Coexpression of Simple Epithelial Keratins and Vimentin by Human Mesothelium and Mesothelioma in Vivo and in Culture

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ABSTRACT

We have determined the intermediate filament proteins present in normal and malignant mesothelium in vivo. Pure sheets of normal lung mesothelium were obtained by transfer to agar-coated slides or by gentle scraping and cyto-centrifugation. Cytoplasmic filament networks in the mesothelium were labeled via indirect immunofluorescence both by anti-M, 40,000 keratin and anti-vimentin antisera. Two-dimensional gel electrophoresis of the Triton:high-salt-insoluble proteins of normal lung mesothelium disclosed the presence of vimentin and all but the largest (M, 55,000) of the four simple epithelial keratins synthesized by mesothelial cells in culture. Samples of three peritoneal and three pleural mesotheliomas were found to contain either all four simple epithelial keratins or all but the M, 55,000 keratin. Notably, none of the keratins characteristic of stratified and many glandular epithelia and their malignant forms were present in these mesotheliomas. Two mesothelioma samples from which the tumor cells could be obtained free of other cell types were found to contain vimentin as well as simple epithelial keratins and to synthesize these same proteins during short-term culture. None of the mesotheliomas placed in culture grew progressively in medium optimal for the growth of normal mesothelial cells. These data demonstrate that malignant mesothelial cells preserve the normal pattern of intermediate filament protein synthesis, including coexpression of simple epithelial keratins and vimentin, and suggest the use of this characteristic as an aid in the identification of cells of mesothelial origin.

INTRODUCTION

The mesothelium is the simple squamous epithelium that lines the pleural, pericardial, and peritoneal cavities and covers the outer surfaces of the organs contained therein (6, 7). Unlike most other epithelia, the mesothelium is of embryonic mesodermal origin. Perhaps because of this the malignant form, mesothelioma, can adopt either sarcomatous or carcinomatous forms, often making differential diagnosis difficult (9, 18, 37).

This laboratory has recently determined the cell type-specific requirements of normal, diploid human mesothelial cells for rapid growth in long-term, serial culture (4). Mesothelial cells are different from most other normal epithelial cell types in that they express in culture high levels of 2 different types of intermediate filament proteins: keratins and vimentin (42, 44). Because normal mesothelial cells that detach from the mesothelium and float in ascites fluid also contain both vimentin and keratins (4), we wanted to determine whether vimentin is present in the cells of the intact mesothelium also, or whether it is only acquired after cell detachment or cultivation, as has been suggested by others (12, 21, 32, 39). The set of keratin proteins synthesized by normal human peritoneal mesothelial cells in culture is different from that of stratified squamous epithelial and many glandular epithelial cell types (22, 27, 38, 43). We wished to determine whether mesotheliomas express any keratins different from those of normal mesothelium, inasmuch as squamous cell carcinomas often synthesize keratins inappropriate for their cell type of origin (23, 43, 44). We report here that normal mesothelial cells in the intact tissue coexpress keratins and vimentin, that the largest keratin M, 55,000 appears to be induced by conditions of culture, and that mesotheliomas express vimentin and the same set of keratins as does normal mesothelium.

MATERIALS AND METHODS

Preparation of Mesothelial Tissues. Sheets of normal lung mesothelium were obtained free of underlying connective tissue by a modification of the imprining method (42). A molten solution of 1% agarose (Sea Kem) in water was applied to glass slides and dried overnight. The surface of freshly excised human fetal lung was rinsed in isotonic PBS5 and exposed to the air. Just as the mesothelium began to appear dry, an agarose-coated slide was pressed against the lung for several seconds. When the slide was pulled away, parts of the mesothelial cell layer adhered to the agar and were peeled off. Slides were examined under an inverted microscope with phase contrast optics to identify areas containing large sheets of mesothelium. These were rinsed with PBS to remove any blood cells. Slides were then either briefly air-dried and immersed in cold (−20°C) methanol to fix for immunofluorescence microscopy, or the agarose and cell material were scraped into Triton:high-salt extraction buffer and stored frozen for future electrophoretic analysis. Tissues to be used as electrophoresis samples were collected on slides that had been coated with electrophoretically purified agarose in order to reduce the levels of contaminants which would be stained in the subsequent silver staining procedure. As an alternate method for obtaining small sheets and clusters of mesothelial cells for immunofluorescence, the lung surface was gently scraped with a rubber policeman into PBS. The cell suspension was then diluted 1:1 with serum and applied to a glass slide by spinning in a cytocentrifuge. The slides were then air dried and fixed in cold methanol. For these experiments, lungs were obtained from 18 to 22-week-old human fetuses or from adult rats. Surgical specimens of solid peritoneal and pleural mesotheliomas (kindly provided by Dr. J. M. Corson, Brigham and Women’s Hospital) were collected within 1 h after resection and minced into 1 mm pieces. Some fragments from each tumor were placed in culture, and some were frozen in Triton:high-salt buffer until extracted for electrophoresis. Mesothelioma cells were also obtained from a pleural effusion of mesothelioma (MS-7). This effusion was found by cytopathological examination to consist of >90% mesothelioma cells. The cells were concentrated by

