

Intermediate Filament Cytoskeleton of Amnion Epithelium and Cultured Amnion Epithelial Cells: Expression of Epidermal Cytokeratins in Cells of a Simple Epithelium

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ABSTRACT Using immunofluorescence microscopy and two-dimensional gel electrophoresis, we compared the cytoskeletal proteins expressed by human amnion epithelium in situ, obtained from pregnancies of from 10-wk to birth, with the corresponding proteins from cultured amnion epithelial cells and cultures of cells from the amniotic fluid of 16 week pregnancies. Epithelia of week 16 fetuses already display tissue-specific patterns of cytokeratin polypeptides which are similar, although not identical, to those of the corresponding adult tissues. In the case of the simple amnion epithelium, a complex and characteristic complement of cytokeratin polypeptides of M_r 58,000 (No. 5), 56,000 (No. 6), 54,000 (No. 7), 52,500 (No. 8), 50,000 (No. 14), 46,000 (No. 17), 45,000 (No. 18), and 40,000 (No. 19) is present by week 10 of pregnancy and is essentially maintained until birth, with the addition of cytokeratin No. 4 (M_r 59,000) and the disappearance of No. 7 (M_r 54,000) at week 16 of pregnancy. In full-term placentae, the amnion epithelium displays two morphologically distinct regions, i.e., a simple and a stratified epithelium, both of which express the typical amnion cytokeratin polypeptides. However, in addition the stratified epithelium also synthesizes large amounts of special epidermal cytokeratins such as No. 1 (M_r 68,000), 10 (M_r 56,500), and 11 (M_r 56,000). In culture amnion epithelial cells obtained from either 16-wk pregnancies or full-term placentae will continue to synthesize the amnion-typical cytokeratin pattern, except for a loss of detection of component No. 4. This pattern is considerably different from the cytokeratins synthesized by cultures of cells from amniotic fluids (cytokeratins No. 7, 8, 18, and 19, sometimes with trace amounts of No. 17) and from several so-called "amnion epithelial cell lines." In addition, amnion epithelial cells in situ as well as amnion epithelial cell cultures appear to be heterogeneous in that they possess some cells that co-express cytokeratins and vimentin.

These observations lead to several important conclusions: (a) In contrast to the general concept of recent literature, positively charged cytokeratins of the group No. 4–6 can be synthesized in a simple, i.e., one-layered epithelium. (b) The change from simple to stratified amnion epithelium does not require a cessation of synthesis of cytokeratins of the simple epithelium type, but in this case keratins characteristic of the terminally differentiated epidermis (No. 1, 10, and 11) are also synthesized. (c) Our findings in amnion epithelium together with recent demonstrations of cytokeratins of the group No. 4–6 in various other nonstratified epithelia, suggest that the expression of such cytokeratins is not restricted to the differentiated stratified state but may be characteristic for epithelial cell types that have the potential to produce metaplasias and other forms of stratified epithelium. (d) As cultures of amnion epithelial cells continue to express their characteristic cytokeratin polypeptides, we conclude that cell cultures from amniotic fluid, which synthesize a different, more simple set of

cytokeratins, are not derived from amnion epithelium but probably from simple epithelial cells of the fetus. Implications of these findings for interpretations of epithelial differentiation and for prenatal diagnosis are also discussed.

True epithelial cells *in vivo* and *in vitro* possess intermediate-sized filaments (IF)¹ of the cytokeratin type (17–19, 28, 51, 67, 68). However, different kinds of epithelial cells express different combinations of members of this polypeptide family, resulting in cell type-specific cytokeratin patterns (15, 24–26, 28, 51, 70, 78, 80). Most cytokeratin polypeptides have been shown to be the products of different mRNAs apparently coded for by different genes (35, 44, 45, 50, 62). In general, stratified epithelia present more complex cytokeratin polypeptide patterns than one-layered (“simple”) epithelia (1, 24–26, 28, 53, 70, 80) and usually contain a special group of cytokeratin polypeptides that in 9.5 M urea solutions are isoelectric at pH values of 7 and above (“basic cytokeratins”; No. 1–6 of the human cytokeratin catalog of reference 51; for M_r and isoelectric pH values, see Table I). Cytokeratins of this group have also been found in certain complex epithelia, such as tracheal and bronchial linings, skin glands, and mammary gland ducts (7, 51). Sun and colleagues (16, 54, 69, 77) have recently proposed that expression of such “basic” cytokeratins, notably human cytokeratin No. 5 of M_r 58,000, is exclusive to—and characteristic of—stratified epithelia and the corresponding squamous cell carcinomas. This is in contrast to our previous data that amnion epithelium, which is of the simple epithelium type, contains the basic cytokeratins No. 5 and 6 (51). A further source of apparent conflict with our previous data are reports that keratins No. 5 and 6 are not present in various cultured amniotic epithelial cells (8, 9) and in cell cultures from human amniotic fluids (55). To examine these apparent discrepancies and because of the importance of the identification of possible contributions of amniotic epithelium cells to cell cultures from amniotic fluids used in prenatal diagnosis (cf. 61; also, see references 55, 75), we examined in detail the IF cytoskeletons of amniotic epithelium *in situ* and cultured amniotic cells. We show that amnion epithelium cells of various stages of fetal development, including both single-layered and stratified regions, express polypeptides of the basic cytokeratin group No. 1–6 and that expression of cytokeratins of this group is maintained during culture. In addition, we show that amnion epithelium cells *in situ* and in culture are heterogeneous, in that some cells express vimentin in addition to cytokeratins, whereas other cells do not. These findings have important implications for our understanding of the differentiation of epithelial cells as well as for prenatal diagnosis via cells derived from human amniotic fluids.

MATERIALS AND METHODS

Tissue Samples: Amnion epithelia were obtained from placentae of full-term pregnancies within 2 h of birth of normal babies. Amnion tissue of 10-wk pregnancies was obtained during operation in cases in which hysterectomy was indicated due to a diagnosis of myomas. The “amniotic membrane” was stripped off with tweezers from regions near the umbilical cord and any blood present was carefully rinsed off with phosphate-buffered saline (PBS). Fetal tissues from 16-wk pregnancies were obtained from legal abortions, including two cases of spontaneous rupture of the amniotic sac. Tissues were

cut into small pieces (<1 cm³) and frozen in isopentane cooled by nitrogen at –150°C and stored at –70°C.

Cultured Cells: Amniotic fluid cells were obtained by medically indicated transabdominal amniocentesis during week 16 of pregnancy. 1–5 ml of amniotic fluid were centrifuged at 500 g for 10 min. After careful removal of the supernatant, cells were resuspended in culture medium (Ham F 10), transferred to cell culture dishes, and grown in monolayer culture. Between days 17–21 of culturing, cells were harvested for preparations of cytoskeletons. For labeling cytoskeletal proteins with [³⁵S]methionine, cell monolayers were washed twice in methionine-free minimum essential medium and incubated for 12–15 h in the same medium containing 0.2% of the normal methionine concentration and 100 μCi/ml [³⁵S]methionine (cf. reference 25).

Amnion epithelial cells were obtained essentially as described by Valle and Penttinen (71) and Dell’Aquila and Gaffney (14) and cultured in monolayers in RPMI 1640 medium, supplemented with 10% fetal calf serum. The medium was later replaced by Ham F12 medium containing 10% fetal calf serum. Some cells of full-term placentae were grown on coverslips for 72 h and processed for immunofluorescence microscopy. Cells were cultured for various periods of time, from 2 d to 4 wk, labeled with [³⁵S]methionine at a concentration of 85 μCi/ml for 12 h and used for preparation of cytoskeletons.

Amnion epithelial cells from 16-wk pregnancies were cultured under the same conditions. After 4 wk and after 4 mo of culture, the cells were labeled with 50 μCi/ml [³⁵S]methionine as described above. In addition, after several passages cells were grown on coverslips and processed for immunofluorescence microscopy.

Established cell lines HeLa, A-431 (from epidermoid carcinoma), and “Detroit 562” (from pharyngeal carcinoma) were cultured as described (30, 51, 53).

Cytoskeletal Preparations from Cultured Cells: Cells were rinsed three times with PBS, lysed by 2–5-min incubation with 140 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM Tris-HCl (pH 7.6). Lysis buffer was decanted, and cell residues were incubated for 30 min in high salt buffer (1.5 M KCl, 0.5% Triton X-100, other components as in lysis buffer, pH 7.4; cf. reference 26). After carefully decanting high salt buffer, the residual material was washed once in PBS, scraped off in PBS with the help of a rubber policeman, and pelleted by centrifugation for 10 min at ~3,500 g. Some monolayer cells detached during incubation in high salt buffer. These floating cells were diluted with PBS and pelleted at ~13,000 g for 30 min.

