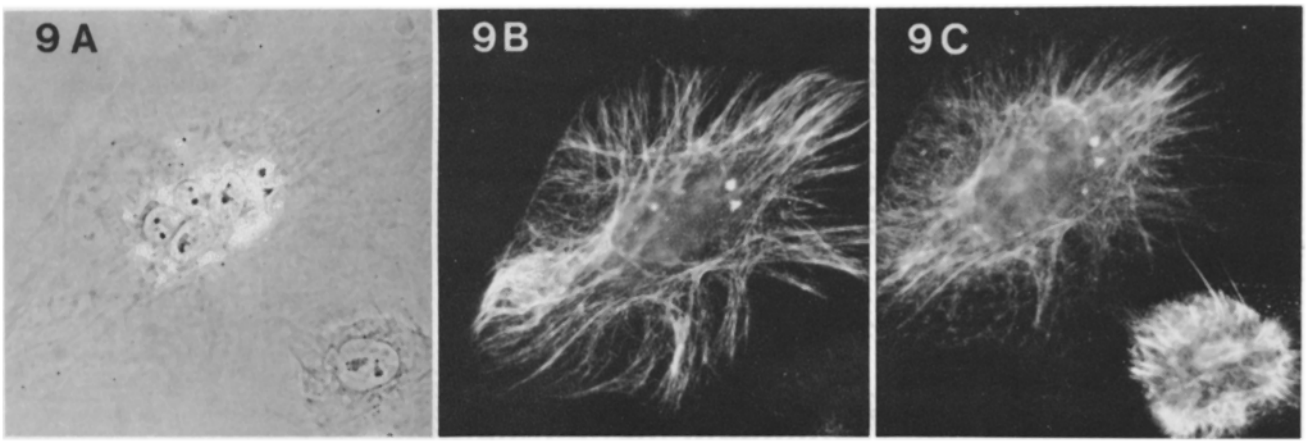

FIGURE 9 (*A*) A heterokaryon 1 d after fusion. (*B*) After vimentin staining a typical fibrillar cytoplasmic staining occupying the entire cytoplasmic area of the heterokaryon is seen. (*C*) After keratin staining a fibrillar fluorescence pattern is seen over the entire cytoplasmic area. × 420.

contact regions appeared less extensive in heterokaryons when compared to those formed by epithelial cells (cf. Fig. 4 with Fig. 15).

Reaction of Intermediate Filaments to Vinblastine Treatment

When the cultures were treated with vinblastine sulphate vimentin filaments characteristically formed perinuclear bundles of filaments both in the fibroblasts and their homokaryons as well as in the vimentin-positive epithelial cells and their homokaryons (Figs. 12 and 13). However, the keratin pattern of the epithelial cells and their homokaryons was nearly unaffected (Fig. 13). In heterokaryons the vimentin filaments were similarly seen to form coiling bundles after vinblastine treatment, whereas the keratin pattern was again only slightly affected (Fig. 14). Accordingly, the keratin-positive cell-to-cell contact areas formed by the heterokaryons were also resistant to vinblastine treatment (Fig. 15).

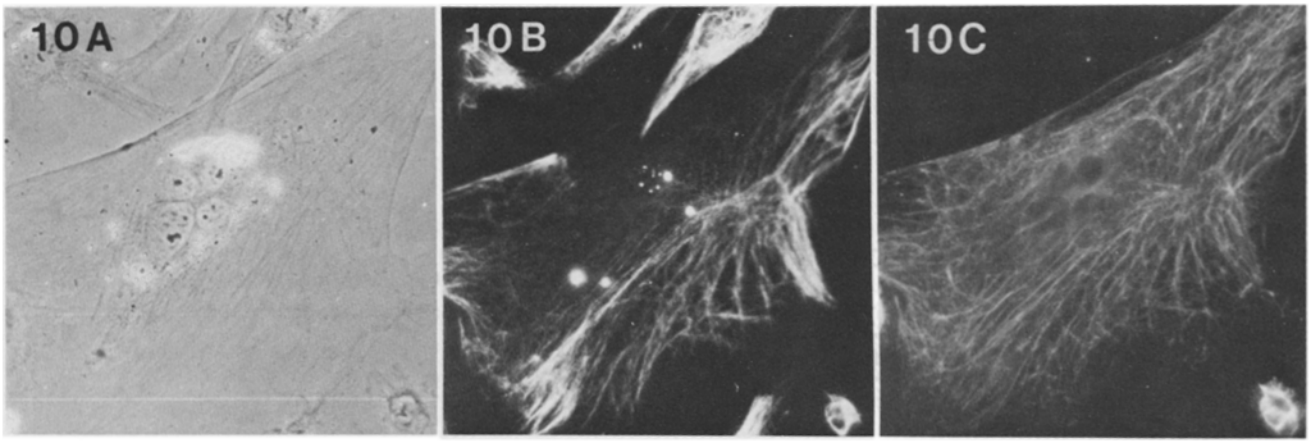

FIGURE 10 (*A*) A heterokaryon 1 d after fusion. After vimentin staining (*B*) and after keratin staining (*C*) the fluorescence patterns do not align. × 420.

