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Coexpression of keratin- and vimentin-type intermediate filaments in human metastatic carcinoma cells

(metastasis/cytoskeleton/immunofluorescence)

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ABSTRACT **Metastatic tumor cells of epithelial origin present in effusions from human serous cavity fluids (ascites or pleural fluid) were examined for their intermediate-sized filament types by using antibodies to keratin, vimentin, and desmin in the indirect immunofluorescence technique. Solid epithelial tumors (both primary carcinomas and their metastases) contain keratin intermediate-sized filaments exclusively. However, when these cells are present in ascitic or pleural fluid, they also express vimentin, which occurs in a fibrillar organization. The possible effects of this additional, but temporary, cytoskeleton on metastatic growth or aggressiveness (or both) are discussed.**

Intermediate filaments (IFs) in eukaryotic cells constitute a considerable part of the cytoskeleton in addition to microfilaments and microtubules (1). They can be visualized in the electron microscope as 7- to 11-nm fibrils (2) and are characterized by specific biochemical and immunological properties (3–7). These properties also distinguish between different types of IFs, whose nature seems to correlate with the embryonic origin of the tissue (1, 6, 8–10). For example, epithelial cells are characterized by keratin IFs, whereas muscle cells contain mainly IFs of the desmin type. Another, fibroblastic, type of IF constituent is vimentin, a M_r 57,000 protein (6). In the adult animal this type of IF protein seems to be characteristic for cells of mesenchymal origin (11, 12) but also is demonstrated in many cells in culture (13); for example, this type of IF protein occurs in cultured epithelial cells in addition to their specific keratin IFs (14–17), suggesting that vimentin IFs are induced by *in vitro* growth (16, 17). The function of this additional cytoskeletal element in cells in monolayer or suspension cultures is unknown, although a role in mitosis (18, 19) or organelle anchorage (20), or both, has been suggested. However, it has been proposed that the appearance of vimentin in epithelial cells in culture is not a result of enhanced mitosis, motile functions, or neoplastic transformation but rather is correlated with physiological adaptation to the conditions of cell growth *in vitro*.

In this report we describe the coexistence of both vimentin and keratin IFs in metastatic human epithelial cells present in ascites and pleural fluids, and we discuss their possible effect on metastatic activity of tumor cells in general.

MATERIALS AND METHODS

Cell Preparations. Thirty-four samples of human ascites and 32 samples of human pleural fluids were used in the studies reported here. Some of the preparations used in these experiments had been stored at −40°C for up to 3 yr. In total, 120 preparations, including control preparations (sputum, etc.), were used for this study.

Antisera. The following antibody preparations were used in this study: (*i*) an antiserum directed against human foot callus keratin raised in rabbits as described (12, 21); (*ii*) a monoclonal antibody directed against PtK1 keratin (ref. 22; provided by E. B. Lane, Imperial Cancer Research Fund, London); and (*iii*) a rabbit antiserum directed against calf lens vimentin. Preparation and testing of this serum has been described (12). For specificity of its reactions, see also refs. 21, 23, and 24.

Next to the sera described above, preimmune sera and an antiserum directed against desmin were tested in parallel control experiments. The desmin antibodies were raised in rabbits and were directed against muscle desmin from chicken gizzard prepared by using modifications of the methods described by Geisler and Weber (25) and Franke *et al.* (26).

Indirect Immunofluorescence Technique. The single-antibody labeling technique was performed essentially as described (12), with the exception that washing buffers contained 0.25% Triton X-100. The double-antibody labeling technique was performed as follows. Conventionally prepared cell smears, fixed in cold methanol and acetone, were incubated with the first antibodies, with a subsequent washing step between the incubations. The antibodies used in this double-label experiment were: (*i*) a monoclonal antibody preparation from mouse directed against PtK1 keratin (LE 65; described in ref. 22) kindly provided by E. B. Lane, and (*ii*) the antiserum raised in rabbits and directed against calf lens vimentin.

After an incubation step of 30 min in a humidified box at room temperature, the slides were washed with phosphate-buffered saline containing 0.25% Triton X-100, in two subsequent washing steps of 10 min each, and thereafter were washed with phosphate-buffered saline alone. The cell smears then were incubated for another 30 min with the second antibodies, again with a subsequent washing step between the incubations. The labeled second antibodies (Nordic Immunology, Tilburg, The Netherlands) were: (*i*) fluorescein-conjugated goat anti-mouse IgGs (diluted 1:20) and (*ii*) rhodamine-conjugated goat anti-rabbit IgGs (diluted 1:25).