Pellets obtained were washed twice by resuspension in PBS and re-centrifugation. Final cytoskeletal pellets were analysed directly or frozen at –20°C and stored until use. Cultures amnion epithelial cells growing at a very low density were labeled with [³⁵S]methionine, mixed with an excess of unlabeled cells of monolayer cultures of lines A-431 and “Detroit 562” cells obtained from American Type Culture Collection (Rockville, MD).

Antibodies: The following antibodies to cytokeratin polypeptides were used: (a) Murine monoclonal antibody K₆ 8.13, which reacts with a relatively large number of cytokeratin polypeptides, notably those of the basic subfamily (No. 1 and 5–8) but also with the “acidic” cytokeratin No. 18 (37). (b) Murine monoclonal antibody K₆ 18.18, which reacts exclusively with human cytokeratin polypeptide No. 18, stains cells of simple and complex epithelia but is negative on stratified epithelial cells, except for the basalmost cell layer in several epithelia such as esophagus, tongue, and vagina (W. W. Franke, R. Nagle, S. Winter, E. Schmid, C. Kuhn, R. Moll, and G. Haemmerling, manuscript in preparation). (c) Murine monoclonal antibody CK-4, which also reacts with human cytokeratin polypeptide No. 18, but, in contrast to antibody K₆ 18.18, does not stain the basal cell layers of stratified epithelia (12, 13). (d) Guinea pig antibodies raised against total epidermal prekeratin from bovine muzzle which display a broad spectrum of reactivity on diverse epithelial cells (18, 23, 26). (e) Guinea pig antibodies (KVI) prepared against and reacting with bovine muzzle prekeratin No. VI, which cross-reacts with human cytokeratins No. 10 and 11 of epidermal keratinocytes (31, 44). (f) Antibodies to desmoplakins from bovine muzzle epidermis, which also stain desmosomal plaques of human tissues (27, 29). (g) Guinea pig antibodies to vimentin (17, 22).

Immunofluorescence Microscopy: Colonies of amnion epithelium cells grown on coverslips were briefly rinsed with PBS, fixed in –20°C cold methanol (5 min), dipped 6 × 1 s in –20°C cold acetone, and allowed to air-dry. These cells were processed for immunofluorescence microscopy as described for other cultured cells (cf. references 17, 18, 22). Cryostat sections of fetal tissue (4 μm) were air-dried, fixed in acetone for 10 min at –20°C, and allowed to dry again. 3-μm sections of amnion epithelium of full-term placenta

¹ Abbreviation used in this paper: IF, intermediate-sized filaments.

were treated with one of two modifications: (a) Sections obtained at -35°C instead of -20°C were not allowed to air-dry but immediately dipped into -20°C cold acetone three times for 1 s each. Directly after washing in PBS for 1 min, the first antibody was applied to the wet section. (b) Sections were air-dried, fixed in cold acetone for 10 min, and washed in PBS for 5 min; antibodies were applied to the wet section. Indirect immunofluorescence microscopy was performed as described (17, 19, 23). After brief air-drying, specimens were embedded in Moviol (Hoechst Co., Hoechst, Federal Republic of Germany [FRG]). Double immunofluorescence microscopy with murine monoclonal antibodies to cytokeratin and guinea pig vimentin antibodies was essentially as described (cf. references 31, 58).

Microdissection of Tissue Sections of Fetal Tissues and Amniotic Epithelia and Preparation of Cytoskeletal Material: 20–40- μm sections through amnion epithelium and diverse fetal tissues (e.g., urinary bladder, colon, trachea, esophagus, and epidermis) were obtained at -20°C with a cryostat (Frigo Cryocut, Reichert-Jung, Nussloch, FRG) and immediately dried with a cold air-stream using a hair dryer. Using a binocular microscope ($\times 50$) equipped with a cold-light source, the epithelial structures were circumscribed and separated from the underlying connective tissue by dissecting with a fine needle (51–53). Care was taken to minimize contaminations by blood vessels and connective tissue, but usually small amounts remained adhering to the dissected epithelia, resulting in the appearance of vimentin from mesenchymal cells in the gel electrophoretic analyses. Regions of nonkeratinized, nonsquamous single-layer amnion epithelium, and those containing squamous stratified epithelium, were distinguished by staining of consecutive sections and extracted separately. A few drops of buffer A (1.5 M KCl, 0.5% Triton X-100, 5 mM EDTA, 0.4 mM phenylmethylsulfonylfluoride, 10 mM Tris-HCl, pH 7.2) were applied to the section while it is still attached to the slides, and the separated epithelium was immediately lifted from the slide with a fine needle, aspirated with a 1-ml syringe, and transferred to a 1.5-ml centrifuge tube and extracted for 30 min in 1.5 ml of the same buffer at 0°C with occasional shaking. The residual cytoskeletal material was pelleted by centrifugation for 5 min at 8,000 *g*, resuspended, and washed once with 1 ml buffer B (5 mM EDTA, 10 mM Tris-HCl, 0.4 mM phenylmethylsulfonylfluoride; pH 7.2) and pelleted by centrifugation. Pellets were immediately frozen and stored at -20°C until use.

Gel Electrophoresis: For two-dimensional gel electrophoresis of cytoskeletal proteins, both nonequilibrium pH gradient electrophoresis (57; ampholine range pH 2–11; lysis buffer containing 0.25% SDS) and isoelectric focusing (56; same ampholine range) were used for separation in the first dimension. For electrophoresis in the second dimension in the presence of SDS, 12% polyacrylamide gels (stacking gels 5% polyacrylamide) were used with a

modified electrode buffer and an acrylamide (51). Gels were stained with a silver technique (3). Gels containing [^{35}S]methionine labeled proteins were processed for autoradiofluorography. Cytokeratin polypeptides and vimentin were identified by co-electrophoresis with cytoskeletal proteins from established cell lines.

RESULTS

Cytokeratins of Fetal Tissues

We studied epithelia from fetuses of various stages of development. In the context of the present study we analyzed in special detail fetuses from 16-wk pregnancies as prenatal diagnostic amniocenteses are usually performed between weeks 16 and 20 of pregnancy.

Different epithelial tissues of week-16 fetuses were examined by immunofluorescence microscopy and gel electrophoresis of cytoskeletal proteins, with special attention given to epithelia that are in contact with the amniotic fluid. Using different antibodies to cytokeratins on frozen sections, we found that all major epithelia, including stratified ones such as esophagus (Fig. 1a), epidermis (Fig. 1d), and tongue mucosa (not shown) as well as the transitional epithelium of the urinary tract (Fig. 1c), the complex epithelium of trachea (Fig. 1e) and various simple epithelia such as intestinal mucosa (Fig. 1b) were all strongly positive (Fig. 1 shows the reactions with monoclonal antibody K_G 8.13). This shows that cytokeratins are expressed in the diverse epithelia during early stages of fetal development, and is in agreement with previous studies on human skin development (52) and mouse embryogenesis (42, 43, 64). All cytokeratin-positive cells also showed, in punctate arrays of fluorescence along cell-to-cell borders, with antibodies against desmoplakins, i.e., marker proteins for the desmosomal attachment plaques of the cytokeratin IF (not shown; cf. reference 55).

Examination of cytoskeletal proteins from microdissected fetal tissues by two-dimensional gel electrophoresis (Fig. 2, a–