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The abbreviation used is: PBS, phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 16 mM Na2HPO4, 2 mM KH2PO4, pH 7.3).
of pure tumor cells were placed into culture in the growth medium described above.

**Keratin Nomenclature.** Keratins 40Kw, 44Kw, 52Kw and 55Kw, the "mesothelial keratins" described in Wu et al. (43), are keratins 19, 18, 8, and 7, respectively, independently identified as "simple epithelial keratins" by Moll et al. (22).

### RESULTS

**M. 40,000 Keratin Immunofluorescence of Mesothelia.** Normal mesothelium was transferred from the outer surface of a fetal human lung to an agarose-coated slide by an imprinting technique described by Whitaker et al. (42). Cell shape, nuclear position, and intermediate filament content was examined by phase-contrast microscopy (Fig. 1a) and indirect immunofluorescence with anti-40Kw keratin. Favorable areas of the transferred mesothelium were found to contain this keratin in a fibrous network (Fig. 1b) similar in appearance to that of cultured epithelial cells. Heterogeneity of staining presumably resulted from variability in the amount of cytoskeleton transferred to the slide. In these preparations, the apical portion of the plasma membrane adheres to the agarose, and the cells apparently fragment such that variably abundant contents of the cytoplasmic matrix are removed to the slide, even though the nucleus (or its outline) can be seen in each transferred cell (Fig. 1a).

Small clusters to large sheets of mesothelial cells could also be dislodged by scraping the lung surface with a rubber policeman and depositing the PBS:serum suspension of these cells on a slide with the aid of a cytocentrifuge (Fig. 1c). Clusters of easily identified mesothelial cells stained positively for the 40Kw keratin and also revealed details of the cytoplasmic filament network (Fig. 1d). This was consistent with the results of our preliminary examination of paraffin sections of mesothelium by immunoperoxidase staining (44). The mesothelium was also stained strongly by anti-total stratum cornue keratin, an antiseraum which cross-reacts with most of the keratins (43, 44) (data not shown), as had been reported previously (5, 36).

The M, 40,000 keratin was also detected by immunofluorescence in secondary cultures of mesotheliomas MS-7 (Fig. 1h) and MS-10 (data not shown). The mesothelioma cells had a morphology in culture (Fig. 1g) distinct from that of normal mesothelial cells (Fig. 1e). MS-7 and MS-10 could not grow progressively in the culture medium permissive for long-term growth of normal pleural and peritoneal mesothelial cells and ceased dividing in secondary culture. We initiated 5 other cultures of solid pleural and peritoneal mesotheliomas, but the only cells that grew had morphologies and growth requirements of either normal mesothelial cells or of normal connective tissue fibroblasts and were not tumorigenic in nude mice. Apparently the mesothelioma cells present in those 5 tumor specimens could not grow at all under the conditions we used.