After a second series of washes the slides were mounted with 50% glycerol in phosphate-buffered saline (pH 7.4). In some cases, washing steps with phosphate-buffered salines containing either 100 mM $MgCl_2$ or 100 mM KCl were applied to minimize nonspecific binding. No significant decrease in keratin or vimentin staining was observed in these control experiments. When the vimentin antiserum was absorbed with a crude vimentin preparation from bovine lens, fibrillar staining was diminished. Absorption of the vimentin antiserum with a keratin preparation from human skin had no such effect. Cells were viewed with a Leitz Dialux EB 20 microscope equipped with epi-fluorescent illumination (HBO 100 W bulb), by using the

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Abbreviation: IF, intermediate filament.

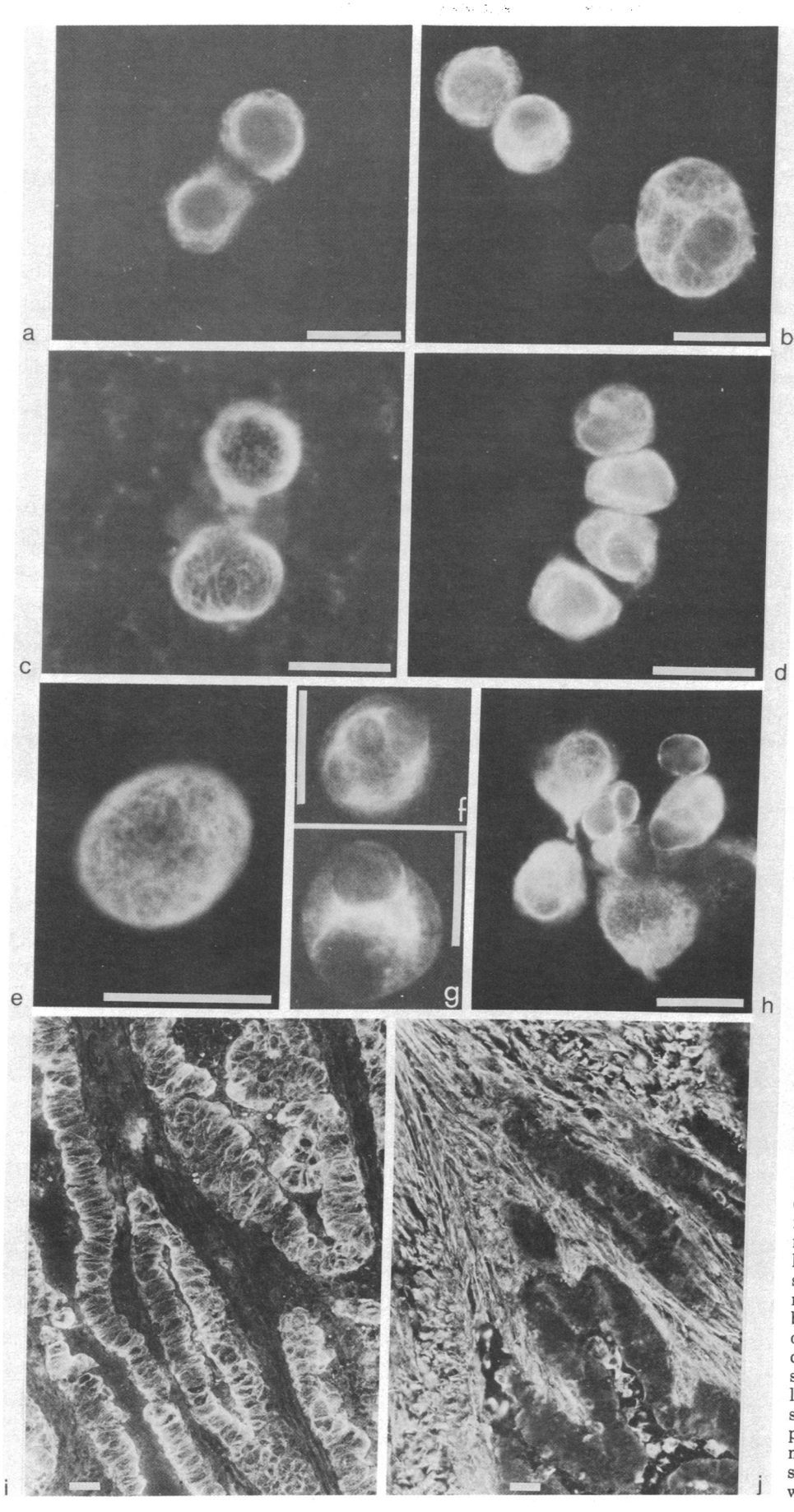

FIG. 1 Indirect immunofluorescence microscopy of human tumor cell preparations incubated with antibodies to the IF protein keratin or vimentin, as indicated. Cells from metastatic breast carcinoma obtained from pleural fluid, incubated with antikeratin (*a* and *c*) or antivimentin (*b*) as described elsewhere (12). Washing buffers contained 0.25% Triton X-100 to increase specificity. (*d*) Metastatic cells of a thyroid carcinoma in ascites positive for vimentin. Adenocarcinoma metastases in ascites incubated with keratin (*e–g*) or vimentin (*h*). Frozen sections from a colon adenocarcinoma metastatic to the omentum and incubated with the antibodies to keratin (*i*) or vimentin (*j*). Note the mutual exclusiveness of the reactions with these sera: keratin stained only the epithelial tumor cells, whereas vimentin stained only the stroma that accompanies the tumor cells. Neither preimmune serum nor desmin antibodies showed significantly strong reactions with the cells. (Bars = 20 μm.)

appropriate filter systems for fluorescein and rhodamine fluorescence.

Overlap between the two channels was checked by using cells labeled with a single antibody. Pictures were taken with a Leitz Fluotar ×40 objective by using an automatic camera (ASA setting of 400 or 800) and Kodak Tri-X film.

RESULTS

Serous cavity effusions (ascites and pleural fluids), received for cytologic examination, were evaluated by conventional smears. These cell preparations, as well as smears from sputum (containing bronchial epithelial cells) and from cervix (containing both squamous and glandular epithelial cells) and thin-needle aspirates from solid breast tumors, were fixed with ethanol and methanol and incubated with antibodies to keratin, vimentin, and desmin. All preparations described here were made from representative samples known to contain tumor cells in addition to mesothelial cells or blood cells (or both), as judged from routine cytology.