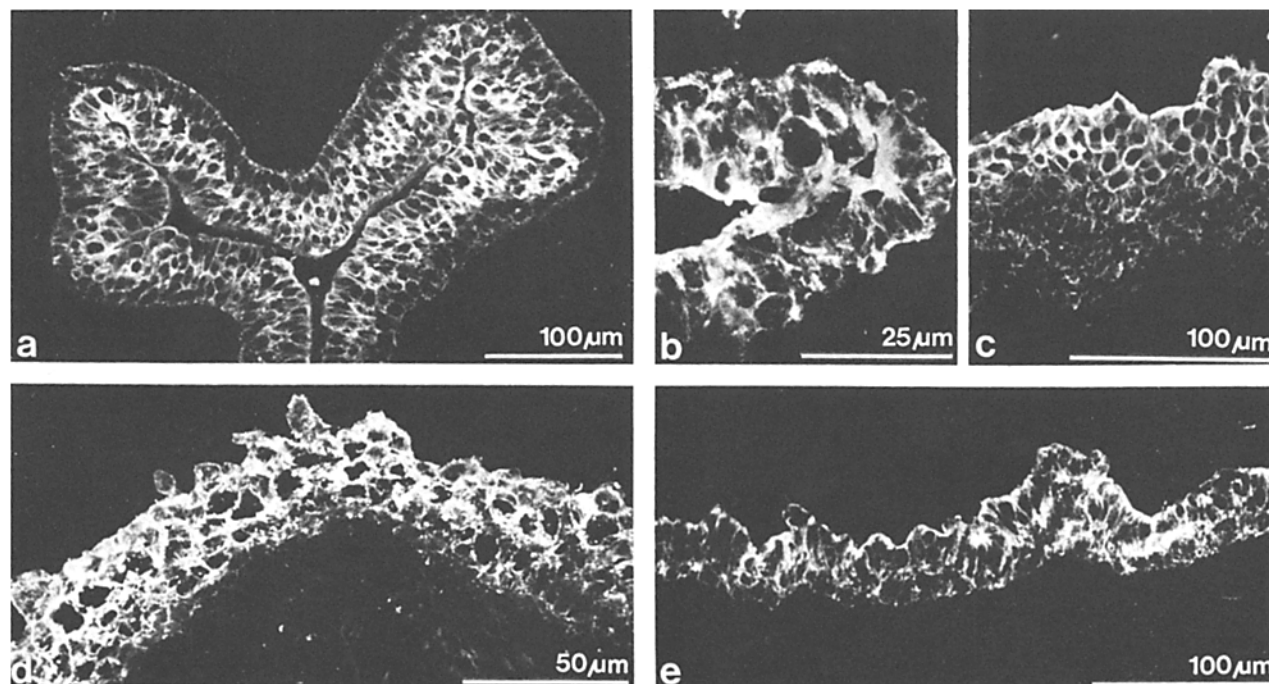


FIGURE 1 Immunofluorescence microscopy showing the reaction of monoclonal murine cytokeratin antibody K_G 8.13 on frozen sections of various human fetal epithelial tissues of wk 16 of pregnancy. (a) Esophagus; (b) small intestine; (c) urinary bladder; (d) epidermis; (e) trachea. Note that all epithelia show intense staining.

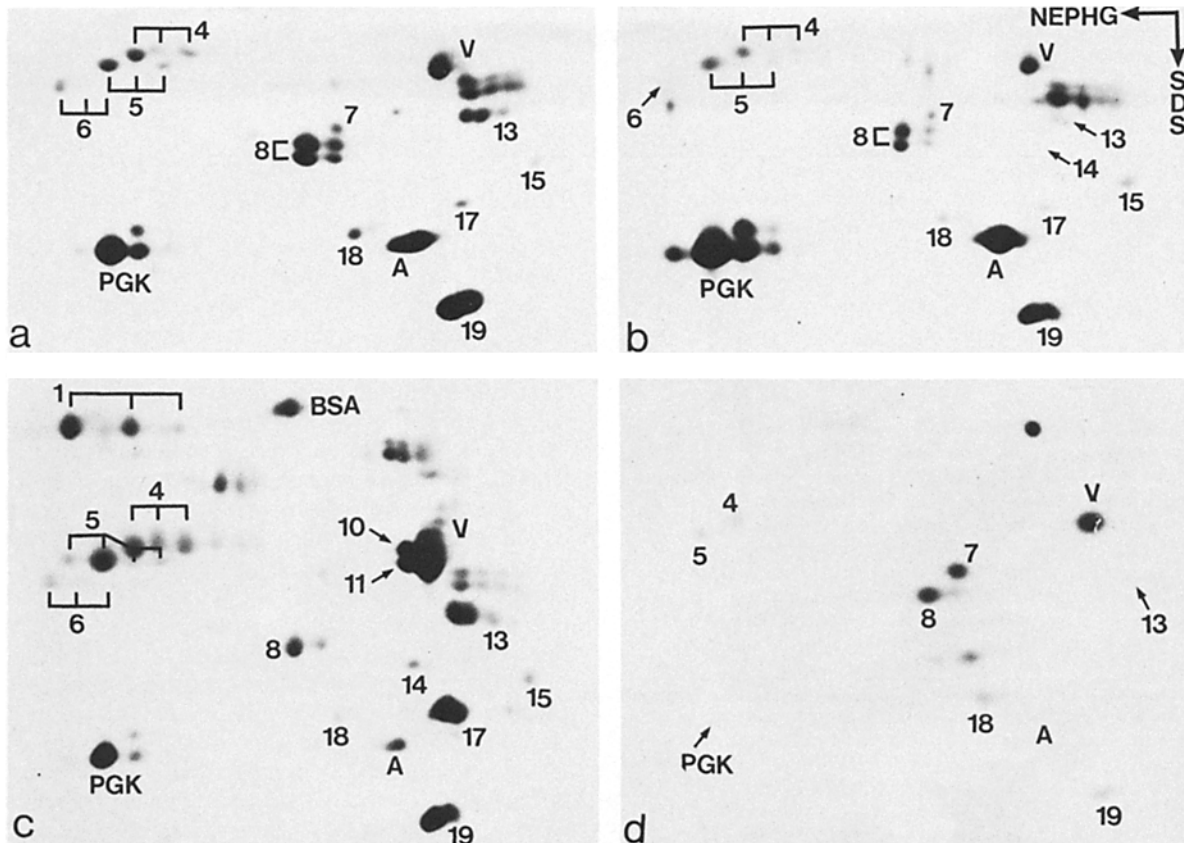


FIGURE 2 Two-dimensional gel electrophoresis of cytoskeletal proteins of microdissected fetal epithelial tissues from wk 16 of pregnancy. First dimension of electrophoresis was nonequilibrium pH gradient electrophoresis (basic polypeptides to the left); second dimension of electrophoresis was SDS PAGE. Reference proteins used for co-electrophoresis are PGK (3-phosphoglycerokinase; M_r 43,000, isoelectric at pH 7.4), A (α -actin from rabbit skeletal muscle: M_r 42,000, isoelectric at pH 5.4); BSA (bovine serum albumin: M_r 68,000; major variant, isoelectric at pH 6.34); V (vimentin detected in the gels due to contamination by mesenchymal cells, e.g., from connective tissue). Cytokeratins are numbered according to Moll et al. (51). (a) Esophagus; (b) trachea (cytokeratin No. 14 of trachea is detected only in very small amounts but has been positively identified in overloaded gels); (c) epidermis; (d) urinary bladder. Gels were stained with the silver technique (3). In a and b, cytokeratin No. 8 appears as a pair of vertically aligned spots, the lower one representing a proteolytic breakdown product. For summary of data, see Table I.

d) showed that cell type-specific patterns of expression of different members of the cytokeratin family was established already at this time of development, revealing polypeptide patterns similar to, but not identical with, those of the corresponding adult tissues. Table I summarizes the results of such analyses. Fetal colon mucosa is essentially identical in its cytokeratin polypeptide pattern (component No. 8, 18, and 19) to that of mucosa of small intestine and colon of adults. In addition, however, we found trace amounts of component No. 7 in the fetal tissue which was not detected in adult colon mucosa (cf. references 51, 53). A similar correspondence was found in fetal and adult urinary bladder. In fetal transitional epithelium (Fig. 2d) we noted, in addition to the common cytokeratins No. 5, 7, 8, 13, 18, and 19, also a cytokeratin of M_r 59,000 identified as No. 4. Similarly we observed cytokeratin No. 4 in fetal trachea but not in trachea of adults (cf. references 7, 51), while all other cytokeratins of trachea (No. 5-8, 13-15, and 17-19) were common to both stages of development. Stratified epithelia of esophagus and epidermis showed a more substantial difference in the fetal and adult tissues. In esophageal epithelium of week-16 fetuses we found cytokeratins No. 4, 5, 6, 7, 8, 13, 15, 17, 18, and 19 (Fig. 2a), whereas cytoskeletons from adult esophagus lacked components No. 7, 8, and 18 but contained components No. 14 and

16 which were not found in the 16-wk fetuses. In epidermis of 16-wk fetuses (Fig. 2c), we observed a pattern of cytokeratin polypeptides (No. 1, 4, 5, 6, 8, 10, 11, 13, 14, 15, 17, 18, and 19), which was clearly more complex than that found in interfollicular adult epidermis (No. 1, 2, 5, 10, 11, 14, and 15). The reduction of complexity of the epidermal cytokeratin pattern during human fetal development has already been noted by Moll et al. (52).