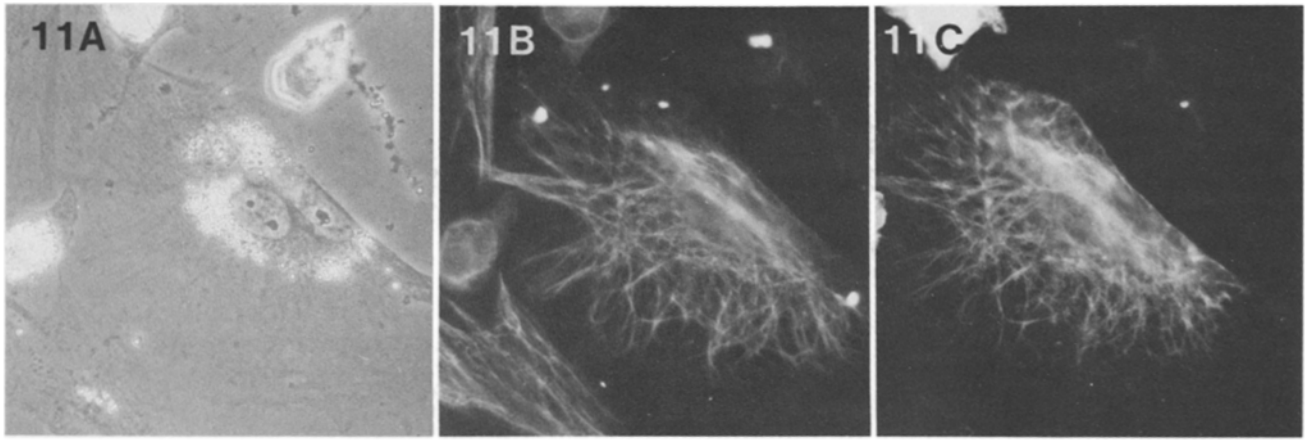

FIGURE 11 (*A*) A binucleated heterokaryon fixed 2 d after fusion. Both large and small cytoplasmic polystyrene particles are seen. (*B*) After staining for vimentin a fibrillar, cytoplasmic fluorescence is seen. (*C*) After staining for keratin a similar fibrillar staining pattern occupying the entire cytoplasmic domain is seen as after vimentin staining. This indicates that both the filament systems are incorporated into the cytoskeleton of the heterokaryons. × 420.

FIGURE 12 (*A*) A fibroblast homokaryon and a single epithelial cell after vinblastine treatment 1 d after fusion. In phase contrast, typical tubulin-containing paracrystals are seen in the cytoplasm of the cells. (*B*) The vimentin filaments have coiled into perinuclear bundles. The epithelial cell shows a small bundle of vimentin filaments. (*C*) After staining for keratin the fibroblast homokaryon shows no staining. The keratin filaments of the epithelial cell are only slightly affected. × 420.

DISCUSSION

To investigate the regulation of phenotype in heterokaryons, we have fused human amnion epithelial cells with human fibroblasts. As structural markers for the fibroblastic and the epithelial cell phenotypes we have used the expression of vimentin and keratin-type of intermediate filaments. The results show that both fibroblastic and epithelial cell characteristics are coexpressed in such heterokaryons.

Keratin polypeptides are constituents of tonofilaments found exclusively in epithelial cells (5, 9, 25, 29). These filaments are associated with the desmosomal junctions which are typically formed only between epithelial cells (4) and they can therefore be regarded as markers for epithelial cells (7, 9, 25, 29). Vimentin-type intermediate filaments, on the other hand, occur primarily in mesenchymal cells, although most cells in culture