Fig. 1 shows the immunofluorescent appearance of a series of cells from these smears. A strong positive fibrillar reaction in the cells was seen only when smears were incubated with antibodies to keratin or vimentin. The preimmune serum used in this study, as well as a desmin-specific antiserum, gave no, or only very weak, staining. In Fig. 1 it can be seen that cells of similar morphology occurring in the same preparation, and diagnosed as being derived from adenocarcinoma with Papanicolaou staining, contain filamentous structures that were stained with antibodies to keratin or vimentin, or both. From previous experiments (12, 21) we know that both sera recognize different types of intermediate-sized filaments present either in epithelial or mesenchymal tissues from the human body. Fig. 1 *i* and *j* illustrate the mutual exclusiveness of the reactions of these sera on frozen sections from solid human adenocarcinoma.

Coexpression of keratin and vimentin IF in the same epithelial tumor cells present in serous cavity fluids was confirmed as follows.

(*i*) Epithelial tumor cells present in smears were stained with antibodies to keratin or vimentin. After screening and photographing of the fluorescent preparations, the cells were stained by cytologic techniques (Papanicolaou stain). Thereafter, cell groups known to contain keratin or vimentin were diagnosed cytologically. In this way it could be shown that carcinoma cells present in ascites and pleural fluids contain keratin as well as vimentin IFs (see Fig. 2 *a* and *b*). Also, mesothelial cells (in many cases slightly atypical ones) present in body fluids may contain vimentin IF next to keratin IF.

(*ii*) Cells present in smears were examined by double-immunofluorescence labeling by using vimentin antibodies raised in a rabbit and a monoclonal antibody preparation directed against keratin (22). Fig. 3 *a–f* show some typical examples of metastatic epithelial tumor cells stained by the double-label technique, illustrating the coexistence of keratin and vimentin IFs in malignant epithelial cells from ascites and pleural fluids. The specificity of antibody reactions could be seen clearly in these preparations. In all preparations vimentin-positive as well as vimentin-negative cells could be demonstrated. The same was true for keratin. In addition, cells positive with both sera were clearly visualized. It also was obvious that keratin and vimentin are distributed differently throughout the cytoplasm of the cell. Although this phenomenon was observed in many cells, several cells gave the impression that both IF systems colocalize.