Cytokeratins and Vimentin in Amnion Epithelium

Broad range cytokeratin antibodies, including monoclonal antibody K_G 8.13, stained all amnion epithelial cells in frozen sections of amniotic tissue from all stages of pregnancy up to full-term placenta (Fig. 3, a-d; cf. reference 11). In full-term placenta, both types of amnion epithelium, the simple, non-keratinized, (Fig. 3a) as well as the stratified, partially cornified (Fig. 3b) epithelium near the insertion of the umbilical cord, showed positive reaction with the cytokeratin antibodies. At higher magnification, fibrillar structures were resolved within the simple epithelial cells, particularly with monoclonal antibody K_s 18.18 (Fig. 3c). In the multi-layered regions of amnion epithelium, antiserum KVI showed no significant staining of the basal cell layer but stained the upper cell layers brightly (Fig. 3d), indicating that keratin polypep-

TABLE 1
Distribution of Cytokeratin Polypeptides in Various Human Fetal Tissues and Cultured Fetal Cells

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Molecular Weight ($\times 10^{-3}$)	68	65.5	63	59	58	56	54	52.5	64	56.5	56	55	54	50	50	48	46	45	40
Isoelectric pH	7.8	7.8	7.5	7.3	7.4	7.8	6.0	6.1	5.4	5.3	5.3	4.9	5.1	5.3	4.9	5.1	5.1	5.7	5.2
Fetal and Adult Tissues																			
Epidermis, fetus, week 16	++			++	++	+		++		++	++		++	+	+		++	+	++
Epidermis, adult	++	+			++	++				++	++			++	+				
Esophagus, fetus, week 16				+	+	+	+	++					++				+	+	++
Esophagus, adult				++	++	+							++		+	+	+	+	+
Trachea, fetus, week 16				++	++	+	+	++					+	(+)	+		+	+	++
Trachea, adult					++	++	+	++					++	++	++		++	+	++
Bladder, fetus, week 16				+	+		++	++					++	++	++			++	++
Bladder, adult					+		++	++					+					+	++
Colon, fetus, week 16							(+)*	++										++	++
Colon, adult								++										++	++
Amnion Epithelium In Situ																			
Week 10, simple epithelium					+	+	++	++						+			++	++	++
Week 16, simple epithelium				++	++	+		++						+			++	+	++
Full-term, simple epithelium				+	+	+		++						++			++	++	++
Full-term, stratified regions	++			+	++	++		++		++	++			+			++	+	++
Cell cultures																			
Amnion epithelial cells derived from week-16 fetus					+	++	++	++						+			++	++	++
Amniotic epithelium cells derived from full-term placenta					++	++	++	++						++			++	++	++
Cells from amniotic fluid at week 16 of pregnancy							++	++									+	++	++

++, Components always observed in substantial amounts. +, Components present in minor amounts. (+)*, In fetal colon, cytokeratin No. 7 has been found in trace amounts and not in all samples. (+), In fetal trachea, cytokeratin No. 14 has been found in trace amounts.

tides No. 10 and 11 are not present in basal cells (for related observations in adult epidermis and penile mucosa, see references 5, 32, 33, 38, 39, 44, 69, 77, 79). At week-16 pregnancy, amnion epithelium consisted of a single layer of cells that all showed a bright reaction with antibodies to cytokeratins, including monoclonal antibodies K_G 8.13, K_s 18.18.

Antibodies against cytokeratin No. 18 displayed a remarkable heterogeneity of staining. Antibody K_s 18.18 strongly stained all cells of simple amnion epithelium (Fig. 3c) but showed a rather diffuse and weak staining of the stratified regions, often with an accentuation of the basalmost or apical areas. A similar homogeneous positive staining of the simple amnion epithelium has been reported for antibody CK-3 (12). Antibody CK-4 stained certain regions of simple epithelium, whereas other regions were totally negative (not shown; see also reference 12). In the stratified regions, most cells were negative with CK-4, but isolated groups of positively stained cells were occasionally noted within the stratified tissue. At present, we cannot explain these different staining patterns with different monoclonal antibodies to the same cytokeratin (No. 18). The phenomenon may be due to differences of accessibility of the determinants, recognized by the specific antibodies.

Vimentin antibodies applied to sections of amnion epithelium of 16-wk pregnancies and full-term placenta stained the mesenchymal cells of adjacent amnion connective tissue (Fig. 3e). In addition, however, a few individual cells or groups of cells within the epithelial cell layer were positively stained (Fig. 3e), suggesting that a certain subpopulation of amnion epithelial cells expresses both types of IF proteins simultaneously. The amnion epithelium lining the umbilical cord at week-16 pregnancy is composed of two layers of cells with flattened, partly overlapping cells in the basal layer and polygonal ones in the upper layer. Full-term amnion epithelium of this region also contained some isolated groups of individual cells reacting positively with antibodies against vimentin,

displaying a finely fibrillar meshwork (data not shown). This was confirmed by double immunofluorescence microscopy with guinea pig antibodies to vimentin and monoclonal murine antibodies K_G 8.13 or K_s 18.18 (data not shown). Double antibody staining also showed that, within the same cell, the display of vimentin fibrils was usually different from that of the cytokeratin fibrils.

Gel Electrophoresis of Cytoskeletal Proteins from Amnion Epithelium

Two-dimensional gel electrophoresis of cytoskeletal proteins from microdissected amnion epithelium in fetuses at various stages of development (Fig. 4a-c; Table I) showed the major cytokeratin polypeptides No. 5-8, 14, 18, and 19, and, in addition, some vimentin, which partly may also have come from adherent mesenchymal cells. Cytokeratin No. 4 was not detected in samples from 10-wk pregnancies but was present at later stages, and the relative amount of component No. 14 appeared to increase between weeks 10 and 16 (cf. reference 51).

In advanced pregnancy and full-term placenta, amnion epithelium displays plaques of stratified, partially cornified epithelium (for light and electron microscopy, see references 40, 65). Therefore, to examine their cytokeratin composition, we microdissected and separated the epithelium of such stratified plaque regions and adjacent simple epithelium regions. Two-dimensional gel electrophoresis revealed that the stratified amnion epithelium possessed, in addition to the cytokeratins found in the simple epithelium, cytokeratins No. 1, 10, and 11 (Fig. 4, c and d).

Cytoskeletal Fibrils in Cultured Amnion Epithelial Cells

The IF cytoskeleton of amnion epithelial cells obtained at various stages of development and grown in culture for dif-

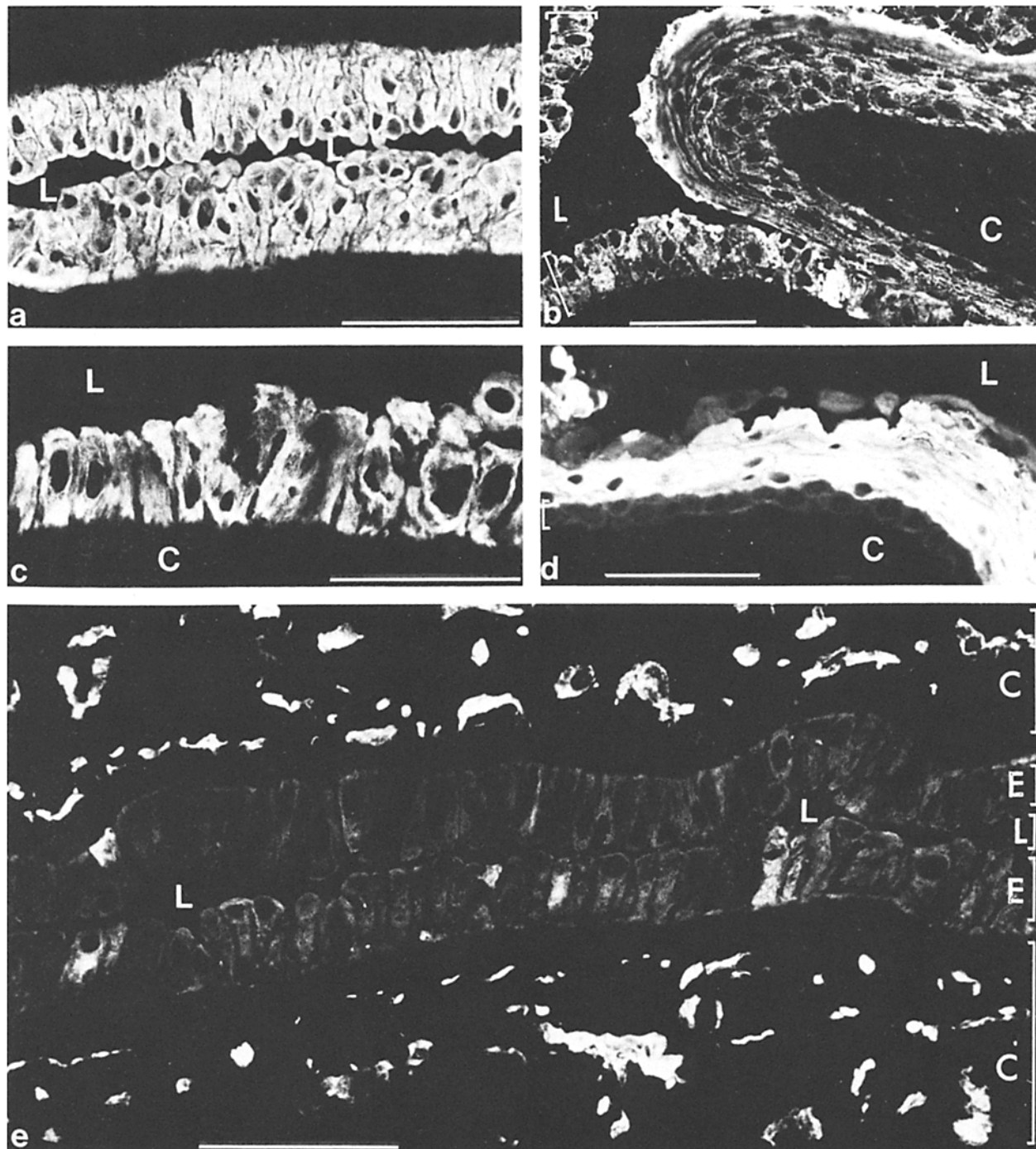
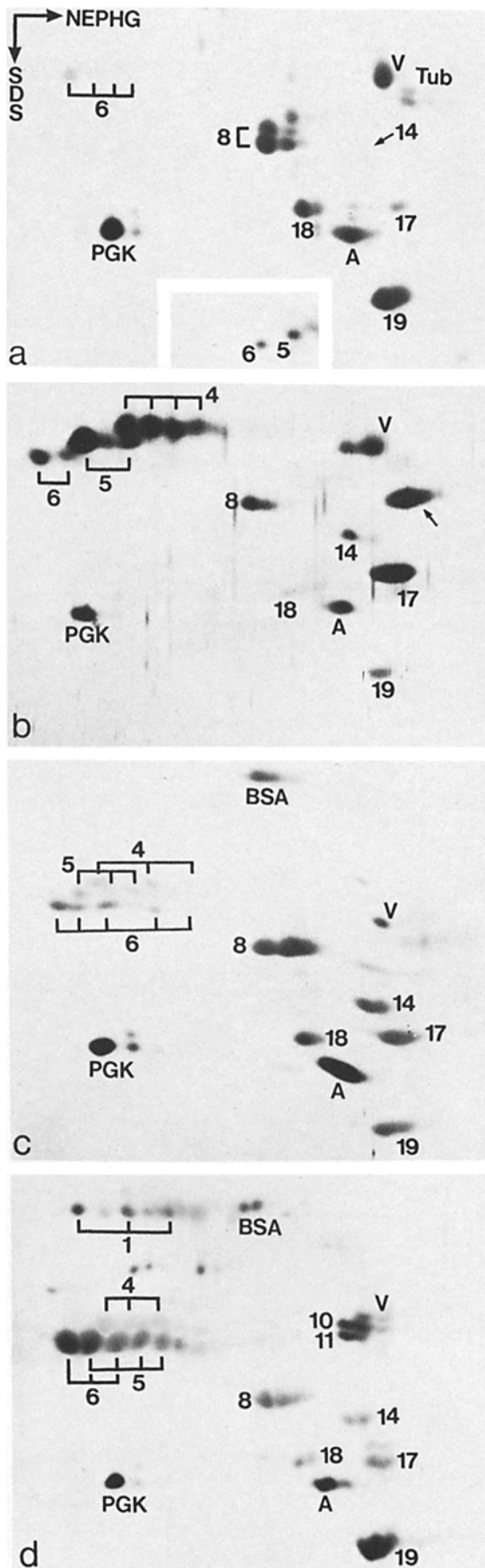


