

Parietal and visceral endoderm differ in their expression of intermediate filaments

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Two layers of extra-embryonic endoderm, viz. the parietal endoderm (PE) and the visceral endoderm (VE), arise in the mouse embryo shortly after implantation. Both cell populations apparently originate from the primitive endoderm of the blastocyst. While the endoderm differentiation has been studied both in the embryo and in the embryonal carcinoma model system, the investigation has been hampered by the paucity of unequivocal markers of differentiation, especially in the case of the PE. Here we show that the PE and VE of mouse conceptuses differ in their expression of intermediate filaments: while both cell types contain cytokeratin, expression of vimentin was only revealed in the cells of the PE. The association between the differentiation of PE and the appearance of vimentin filaments is discussed.

Key words: differentiation/endoderm/foetal membranes/intermediate filaments/teratocarcinoma

Introduction

Different intermediate filament (IMF) proteins show tissue-type specificity and therefore serve as markers for different cell types and lineages (Lazarides, 1982; Franke *et al.*, 1982b; Osborn *et al.*, 1982). During the early stages of mouse development, the first IMF proteins expressed seem to be cytokeratins, reported to appear during blastocyst formation (Jackson *et al.*, 1980; Paulin *et al.*, 1980). In early post-implantation embryos, desmosomes with attached cytokeratin filaments have been demonstrated in the embryonic ectoderm and visceral endoderm (VE) (Jackson *et al.*, 1981). During further development, monoclonal antibodies, suggested to react with cytokeratin, decorate VE, parietal endoderm (PE) and trophoblast in 4- to 9-day-old embryos, and epithelial cells, regardless of their origin, in 12- and 13-day-old embryos (Kemler *et al.*, 1981). IMFs of types other than cytokeratin have not been observed in mouse embryos before late in day 8. At this stage, disperse cells containing vimentin filaments can be detected in the nascent mesoderm of the embryo (Jackson *et al.*, 1981; Franke *et al.*, 1982a).

Until now, the studies on IMF expression in murine endodermal cells have concentrated on established cell lines. The results have suggested that teratocarcinoma-derived endodermal cells express cytokeratin (Paulin *et al.*, 1980; Paulin, 1981; Oshima, 1982), and that both PE (Paulin *et al.*, 1980; Paulin, 1981; Oshima, 1982) and VE (Oshima, 1982) cell lines also contain vimentin filaments. Correspondingly, treatment of F9 embryonal carcinoma (EC) cells with retinoic acid leads to the appearance of endoderm-like cells which express cytokeratin (Paulin *et al.*, 1982; Jones-Villeneuve *et al.*,

1982; Lehtonen *et al.*, 1983). However, cultured cells contain vimentin in addition to their original tissue-specific IMF type (Franke *et al.*, 1978; Virtanen *et al.*, 1981), and cells which do not express vimentin *in vivo*, usually start to do so after a few days in culture (Franke *et al.*, 1979; Virtanen *et al.*, 1981). In the present investigation, we therefore used frozen sections of embryonic tissues to study the *in vivo* expression of IMFs in the parietal and visceral yolk sac endoderm of mouse conceptuses.

Results

In immunofluorescence microscopy of frozen sections, the PE cells showed both cytokeratin (Figure 1a,b) and vimentin (Figures 1d,e, and 3c) filaments at all stages studied. Also, after primary culture of 1–2 days, the PE cells consistently showed cytokeratin (Figure 1c) and vimentin (Figure 1f) filaments. Co-expression of both IMF types at a single cell level was also confirmed by double immunofluorescence microscopy (Figure 4 a–c). In contrast to the IMF pattern of PE cells, only cytokeratin filaments (Figure 2a–c) were revealed in frozen sections and primary cultures of VE cells; no vimentin could be seen in these cells (Figure 2 d–f).

In polyacrylamide gel electrophoresis, a distinct polypeptide band with mol. wt. 57 kd, probably representing vimentin, was observed in detergent-resistant cytoskeletal preparations of PE cells but not in those of VE cells (Figure 5). In two-dimensional gel electrophoresis, this polypeptide showed a mobility characteristic for vimentin and could be immunostained with monoclonal vimentin-antibodies (Figure 6, run 3).

A regular co-expression of cytokeratin and vimentin was observed in PYS-2 (Lehman *et al.*, 1974) PE cells (Figure 7a,b) and in PSA5-E (Adamson *et al.*, 1977) VE cells (Figure 7c,d), both cell lines derived from teratocarcinomas. This result is consistent with previous studies on established PE (Paulin *et al.*, 1980; Paulin, 1981; Oshima, 1982) and VE (Oshima, 1982) cell lines.