**Detection of Vimentin in Normal Mesothelial Cells by Immunofluorescence.** This laboratory previously had reported that normal mesothelial cells present among the cells in an ascites fluid specimen, which had been allowed to attach to a coverslip, contained both keratin and vimentin (4). We wished to confirm this coexpression in ascites fluid mesothelial cells that never before has been exposed, even briefly, to culture conditions, and also to determine whether the cells of intact mesothelium contain vimentin. We applied human ascites fluid cells and intact rat lung mesothelium (dislodged by scraping with a rubber policeman) to

**Cell Culture Methods.** A population of normal, diploid, human pleural mesothelial cells (strain HPM-2) was cultured from pleural effusion fluid withdrawn from an adult female. The normal, diploid, human peritoneal mesothelial strain LP-9 has been described previously (43). LP-9 and HPM-2 were grown as described (4), in either M199 or M199/MCD202:15% fetal calf serum (Sterile Systems);hydrocortisone (0.4 µg/ml; Calbiochem):epidermal growth factor (10 ng/ml; Collaborative Research). In order to maximize keratin synthesis (Fig. 3, b to d), cells were grown to confluence on one passage in the absence of epidermal growth factor.

Cells from a pleural effusion mesothelioma (MS-7) and from a solid peritoneal mesothelioma (MS-10) which could be dislodged as clusters

**Cell Labeling, Extraction, and Electrophoresis.** Methods were similar to those previously described (43, 44). Proteins synthesized by cultured cells were labeled by a 4-hr incubation with [35S]methionine (50 µCi/ml) (specific activity, ~800 Ci/mmol, NEN Chemicals) in otherwise methionine-free minimal Eagle's medium (Flow Laboratories) supplemented with 20% dialyzed fetal calf serum. Labeled cultured cells, agarose slide-collected mesothelium, and minced tumor tissue were rinsed with PBS. Dounce homogenized in Triton:high-salt extraction buffer (20 mM Tris-HCl, pH 7.4; 0.6 M KCl; 1% Triton X-100; 1 mM phenylmethylsulfonylfluoride) at 0°C, and then sonicated for 45 sec. Lysates were then centrifuged at 10,000 x g for 45 min at 4°C. The insoluble pellets (containing the keratins and vimentin) were then dissolved in O'Farrell 2-dimensional lysis buffer. The proteins were separated in the first dimension by isoelectric focusing (29) and in the second dimension by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) (20). High-purity agarose (Sea Kem) was used to hold the isoelectric focusing tube gel in place at the top of the sodium dodecyl sulfate-polyacrylamide slab gel. The possible presence of basic (isoelectric points 7 to 8) keratins was examined by separating the proteins by nonequilibrium pH gradient electrophoresis for 3.5 hr in the first dimension, according to the method of O'Farrell et al. (30). Labeled proteins were detected by scintillation fluid-enhanced (EnHance; NEN Chemicals) autoradiography.

**Protein Detection in Gels by Silver Staining.** An ultrasensitive silver method (25) was used with the following modifications. After fixation in 10% glutaraldehyde, the gel was rinsed for at least 48 hr to more completely remove glutaraldehyde and consequently reduce the background staining. Ethanolamine (5 µl/ml) was added to the citric acid fixative to prevent the formaldehyde from overdeveloping the silver (10). Wet gels were then photographed over a light box with Kodak Technical Pan film (ASA 50), which was developed in a 1:36 dilution of HC-110 for 8 min. Gels were placed in a solution of 0.02% sodium carbonate and stored in sealed plastic bag for future examination.

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slides by cytocentrifugation and double labeled them with anti-vimentin and anti-keratin antisera. As shown in Fig. 2, a to c, all mesothelial cells in rat lung mesothelioma coexpressed vimentin and keratin. Consistent with the previous report from this laboratory (4), some of the ascites fluid cells coexpressed vimentin and keratin (Fig. 2, d to f). Vimentin-positive, keratin-negative cells in the ascites fluid were assumed to be macrophages (3, 11); these cells attached and spread under culture conditions but did not divide (4). The keratin-positive, vimentin-negative cells were presumed to be neoplastic cells of ovarian surface epithelial origin because cells in the sample which stained with the ovarian surface-specific monoclonal antibody OC125 (1) were vimentin negative (Fig. 2, g to i) and keratin positive (data not shown). Thus, keratin and vimentin coexpression is a normal characteristic of mesothelial cells and is retained by cells that become detached from the mesothelium.