(*iii*) In addition to our finding that in frozen sections keratin antibodies react only with epithelial tissues and the vimentin

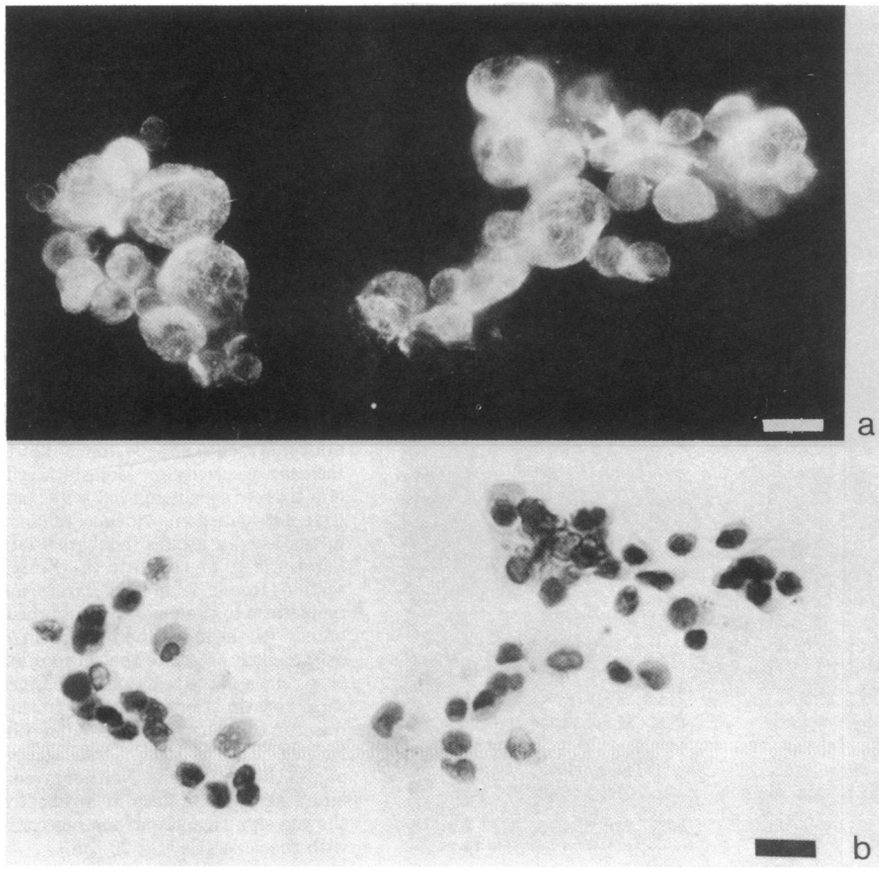

FIG. 2. Indirect immunofluorescence micrograph (*a*) and Papanicolaou staining (*b*) of the same group of papillary adenocarcinoma cells present in an effusion from human ascites. The cells were incubated with antibodies to keratin or vimentin for the indirect immunofluorescence technique, photographed, and thereafter stained (Papanicolaou stain). Keratin-positive epithelial cells were identified as tumor cells after histological staining. Similarly, vimentin-positive cells (*a*) also were identified as epithelial tumor cells after Papanicolaou staining (*b*). Parallel control experiments were performed as described in the legend to Fig. 1. (Bars = 20 μm.)