FIGURE 3 Immunofluorescence microscopy of frozen sections of amnion epithelium of full-term placenta. (a) Regions containing simple amnion epithelium stained with monoclonal cyokeratin antibody K_G 8.13. (b) The same antibody (K_G 8.13) applied to a region containing stratified amnion epithelium. Brackets denote simple amnion epithelium, as opposed to the folded stratified epithelium in the upper right. In the flattened, partially cornified cells of the stratified regions, the staining often appears to be restricted to the cell periphery, probably reflecting local differences of accessibility of antigens. (c) At higher magnification, monoclonal antibody $K_18.18$ reveals fibrillar organization within cells of simple amnion epithelium. (d) Antibodies of serum KVI show a very bright staining of the squamous cell layers, but do not significantly react with cells of the basal layer (bracket) and the uppermost desquamating cells. (e) Antibodies to vimentin stain mesenchymal cells in the adjacent connective tissues (C, brackets) but also react with a few isolated cells within the amnion epithelium (E, brackets). L, Lumen. Bars, (a, b, d, and e) 100 μm ; (c) 50 μm .

ferent periods of time was examined by indirect immunofluorescence microscopy and by two-dimensional gel electrophoresis of cytoskeletal proteins. Amnion epithelial cells obtained from 16-wk pregnancies which had been grown in culture for 4 mo (Fig. 5, *a-d*) or more showed bright fibrillar immuno-

fluorescent staining after reaction with various cyokeratin antibodies, including monoclonal murine antibodies of broad spectrum reactivity, such as K_G 8.13 (Fig. 5*a*), and others which react only with a single polypeptide, such as $K_18.18$ (Fig. 5*b*), CK 4 (Fig. 5*c*), and conventionally produced guinea



pig antisera raised against cytokeratins (Fig. 5d). Desmosomal attachment plaques were recognized with antibodies against desmoplakin as dotted lines at cell-to-cell borders (data not shown). Even during primary culture, the amnion epithelium cells displayed extended cytokeratin fibril arrays (Fig. 6, a and b, present examples of a 72-h culture), which often projected to distinct points of cell-to-cell contacts, probably corresponding to desmosomes (Fig. 6b). In contrast, staining with vimentin antibodies did not show significant fibrillar structures in the majority of cells, except for small perinuclear aggregates of vimentin fibrils seen in a few cells (e.g., Fig. 6c). This cell heterogeneity detected by vimentin antibody staining was also observed on prolonged culturing of amnion epithelium cells, and it was likewise found in cells derived from full-term placentae, as well as in cells from 16-wk pregnancies. Fig. 7, a-d, present immunofluorescence microscopy of colonies of 16-wk pregnancies with antibodies against vimentin. Most cells showed no significant reaction (Fig. 7, a and b). In certain colonies, however, cells with positive fibrillar fluorescence, often showing some concentration in peripheral regions, were found (Fig. 7, c and d).

Gel Electrophoresis of Cytoskeletal Proteins from Cultured Amnion Epithelium Cells

Two-dimensional gel electrophoretic analyses of proteins of cytoskeletal structures resistant to extraction with high salt buffer and detergent were performed after metabolic labeling by [³⁵S]methionine. To compare and identify polypeptides, we mixed labeled cultures of amnion epithelium cells obtained from full-term placenta (Fig. 8a) or 16-wk pregnancies (Fig. 8, b-d) with an excess of unlabeled cultured carcinoma cells of lines "Detroit 562" (Fig. 8e) or A-431 and used for co-electrophoresis. In both full-term and 16-wk amnion epithelium cells, the same cytokeratin components (No. 5-8, 14, and 17-19) were found, although in somewhat variable amounts. Thus, except for an absence of the basic cytokeratin No. 4, the pattern resembled amnion epithelium in situ in 16-wk fetuses. Amnion epithelial cells of 16-wk pregnancies that had been cultured for 4 wk (Fig. 8b) revealed the same cytokeratin pattern as those cultured for 4 mo (Fig. 8c). Vimentin was detected regularly in cultures of amnion epithelium cells, albeit in relatively low and somewhat variable amounts (Fig. 8, a-d).

FIGURE 4 Two-dimensional gel electrophoresis of cytoskeletal proteins from microdissected human amnion epithelium obtained from various stages of fetal development and from different regions (for designations see Fig. 2). (a) Amnion epithelium of 10-wk pregnancy; (b) amnion epithelium of 16-wk pregnancy; (c) regions of full-term placenta containing only simple (i.e., one-layered) epithelium; (d) stratified regions of amnion epithelium of full-term placenta (similar to that shown in the upper right of Fig. 3b). Note that the typical cytokeratin pattern of amnion epithelium is already recognized at early stages of development (a). Increased amounts of cytokeratins No. 5, 6, and 14 (No. 5 is clearly seen in the central bottom of inset a) are expressed in fetuses of wk 16 (b). The pattern of amnion epithelium of wk 16 does not show a significant difference in cytokeratin expression in comparison to simple amnion epithelium of full-term placenta (c) but stratified and partially cornified amnion epithelium expresses, in addition, cytokeratins No. 1, 10, and 11, typical of certain stratified epithelia such as epidermis. The arrow in b denotes an unidentified, nonkeratinous protein, possibly β -tubulin.