Electrophoretic Analyses of Mesothelial and Mesothelioma Cytoskeletal Proteins. The Triton-X-100-soluble protein fraction extracted from normal and malignant mesothelial samples were analyzed by 2-dimensional gel electrophoresis to determine precisely the intermediate filament proteins present (Table 1). Fetal human pleural mesothelioma collected by stripping with an agar-coated slide, precluding contamination with underlying connective tissue, contained vimentin, keratins 40Kw and 44Kw, and, in greatest abundance, keratin 52Kw (Fig. 3a). Keratin 55Kw, synthesized by adult peritoneal mesothelial cells in culture (4, 43) (see Fig. 3, c and d) was not detectable. Normal pleural mesothelial cells (HPM-2) synthesized vimentin and all 4 simple epithelial keratins in culture, negating the possibility that cells of pleural origin are unable to synthesize keratin 55Kw. A rapidly turning over, basic M, 46,000 keratin which is expressed at variable levels by mesothelial cells growing in culture (4, 43) (see Fig. 3d), also was not detected in the in vivo mesothelium.

A relatively pure cell suspension of pleural mesothelioma (MS-7) and clusters of cells teased from a solid peritoneal mesothelioma (MS-10) were extracted directly and their intermediate filament proteins compared with those synthesized by cells from these same tumors after 1 to 2 weeks in culture. MS-7 contained vimentin and keratins 40Kw, 44Kw, and 52Kw in vivo and also synthesized these same proteins in secondary culture (Fig. 3, e and g). MS-10 contained vimentin, keratins 40Kw and 44Kw, a small amount of keratin 52Kw, and what appeared to be proteolytic digestion products of 52Kw (Fig. 3f). Cultured MS-10 cells synthesized vimentin and all 4 simple epithelial keratins (Fig. 3h). Thus, malignant mesothelial cells may also increase their expression of 55Kw in culture.

Samples of 3 other solid peritoneal mesotheliomas and 4 other pleural mesotheliomas were extracted and analyzed by 2-dimen- sional gel electrophoresis. Three samples gave no interpretable spots due to failures of isoelectric focusing or to paucity of mesothelioma cells in a predominantly fibrous tumor. Four samples could be scored (Fig. 4). Keratins 40Kw, 44Kw, and 52Kw were present in all samples, with keratin 55Kw present in all but one sample (Fig. 4b). One tumor (Fig. 4d) also contained a protein migrating in the position of keratin 46Kw. Vimentin was detected in 3 of the samples, but since these contained connective tissue, the vimentin content of the tumor cells themselves was indeterminate.

Proteolysis of vimentin (14, 28) and of keratin 52Kw (35) was extensive in some tumor extracts (Fig. 3f, Fig. 4, a, b, and d), precluding quantitative estimation of these proteins when it occurred. Evaluation of gels made from extracts of small tissue samples was also complicated because the silver staining method used to detect very low levels of protein also stained minor impurities in the buffers and in the agarose used to immobilize the tube gel during the second dimension of electrophoresis. These impurities were most common in the M, 56,000 to 70,000 range (eg., cf. Fig. 3c and d, and see Fig. 3a and Fig. 4b, c, and d). Despite the staining background, no proteins migrating at the position of any known keratin (22, 43) other than the simple epithelial keratins were identified in any of the mesothelioma extracts.

DISCUSSION

We previously had identified vimentin in the normal mesothelial cells present in ascites fluid and rising to higher levels in these cells during serial culture (4). Nevertheless, it was necessary to
examine mesothelial cells in situ, since it has been proposed that vimentin may become exposed as a consequence of loss of cell-cell contact after detachment from an epithelial sheet (21, 32). It is also known that some epithelial cell types (12, 39) but not others (4, 34) abnormally synthesize vimentin under certain conditions of culture.

We have found by indirect immunofluorescence microscopy of scraped mesothelium and by 2-dimensional gel electrophoresis analysis of pure, agar slide-transfer preparations of mesothelium that this tissue contains both keratins and vimentin in vivo. Previous reports of an absence of detectable vimentin in cross-sections of chick (36) and mammalian (33) mesothelium by immunofluorescence microscopy may have been due to the relatively low vimentin content in mesothelial cells compared to the high levels present in fibroblasts and endothelial cells and because the mesothelium is very thin and easily damaged during handling and fixation. Mesothelial cells may express keratins and vimentin because they are derived from the embryonic mesoderm (this subject is reviewed in Refs. 9, 18, and 37), inasmuch as most other cell types of mesodermal origin, including mesenchyme-derived cells and cells of some hematopoietic lineages, express vimentin (2, 8, 12, 13, 15, 31, 36).