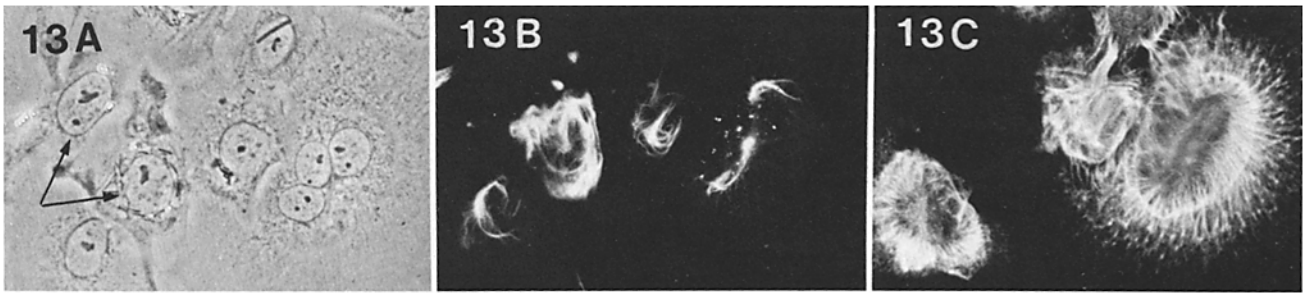

FIGURE 13 (*A*) An epithelial cell homokaryon (right), single epithelial cells and two fibroblasts (arrows) after vinblastine treatment 1 d after fusion. (*B*) Small vimentin bundles are seen in the homokaryon and in some of the single epithelial cells. The fibroblasts show extensive vimentin bundles. (*C*) The keratin filaments of the epithelial cells are nearly unaffected. × 420.

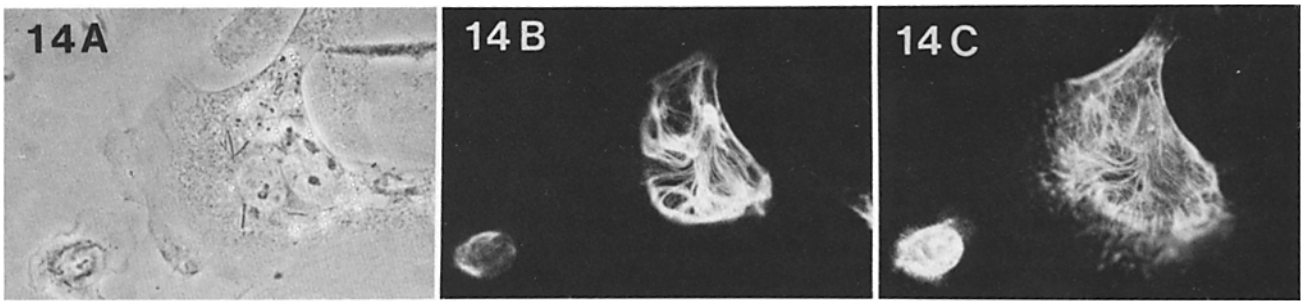

FIGURE 14 (*A*) A heterokaryon and an unfused epithelial cell after vinblastine treatment. (*B*) The vimentin filaments of the heterokaryon are coiled similarly as in the fibroblast homokaryon. (*C*) The keratin filaments, on the other hand, are almost unaffected both in the heterokaryon and in the single epithelial cell. × 420.