Discussion

In normal tissues *in vivo*, cells generally seem to express only one type of IMF (Lazarides, 1982; Osborn *et al.*, 1982). However, co-expression of two different IMFs has been shown in some vascular smooth muscle cells, which apparently contain vimentin and desmin (Schmid *et al.*, 1982), and in astrocytes, which seem to contain vimentin and glial fibrillary acidic protein (GFA) (Yen and Fields, 1981; Schnitzer *et al.*, 1981). Furthermore, certain cell types in pleomorphic adenomas of the parotid gland appear to react with antibodies against vimentin as well as with antibodies against cytokeratin (Caselitz *et al.*, 1981; Krepler *et al.*, 1982).

Here we show that PE cells of mouse conceptuses contain cytokeratin and vimentin filaments both *in vivo* and in culture. VE cells from the same conceptuses, on the other hand, contain only cytokeratin and no vimentin filaments. The presence of vimentin in the PE was also confirmed by one- and two-dimensional gel electrophoresis of detergent-resistant cytoskeletal preparations followed by immuno-

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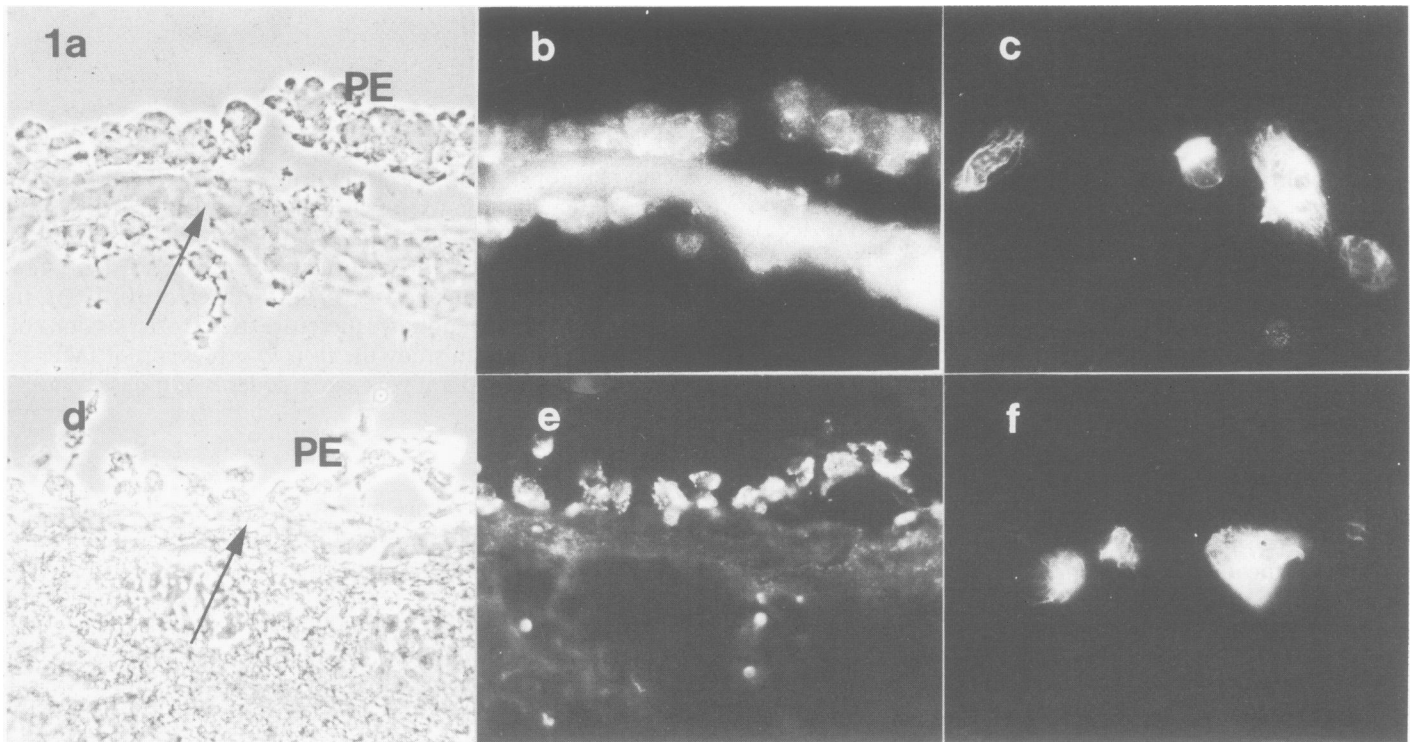


Fig. 1. The expression of IMF in the PE of 11-day mouse embryos. In frozen sections of the PE, indirect immunofluorescence microscopy reveals a positive reaction both with antibodies against cyokeratin (**b**) and with antibodies against vimentin (**e**). After 20 h in primary culture, the PE cells show a distinct fibrillar organization of both cyokeratin (**c**) and vimentin (**f**). **a,d**, phase contrast micrographs. Arrow, Reichert's membrane. **a–c, f**, x 600; **d–e**, x 380.