The types of keratins in normal mesothelial tissue are restricted to the simple epithelial keratins, as we had predicted from the previous results of this laboratory on peritoneal mesothelial cells in culture (4, 43). Samples of human pleural mesothelium contained the 40 K, 44 K, and 52 K simple epithelial keratins, but very little or no keratin 55 K. This lack of keratin 55 K was not simply a regional difference between pleural and peritoneal mesothelial cells, however, because pleural mesothelial cells expressed the keratin 55 K in culture. We do not know the mechanism of this specific regulation of keratin 55 K but it could be the result of very rapid cell division or the abnormal culture substratum. We had found previously that synthesis and content of all 4 keratins are controlled coordinately in mesothelial cells in culture as a function of growth rate (4, 34).

Solid and effusion mesotheliomas and 2 short-term cultured mesothelioma populations contained 3 or 4 of the simple epithelial keratins and vimentin. Six of 9 surgical samples were successfully analyzed by 2-dimensional gel electrophoretic separation and silver staining. Qualitative determination of the intermediate filament proteins was possible by this method, but quantitative estimation of the M, 52,000 simple epithelial keratin and vimentin was often prevented by substantial amounts of proteolysis. These 2 intermediate filament proteins are particularly susceptible to digestion by an endogenous protease in some tissue types and cell lines (14, 28, 35). Furthermore, the presence of vimentin in the mesothelioma cells of most samples could not be confirmed by electrophoretic analysis because of the presence of connective tissue, including vimentin-containing fibroblasts and endothelial cells. Immunofluorescence or immunoperoxidase staining with antivimentin will undoubtedly be a more generally useful method for screening tumors for vimentin expression.

We tried to culture mesothelioma cells in order to compare their growth requirements with those of normal mesothelial cells and to identify selective markers for malignant transformation of this cell type. Only 2 of 7 tumours placed in culture yielded a viable population of cells other than normal fibroblasts and mesothelial cells, and these did not grow progressively in a medium permissive for rapid, long-term growth of normal mesothelial cells. This suggests that mesothelioma cells have significantly altered growth requirements which are difficult to satisfy in culture. We suspect that this is the reason that we have been unable to find any published reports of mesothelioma cell lines. Normal mesothelial cells are anchorage independent, forming large colonies in semisolid medium. Thus, there is no basis at present for culturing mesothelioma cells or selectively identifying them.

In addition to the coexpression of keratins and vimentin in mesothelioma, the specific subset of keratins expressed by mesotheliomas could be used to aid in the differential diagnosis of tumors in the pleural and peritoneal cavities. Tumors of stratified epithelial and mammary epithelial origin contain 2 or more keratins from a set of more basic (M, 58,000 and 56,000) and more acidic (M, 52,000, 50,000, and 46,000) keratins (keratins 5 and 6 and keratins 13, 14, and 17, respectively) (22–24, 26, 44). Carcinomas of the colon, stomach, and ovary would not be distinguishable from mesothelioma by the keratin pattern alone because these tumors also are restricted to simple epithelial keratins (22–24). Nevertheless, mesotheliomas are carcinoma-byoncogenic antigen negative or very weakly positive (5, 19, 40, 41), while adenocarcinomas of the colon are strongly carciinomogenic antigen-positive (17). Ovarian epithelial neoplasms can be recognized by a specific antibody against a surface antigen (1). Our results indicate that vimentin is likely to be present in mesotheliomas, in contrast to its absence in tumors of all other epithelial cell types (31). Vimentin has also been found recently in several renal carcinomas (18) which originate from another mesoderm-derived epithelial cell type. The data we have presented here suggest that coexpression of simple epithelial keratins and vimentin is a useful marker for distinguishing normal and malignant cells of mesothelial origin from those of most other cell types.