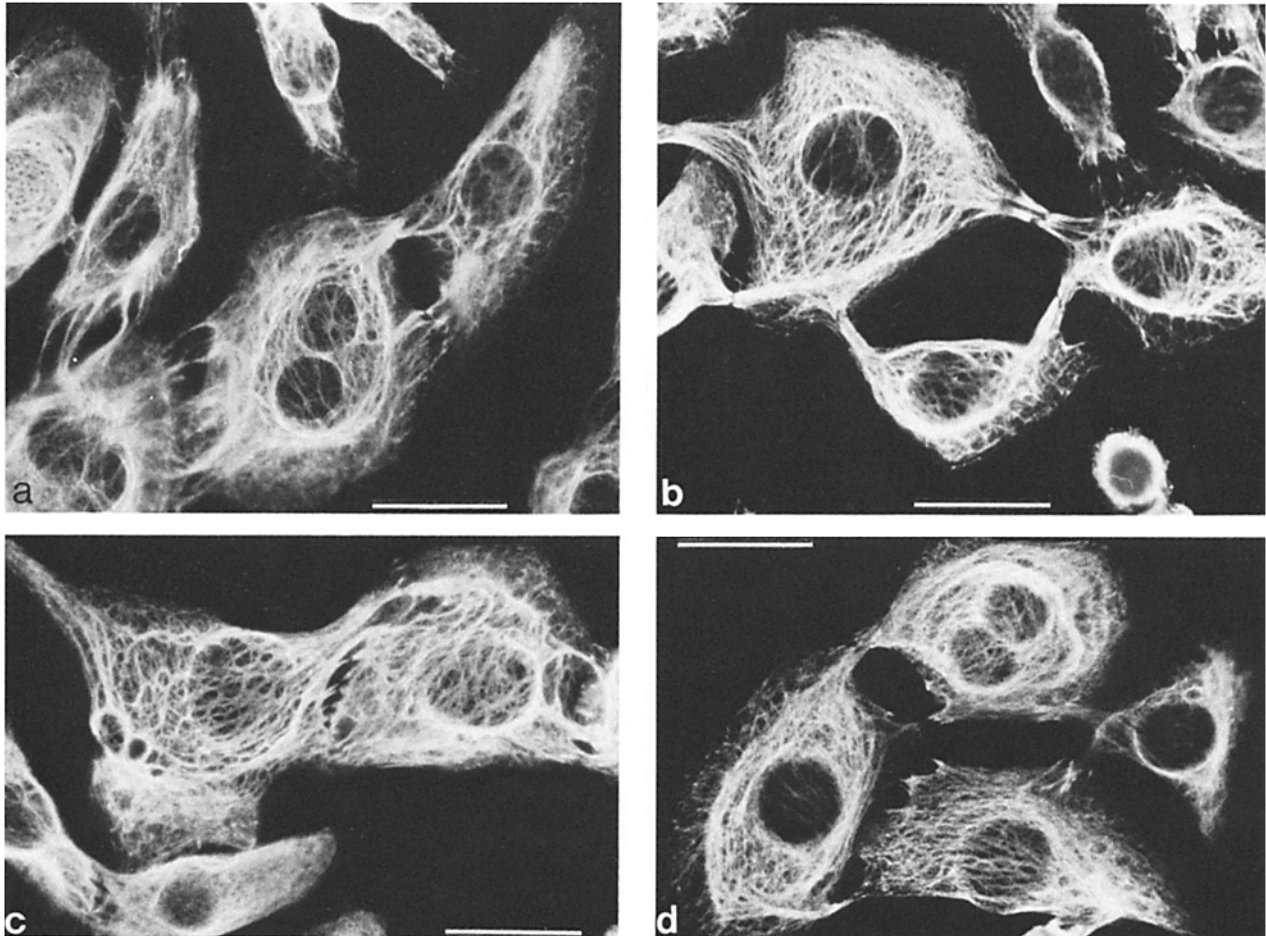


FIGURE 5 Immunofluorescence microscopy of cultured human amnion epithelial cells derived from fetuses of wk 16 of pregnancy after reaction with different cytokeratin antibodies. Cells were cultured for 4 mo. Note that all cells show a fibrillar staining throughout the cytoplasm with typical tonofibrillar correspondence at points of intercellular contact which probably correspond to desmosomes. (a) Broad spectrum cytokeratin monoclonal antibody K_C 8.13; (b) monoclonal antibody K, 18.18; (c) monoclonal antibody CK-4; (d) guinea pig antibodies raised against total prekeratin from bovine muzzle epidermis. Bars, 25 μ m.

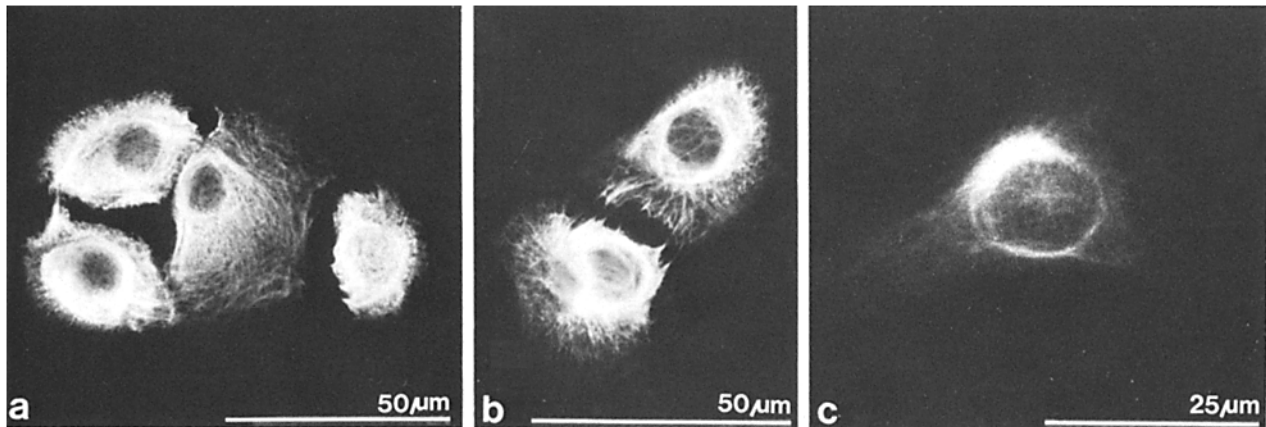


FIGURE 6 Immunofluorescence microscopy of amnion epithelial cells obtained from full-term pregnancies and grown in primary culture for 72 h, showing in all cells intense fibrillar staining with cytokeratin antibodies (a and b; K_C 8.13), displaying in many cells tonofibrillar correspondences at desmosome-containing bridges (b). In most cells of the same culture, vimentin antibodies do not decorate any fibrillar structure, but some individual cells reveal vimentin "caps" in the perinuclear region (c).

Cytoskeletal Proteins of Cultured Cells of Amniotic Fluids

The vast majority of cells of cultures obtained from amniotic fluids collected by amniocentesis are positive for anti-

bodies to cytokeratins and desmoplakins (data not shown; cf. references 11, 55, 72, 75, 76), but many colonies of such cultures also express IF of the vimentin type as their second IF system (55, 73, 75). In the context of the present study, we compared the cytoskeletal protein pattern of amnion epithelial