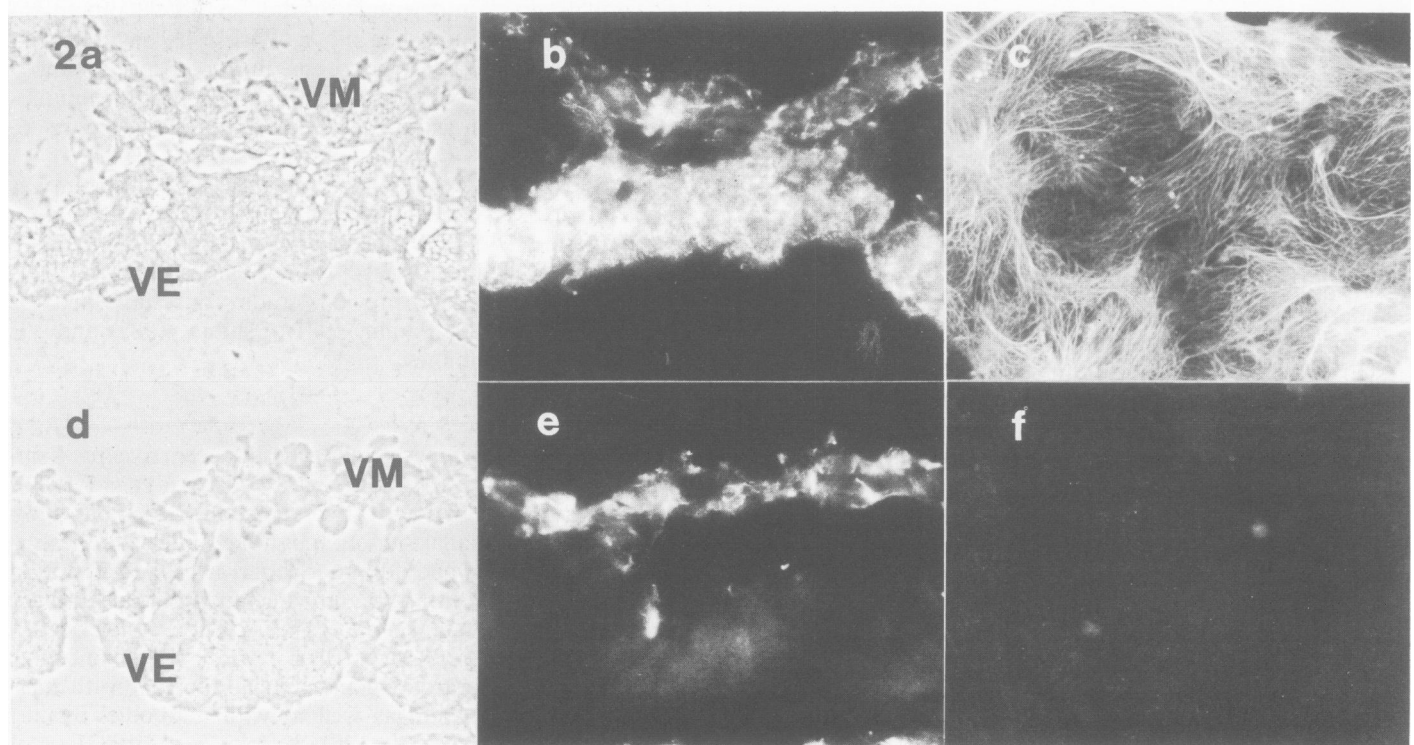


Fig. 2. The expression of IMFs in the VE and visceral mesoderm (VM) of 11-day mouse embryos. In frozen sections, a positive reaction can be seen in VE with antibodies against cyokeratin (**b**). Staining with vimentin antibodies results in a positive reaction in the VM while no reaction can be seen in VE cells (**e**). After 48 h in primary culture, the VE cells show abundant cyokeratin filaments (**c**) but no vimentin-specific fluorescence (**f**). **a,d**, phase contrast. **a–b, d–e**, x 600; **c,f**, x 380.