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Intermediate Filament Proteins of Mesothelium and Mesothelioma


Fig. 1. Morphology and M. 40,000 keratin immunofluorescence of human mesothelium in vivo and of mesotheloma cells in culture. a, fetal lung mesothelium transferred to an agarose-coated slide; b, immunofluorescence of same field as a; c, scraped and cytocentrifuged fetal lung mesothelium; d, immunofluorescence of same field as c; e, normal peritoneal mesothelial strain LP-9 in secondary culture; f, immunofluorescence of a different LP-9 culture; g, pleural mesotheloma strain MS-7 in secondary culture; h, immunofluorescence of a different culture of MS-7. a, c, e, and g are phase contrast; b, d, f, and h are anti-M, 40,000 keratin immunofluorescence. Bar in a is 50 μm and is the same magnification for b; bar in c is 50 μm and is the same magnification for d; bar in e is 200 μm and is the same magnification for g; bar in f is 50 μm and is the same magnification for h.
Fig. 2. Vimentin and keratin immunofluorescence of intact lung mesothelium and of mesothelial cells in ascites fluid. a to c, intact mesothelium scraped from rat lung. Double labeling with guinea pig antivimentin and fluorescein (b) and with rabbit anti-M, 40,000 keratin and rhodamine (c). Small, refractile cells in a are erythrocytes. d to f, human ascites fluid cells from patient with ovarian epithelial neoplasia, double labeled with guinea pig antivimentin and fluorescein (e) and with rabbit anti-stratum corneum keratins and rhodamine (f). g to i, human ascites fluid cells from the same patient, double labeled with rabbit antivimentin and rhodamine (h) and with mouse monoclonal anti-ovarian carcinoma cell surface and fluorescein (i). Bar in a is 50 μm and is the same magnification for b to i. In d, arrows indicate mesothelial cells. In d and g, arrowheads indicate neoplastic ovarian epithelial cells. Vimentin-positive, keratin-negative cells in d to f are presumably macrophages.
Fig. 4. Two-dimensional gel electrophoretic separation of mesothelioma cytoskeletal proteins. Triton-high-salt-insoluble proteins of solid tumor samples separated horizontally by charge (left, basic; right, acidic) and vertically by sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis. Gels a and c were focused isoelectrically between pH 6.5 and 5.0. Gels b and d were separated by nonequilibrium pH gradient gel electrophoresis to permit detection of more basic proteins. a, peritoneal mesothelioma MS-4; b, pleural mesothelioma MS-2; c, peritoneal mesothelioma MS-5; d, pleural mesothelioma MS-11. All gels are silver stained. Lower case letters and arrows within each frame, position of keratins, vimentin, and actin, as in the legend of Fig. 3; arrowheads, proteolytic breakdown products of vimentin and the 52K protei. * in frame a, prominent species of size and charge different from that of any known keratin. v, vimentin.

Fig. 3. Two-dimensional gel electrophoretic separation of the cytoskeletal proteins of normal human mesothelium and mesotheliomas. Triton-high-salt-insoluble protein fractions of tissues, tumors, and cultured cells separated horizontally by their charge (left, basic; right, acidic) and vertically by sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis. Gels a, c, e, and g were isoelectrically focused between pH 6.5 and 5.0 in the horizontal dimension. Gels b, d, f, and h were separated by nonequilibrium pH gradient gel electrophoresis in the horizontal dimension in order to permit detection of more basic proteins. a, human fetal lung mesothelium collected by slide transfer (spots marked with * are agarose contaminants detected by silver staining); b, cultured adult lung mesothelium strain HPM-2; c and d, cultured adult peritoneal mesothelial cell strain LP-9; e, pleural effusion mesothelioma MS-7; f, peritoneal mesothelioma MS-10; g, secondary culture of MS-7; h, primary culture of MS-10. Proteins in a, d, and e were revealed by silver staining, in frame f by Coomassie blue staining, and in b, c, g, and h by autoradiography of [35S]methionine-proteins. Lower case letters within each frame, position of keratins and vimentin. a, 40K[19]; b, 44K[18]; c, 52K[8]; d, 55K[7]; e, 46K. Arrows, actin. c' is presumed to be a proteolytic digestion product of keratin 52K[8]. v, vimentin.