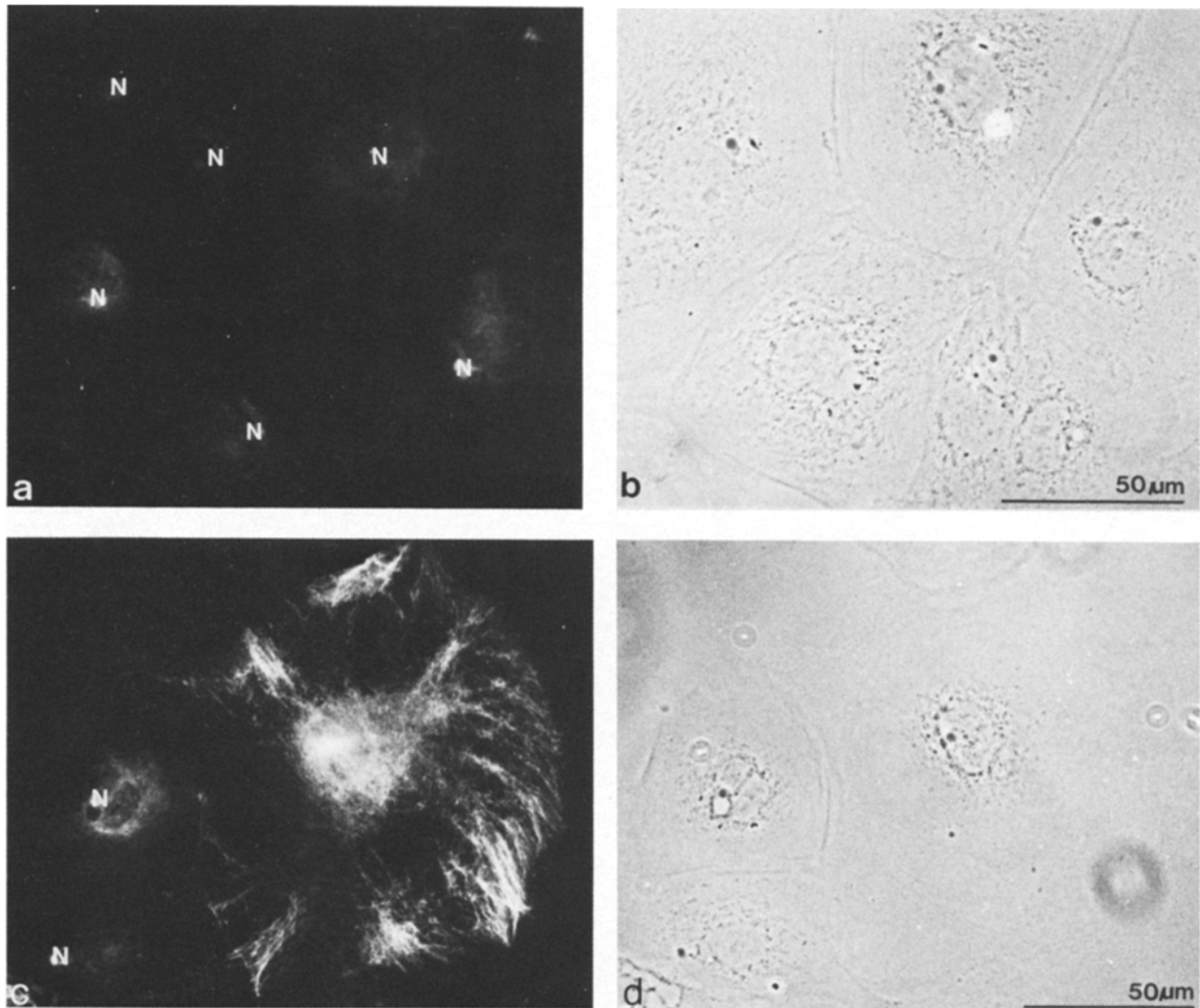


FIGURE 7 Immunofluorescence microscopy (a and c) of cultured amnion epithelial cells from 16-wk pregnancies after 4 mo of culture as seen after reaction with vimentin antibodies, and corresponding phase-contrast micrographs (b and d). (a and b) Most cells do not show any reaction with antibodies against vimentin (N, nuclei of vimentin-negative cells). (c and d) Positive reaction of one cell that is surrounded by cells with negative reaction.

lium cell cultures with that of cultures of cells derived from amniotic fluid. We found cytokeratins No. 7, 8, 18, and 19, together with considerable amounts of vimentin (Fig. 9), independent of the length of culturing. Cytokeratin No. 17 was noted in trace amounts in some but not all cultures (cf. reference 55). This pattern is grossly different from that of the amnion epithelial cells described above.

DISCUSSION

At week 16 of human pregnancy, tissue-specific cytokeratin phenotypes are established in the diverse fetal epithelia, although certain differences between the fetal and the adult stage occur in certain epithelia. Specifically, cytokeratins of the group No. 4–6 are synthesized in diverse stratified epithelia, and the large (M_r 68,000) cytokeratin No. 1 characteristic of terminal differentiation of epidermis is present in fetal epidermis, in agreement with results on human fetuses (52; for corresponding data in rabbits and mice, see references 6, 64). Preliminary results in this laboratory and previous data of Moll et al. (52) from week-10 fetuses further indicate that some cytokeratins of the group No. 4–6 are already expressed at earlier stages than week 16.

Amnion epithelium from week 10 of pregnancy is a typical epithelium with polar organization, desmosomes, and IF (for review, see reference 40), and the cytokeratin content of the latter has been previously demonstrated (e.g., references 11, 51). Remarkably, the amnion epithelium of week-10 and -16 fetuses, which at this stage is a one-layered, cuboidal-to-columnar epithelium with apical microvilli (for review, see references 40, 65), produces a complex set of cytokeratin polypeptides, including the basic components No. 5 and 6 and the acidic cytokeratin No. 14. In addition, the basic cytokeratin No. 4 is recognized in amnion epithelium from week 16 on. Therefore, amnion epithelium presents a case of a simple, polar epithelium expressing cytokeratins that so far have been assumed to be markers for stratified squamous epithelia, such as polypeptides No. 5 and 14 (54, 69, 77). Thus, our results demonstrate that synthesis of cytokeratins No. 4–6 and 14 is not necessarily correlated with stratification. Such cytokeratins are also synthesized in tracheal and bronchial epithelium (7), prostate gland (1), and ducts of the mammary gland (51, 53), which are complex epithelial linings composed by at least two different cell types, the luminal cells and certain basal cells that are not bordering on the lumen,

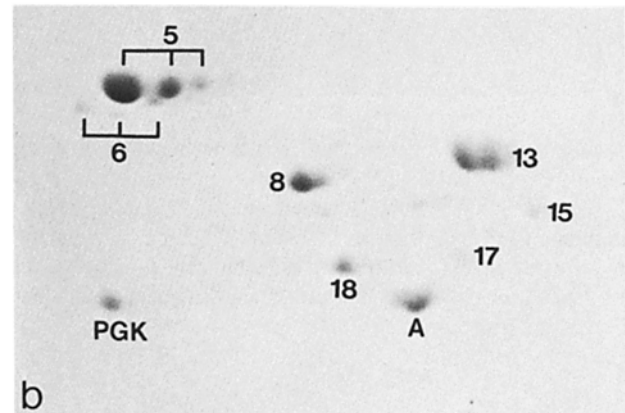
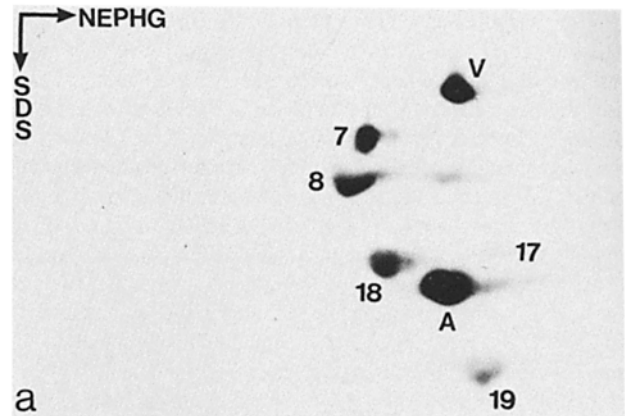
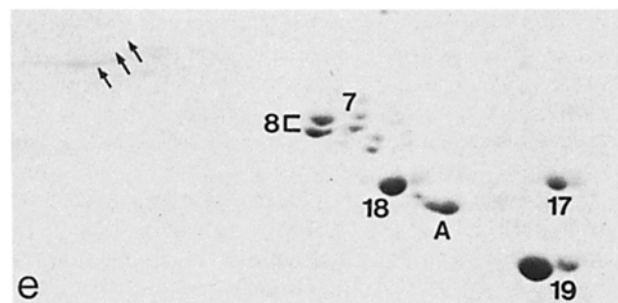
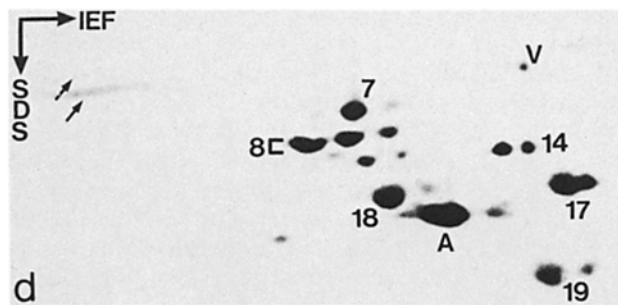
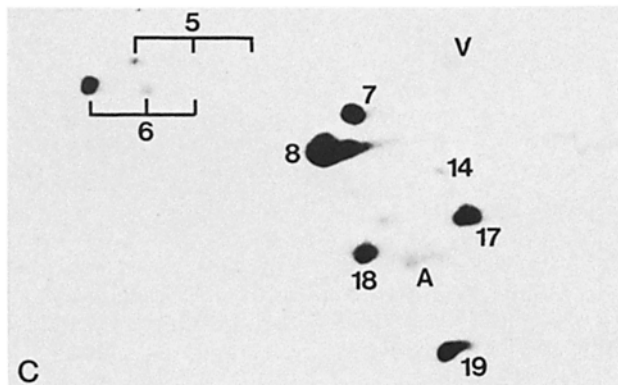
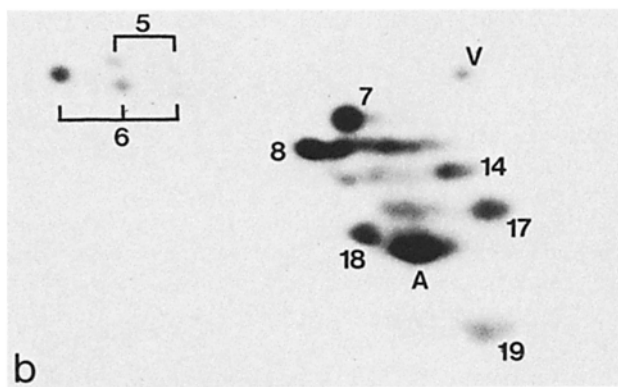
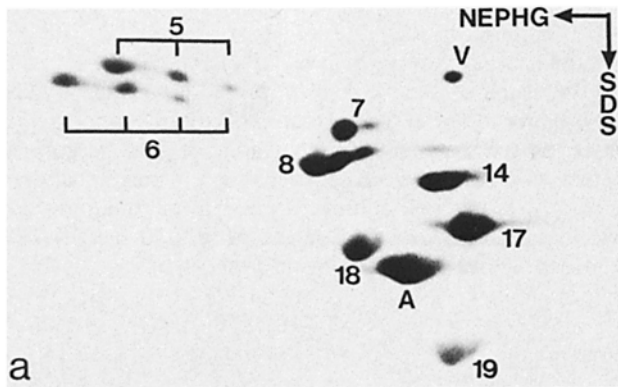


FIGURE 9 Identification of cytokeratins of [³⁵S]methionine-labeled amniotic fluid cells from a 16-wk pregnancy that have been grown in culture for 20 d by co-electrophoresis with an excess of unlabeled cytoskeletal proteins from carcinoma cells of line A-431 (a). (b) Coomassie Blue staining. Abbreviations as in Fig. 2. Cell cultures from amniotic fluid exhibit a simple pattern, showing components No. 7, 8, 18, and 19 (sometimes with traces of cytokeratin No. 17) but no basic cytokeratin No. 4-6. In addition, they contain relatively large amounts of vimentin (V).