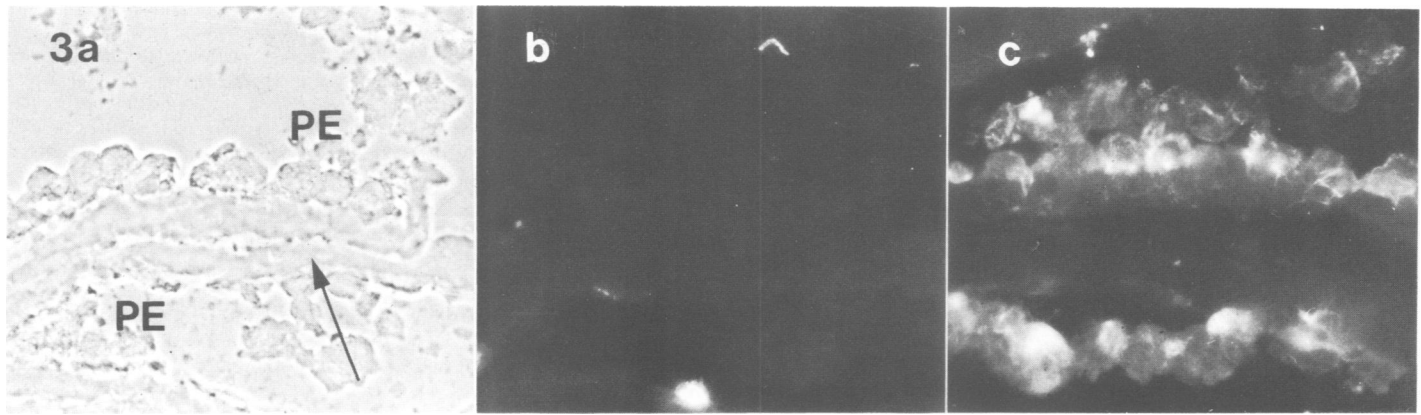


Fig. 3. PE of 11-day mouse embryos, frozen sections. Staining with preimmune rabbit serum gives a negative result (**b**) while rabbit vimentin antibodies reveal vimentin filaments (**c**) in the PE cells. **a**, phase contrast. Arrow, Reichert's membrane. $\times 600$.

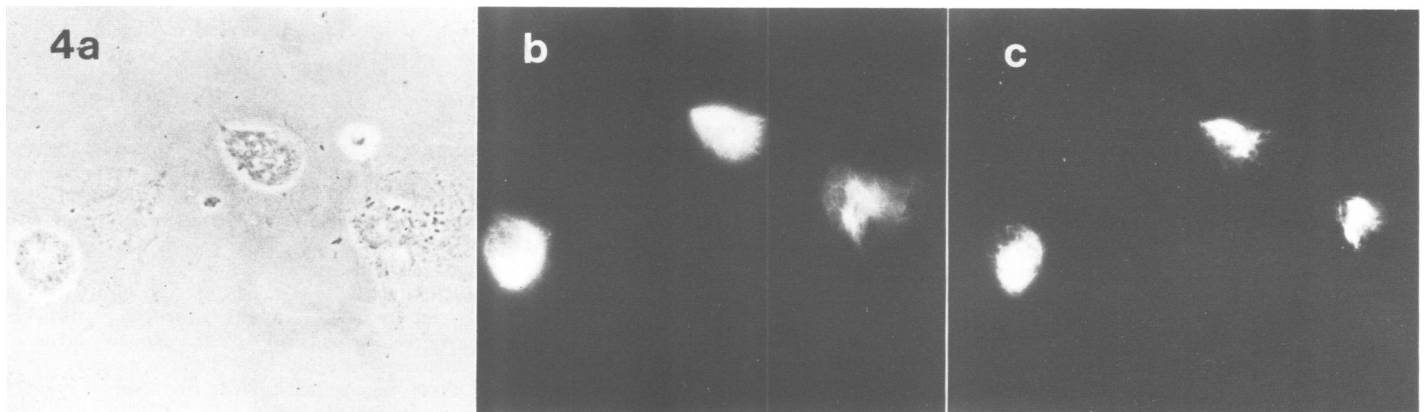


Fig. 4. PE cells of 11-day mouse embryos after 24 h in primary culture. Double immunofluorescence microscopy with rabbit cyokeratin antibodies (**b**) and monoclonal mouse vimentin antibodies (**c**). Both cyokeratin (**b**) and vimentin (**c**) filaments can be seen in PE cells. **a**, phase contrast micrograph. $\times 600$.

blotting. The cytoskeletal preparations (Figure 5) also contained polypeptide bands (mol. wt. ~ 46 and 54 kd) comparable to the X and Y cyokeratins that are found in early mouse embryo tissues (Jackson *et al.*, 1980, 1981; Franke *et al.*, 1982a). These polypeptides have been suggested (Howe *et al.*, 1982) to correspond to the Endo B and Endo A proteins, respectively, found in teratocarcinoma-derived endoderm cells (Oshima, 1981, 1982).