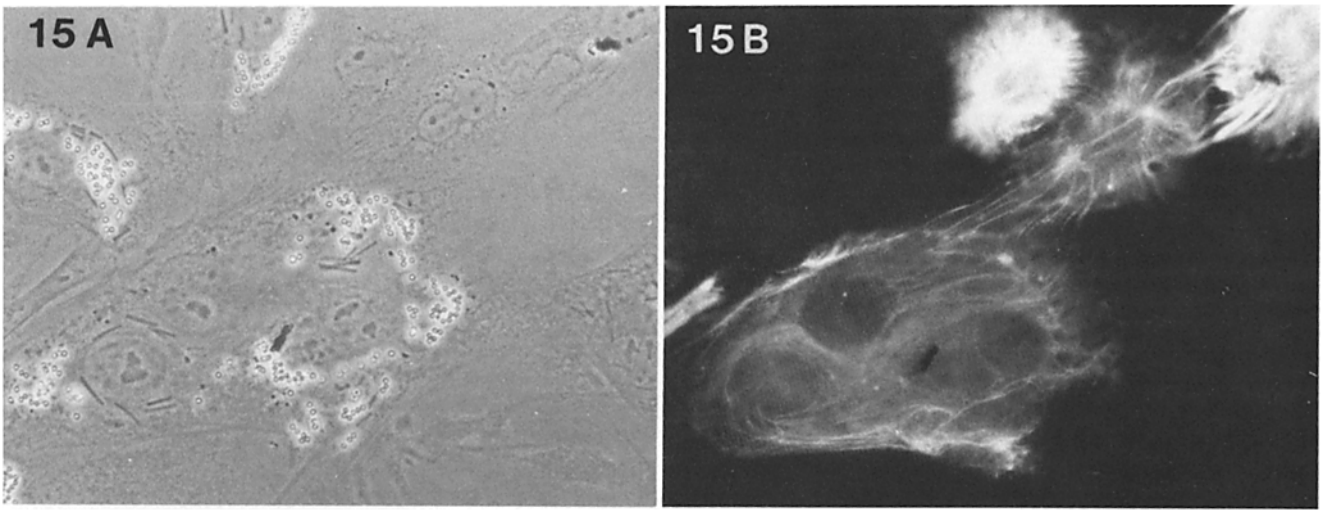

FIGURE 15 (*A*) A heterokaryon, labeled with both large and small cytoplasmic polystyrene particles, after vinblastine treatment. (*B*) After staining for keratin a fibrillar fluorescence is seen to form typical cell-to-cell connections analogous to those seen in the epithelial cell homokaryons. × 540.

conditions appear to express vimentin filaments in addition to their tissue-specific filament protein (cf. refs. 8, 9, 16, 32). Epithelial cells and cell lines also appear to express vimentin filaments after a few passages in vitro (6, 20, 27). However, the amnion epithelial cells used in the present study showed vimentin filaments as typical perinuclear aggregates (cf. also 32) only in a few cells and most of the cells distinctly lacked vimentin under our culture conditions. The expression of fibrillar arrays of vimentin could therefore be regarded as a phenotypic marker for mesenchymal cells.

An interesting finding of our study is that both the keratin and the vimentin filaments are intermixed in the cytoplasm of the heterokaryon. No cytoplasmic organizing centers could be found. However, our results suggest that the nuclei could have an organizing role, as shortly after fusion the different parental cell nuclei were surrounded by their respective intermediate filaments and complete intermixing of the filaments was seen only after the nuclei had aggregated in the center of the heterokaryon. Both the fibroblastic and the epithelial cell nuclei became equally surrounded by both types of filaments and no separate compartments were formed within the cytoplasm of the heterokaryon. The rate and sequence of this

intermixing process suggests that the filaments of the parental cells are mechanically rearranged. Since it is known that cytoskeletal structures and surface receptors may be associated (34), the intermixing of intermediate filaments in heterokaryons may be associated with the concomitant redistribution of surface receptors known to occur rapidly after fusion (11).

Recent immunofluorescence and immunoelectron microscope studies have indicated a structural independence of vimentin and keratin filaments in epithelial cells (1, 12). In our study both the keratin and the vimentin filaments of the heterokaryons were expressed similarly to those of the parental cells. Their morphology resembled each other, but they did not actually coalign, indicating a structural independence of these filament systems in heterokaryons. This was further supported by the differing behavior of the vimentin and the keratin filaments after vinblastine treatment. Moreover, the keratin filaments of heterokaryons formed typical desmosome-type cell-to-cell arrangements similar to those seen between the parental epithelial cells and their homokaryons. Thus the typical features of the epithelial as well as the fibroblastic cell phenotype were seen in the heterokaryons.

The retention of both the vimentin and the keratin filaments in the heterokaryons for at least 3 d suggests that the filaments are fully integrated into the cytoskeleton of the heterokaryons. This seems to be in contradiction to recent results (22) that suggest that keratin filaments are lost from hybrid cells of fibroblasts and HeLa cells. This discrepancy might, however, be due to differences in experimental systems as chromosome losses almost inevitably take place in hybrid cells (23), whereas heterokaryons retain their genomes intact and the observed phenomena are, therefore, more likely to be of true regulatory nature (23). Furthermore, our finding that sometimes large, multinucleated cells with weak and spotty keratin fluorescence were seen, speaks also in favor of regulatory events in these heterokaryons. Accordingly, only one or two epithelial cell nuclei could be seen in such heterokaryons, suggesting a gene dose effect (cf. ref. 23). Alternatively, these findings could be explained by the simple dilution effect of the keratin filaments in the cytoplasm of the heterokaryons. The first explanation seems more likely, however, as epithelial cell characteristics, such as desmosome formation, were actively expressed by the heterokaryons.

In conclusion, our results show that the cell type specific intermediate filaments are coexpressed and retain their original behavior in the heterokaryons of fibroblasts and epithelial cells. This indicates that fibroblastic and epithelial cell characteristics are coexpressed in these heterokaryons.

The skillful technical assistance of Ms. Hannele Laaksonen, Ms. Raili Taavela, Ms. Ritva Nurme-Helminen and Ms. Pipsa Kaipainen is acknowledged.

This study was supported by grants from the Medical Research Council of the Academy of Finland, The Sigrid Jusélius Foundation, The Finnish Cultural Foundation, and by the Research Contract of the Finnish Life Insurance Companies.

Received for publication 2 December 1981, and in revised form 2 March 1982.

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