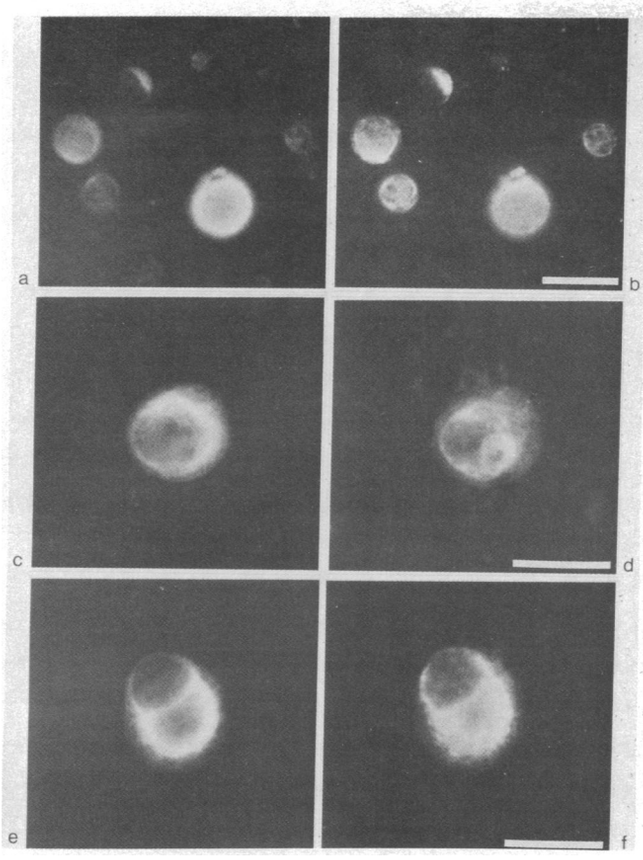

FIG. 3. Double-label immunofluorographs showing the simultaneous presence of keratin (*a*, *c*, and *e*) and vimentin (*b*, *d*, and *f*) in malignant mesothelioma cells in ascitic fluid (*a* and *b*) and metastatic adenocarcinoma cells, also from ascites (*c–f*). (Bars = 20 μm.)

antibodies, only with mesenchymal cells (see Fig. 1 *i* and *j*), we have further tested and confirmed the specificity of the vimentin antiserum reactions in several other ways. To minimize nonspecific binding of the vimentin antibodies to epithelial tumor cells, smears were washed thoroughly after the incubation steps with buffers containing either non-ionic detergent or increased salt concentrations. Vimentin staining persisted despite these treatments.

Furthermore, epithelial cells obtained either by thin-needle aspiration from solid breast tumors (4 samples) or by scraping of uterine cervix (2 samples) or cells from sputum (28 samples) did not show any reaction with the vimentin antiserum, but they gave a strong reaction with the rabbit antiserum raised against human skin keratin. Metastatic carcinoma cells obtained from an individual with acute pleural fluid development were negative for vimentin but were positive for keratin. In this case the ascites containing the metastatic cells existed for only a few days at the time effusions were taken.

Specificity of the keratin staining was shown by screening metastatic cells of mesenchymal origin (for example, in two cases of acute leukemia, one case of melanoma, two cases of lymphoma, two cases of unidentified sarcoma, and normal blood cells). All of these cells gave a positive reaction with antibodies to vimentin only. Finally, when the vimentin antiserum was absorbed with a crude vimentin preparation from calf lens prior to incubation, weak, or no, staining was seen in cells known to react strongly positive for vimentin in parallel experiments. When the antivimentin serum was absorbed with a keratin preparation (from human skin) staining was not affected.

DISCUSSION

The data presented here provide evidence for coexpression of keratin and vimentin in metastatic epithelial tumor cells present in human body fluids. However, we must stress that the two IF systems were not observed simultaneously in the same cell in all cases tested. The case of acute pleural fluid development indicates strongly that epithelial tumor cells obtain vimentin IFs only after they have been shed into body cavities and that it apparently takes some time to develop the vimentin cytoskeleton. Furthermore, no vimentin could be shown in solid primary or metastatic epithelial tumors (12, 21, 23, 27). This indicates that expression of the additional vimentin IF system is not a result of malignant transformation. Preliminary investigation with cultured human epithelial tumor cells that form solid tumors upon inoculation into nude mice (23) suggests that vimentin expression stops as soon as the cells grow in a solid, three-dimensional tissue structure. Therefore, the vimentin cytoskeleton most likely occurs in epithelial cells only during the process of metastasis—i.e., when cells are freely circulating in cavity fluids—a condition that may very well resemble *in vitro* suspension culture conditions.

When carcinomatous tumor cells invade solid tissue, resettle, and form distant solid tumors, the only IF proteins expressed seem to be the keratins. Our material does not suggest that cells containing both keratin and vimentin originate from distinct subpopulations of solid tumor cells, as suggested for proliferating cells in culture by Virtanen *et al.* (13).

Although the integrity of the vimentin cytoskeleton seems not to be a prerequisite for mitosis or motility (28), the presence or absence of an additional vimentin IF cytoskeleton may still influence mitotic as well as motile activity of cells and may therefore well have an effect on growth rate or aggressive behavior (or both) of a metastatic tumor. Therefore, expression of vimentin in nonmesenchymal cells *in vitro* (16) should not be considered merely as a culturing artifact but as a process that also may occur *in situ* when cells lose contact with their neighbors or are removed from solid tissue in the course of metastasis or other pathological events. A thorough study of this phenomenon *in vitro* and *in vivo* may provide valuable information on a possible role of vimentin during the process of tumor metastasis and spreading.

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