FIGURE 8 Radiofluorographies (a-d) of cytoskeletal proteins from cultured [³⁵S]methionine-labeled amnion epithelial cells separated by two-dimensional gel electrophoresis using nonequilibrium pH gradient (a-c) or isoelectric focussing (d and e) for separation in the first dimension (abbreviations as in Fig. 2). The same cytokeratin pattern is seen in amnion epithelial cells from full-term placenta that were grown for 4 wk in culture (a), 4-wk cell cultures from amnion epithelium of 16-wk pregnancy (b), and 4-mo cultures of 16-wk pregnancies (c), independent from the length of culturing. A better separation of the acidic components is obtained after isoelectric focusing in the first dimension. (d) Same preparation as in c. For identification, cytoskeletal proteins from labeled amnion epithelial cells were co-electrophoresed with unlabeled proteins from cells of the Detroit 562 line (d presents the radiofluorograph of the gel shown by Coomassie Blue staining in e). Note coincidence of labeled amnion cytokeratins seen in d with unlabeled cytokeratins of Detroit 562 cells (e; for identification of Detroit 562 cytokeratins, see reference 53). Arrows in d and e denote some material of the basic cytokeratins No. 5 and 6, which has only partially entered the gel.

i.e., short basal cells or myoepithelial cells. Interestingly, many of these epithelia are known to have the potential to stratify under certain pathological conditions ("metaplasia"). Likewise, thymus reticulum epithelial cells, which also produce positively charged cytokeratins of this group, are known to form aggregates of locally "stratified" cells, i.e., the "Hassall bodies" (53). Hence, expression of cytokeratins No. 4–6 and 14 may be related to the potential of a cell to form stratified squamous epithelial structures rather than being a marker of the manifested stratified state.

Amnion epithelium at advanced stages of fetal development and at birth shows two morphologically different forms: regions of simple epithelium border on regions ("plaques") of stratified epithelium, which are especially conspicuous near the insertion of the umbilical cord, revealing vertical changes of cell structure and some indications of cornification ("keratinization"; for review, see references 40, 65). Interestingly, these stratified plaque regions contain, in addition to the cytokeratins found in the simple amnion epithelium, keratin polypeptides No. 1, 10, and 11, i.e., proteins typical of terminally differentiated epidermis, i.e., "keratinization" (16, 32–34, 38, 39, 69, 70, 77, 79). At present, we do not know whether all cells of these stratified plaques contain simple epithelium-type cytokeratins, as does No. 18. In view of the very low amounts of cytokeratin No. 18 in this tissue (Fig. 4d) and the reaction of antibodies to cytokeratin No. 18 with only a few cells, it is conceivable that this cytokeratin, for example, is present only in a few cells of this tissue. Our finding that these proteins occur in cells of a nonepidermal tissue, the amnion epithelium, supports recent studies showing their occurrence in exocervix and vagina (26), in the fossa navicularis, and the glans penis (1), as well as in Hassall body-containing thymus epithelium (51); this finding is not too surprising in view of the demonstrated continuities between amnion epithelium and fetal epidermis. Nevertheless, these studies demonstrate that cytokeratins No. 1, 10, and 11 are not exclusive to epidermis but are also expressed in certain other stratified epithelia. How far the similarity of the amnion epithelial "plaques" to epidermal differentiation extends, specifically in relation to the production of involucrin (4, 39), cornified envelopes, and true keratohyalin (for review, see reference 38) remains to be determined.

Amnion epithelial cells, which constitutively contain cytokeratin IF, are not homogeneous because a few cells also express vimentin (see also reference 11). The simultaneous synthesis of IF of both the cytokeratin and the vimentin type is not uncommon in cultured epithelial and carcinoma cells of various species, including humans (20–22, 25, 28, 58, 63, 72), but is apparently rarely found in tissues. Some examples of such include cells of the parietal (distal) endoderm of mouse embryos (47, 49), developing collecting duct cells of human kidney (41), a certain proportion of cells of amniotic fluids (75), and some special tumors (2, 10, 46, 74; for metastatic cells from ascites fluids, see reference 59). The biological fate and functions of the sparse vimentin- and cytokeratin-positive cells of the amnion epithelium in situ are unknown.

Cultures of amniotic epithelial cells produce IF of the cytokeratin type (cf. references 48, 72). A certain small proportion of cells also synthesized vimentin IF as in amnion epithelium in situ (this study; 48, 72), but we have been unable to relate this co-expression of both types of IF to any morphologically conspicuous feature of these cells. Our present study extends this finding to biochemical data and shows

that the typical pattern of cytokeratins of amnion epithelium cells is maintained in vitro despite several months in culture, including the expression of basic cytokeratins No. 5 and 6 and the distinctive acidic cytokeratin No. 14 (Table I). The stable expression of such cytokeratins in culture is remarkable, insofar as the synthesis of cytokeratins of this category in human cells grown in vitro has been reported only in cultured keratinocytes and cell cultures derived from squamous cell carcinomas (15, 30, 32–34, 38, 39, 51, 53, 60, 66, 77, 80). Continuous expression of bovine tissue type-specific basic cytokeratins corresponding to human cytokeratins No. 4–6 has been shown in a cell line derived from cow's udder (63; for apparently related murine keratin in cultured mouse keratinocytes, see the review in reference 36).

The cytokeratins synthesized in these cultures of authentic amnion epithelial cells are different from the cytokeratin patterns of various other putative amnion epithelial cell cultures, such as those described by Bravo et al. (8, 9), which show a striking resemblance to the typical cytokeratin pattern of HeLa cells (for comparison, see references 26, 28, 51). Moreover, the cytokeratin pattern of the cultured amnion epithelium cells is also grossly different from that of cultures of cells derived from amniotic fluids, i.e., the period of pregnancy at which amniocenteses for prenatal diagnosis are usually performed. Von Koskull and colleagues (75, 76) have suggested that some of the cultured epithelial cells derived from amniotic fluids might be derived from urothelium of fetal bladder as well as from amnion epithelium (a few cells morphologically resembling amnion epithelial cells, notably clone E-3, were observed in a small proportion of the cultures; cf. reference 73). As we have shown in the present study, amnion epithelium cells do not change their complex cytokeratin polypeptide pattern during culturing to the simple (No. 7, 8, 18, and 19; with small amounts of No. 17) pattern characteristic of cultures from amniotic fluid cells. Therefore, we conclude that normal amnion epithelium cells do not contribute to the pool of proliferating cells derived from amniotic fluids. Similarly, we note that the cytokeratin pattern of fetal urothelial cells (No. 4, 5, 7, 8, 13, 18, and 19) and the maintenance of expression of cytokeratins No. 5 and 13 in cultured urothelial cells (80; T. Achtstätter, unpublished data) is hardly compatible with a derivation of the majority of cultured amniotic fluid cells from urothelium, although we cannot exclude an origin from a minor subpopulation of urothelial cells with a different cytoskeletal composition. A cytokeratin pattern essentially identical to that of cultured amniotic fluid cells is known to occur in some fetal epithelial tissues that may release cells into the amniotic fluid, such as the bronchopulmonary tract and the collecting ducts of the kidney. In our opinion such tissues at present cannot be excluded from the list of possible sources of cells floating in the amniotic fluid.

Information of the cell type of origin of cultured cells derived from amniotic fluid would also be of importance in prenatal diagnosis, in particular in the critical evaluation of karyotype disorders that may represent heterogeneities ("mosaicism" or "pseudomosaicism"; for review, see reference 61). One possibility of how false-positive diagnoses, i.e., karyotype disorders observed in some cultured cells which are not observed in the tissues of the fetus *sensu stricto*, might have been explained would be a derivation from the amnion epithelium. Our present results argue against this possible explanation and suggest that proliferative cells present in the am-

notic fluid are derived from other fetal tissues, probably of simple epithelium character.

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