PE and VE cells have different morphologies and synthetic patterns *in vivo* (Dziadek and Adamson, 1978; Hogan and Tilly, 1981), and differences can also be detected in the corresponding established cell lines and even in appropriately treated F9 EC cells (Hogan *et al.*, 1981). The principal differentiation markers introduced in these investigations have included the synthesis of type IV procollagen (Adamson and Ayers, 1979), laminin (Timpl *et al.*, 1979) and plasminogen activator (Strickland *et al.*, 1976) (PE), and the synthesis of α -foetoprotein (Wilson and Zimmerman, 1976; Dziadek and Adamson, 1978) (VE). However, these markers may not be ideal for cell lineage studies, since VE cells also seem to synthesize small amounts of type IV procollagen (Adamson and Ayers, 1979), laminin (Hogan, 1980) and plasminogen activator (Bode and Dziadek, 1979). Furthermore, the synthesis of α -foetoprotein apparently is easily modulated (Dziadek, 1978). The present observations suggest that the differential expression of IMFs in PE and VE cells may provide a useful marker for studies on endodermal differentiation.

In the embryo, the PE cells grow relatively isolated with few intercellular contacts, while the VE cells form an epithelial layer with apical junctional complexes (Solter *et al.*, 1970; Enders *et al.*, 1978). Both cell types apparently arise from a common pool of primitive endoderm cells (Gardner and Papaioannou, 1975) and this differentiation seems to depend on cell interactions: experimental data suggest that, in order to express the visceral phenotype, the endoderm cells have to stay in contact with the underlying ectoderm, while those cells released from this contact differentiate to express the parietal phenotype (Hogan and Tilly, 1981; Gardner, 1982). Our present results show that the differentiation into PE, apparently involving cell migration, is associated with the appearance of vimentin in the endoderm cells. Whether the expression of vimentin is a requirement for cell migration or whether it begins first after the cells have reached the Reichert's membrane is currently unclear. A survey of the cytoskeletal structures in the zone of transition between PE and VE will help in unravelling the processes underlying the differentiation of the two layers of extra-embryonic endoderm.

Materials and methods

Isolation and culture of foetal membrane cells

The embryos were from natural matings and were (CBA/T6T6 \times C57Bl/6)F1. The parietal and visceral yolk sac membranes from 9-day post-coitum (15 somite) to 13-day post-coitum conceptuses were dissected into

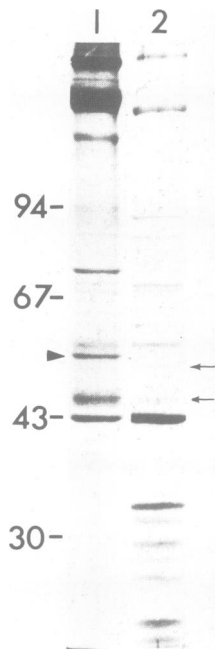


Fig. 5. SDS-polyacrylamide gel electrophoresis of detergent-resistant cytoskeletal preparations from PE and VE. PE cells, together with Reichert's membrane, as well as VE cells were isolated from 11-day mouse conceptuses. In PE cells (**lane 1**), a distinct polypeptide band (arrow head) with mol. wt. 57 kD, probably representing vimentin, can be seen. No polypeptide with similar mol. wt. can be seen in the VE cells (**lane 2**). Two polypeptide bands (mol. wt. ~46 kD and 54 kD), indicated by arrows, may correspond to the X and Y cytokeratins found in early embryonic mouse cells (see Discussion). Coomassie blue staining. The mol. wt. markers are indicated ($\times 10^3$): 30, carbonic anhydrase; 43 ovalbumin; 67, human serum albumin; 94, phosphorylase B.

NaCl-P buffer (140 mM NaCl, 10 mM sodium phosphate, pH 7.2). To separate the VE from the mesoderm, the membranes were incubated in 2.25% pancreatin, 0.75% trypsin for 20 min at 4°C. After ~60 min in Eagle's minimum essential medium (MEM), supplemented with 10% foetal calf serum (FCS) (Flow), the layers were teased apart and washed in the medium. For monolayer cultures, the PE and VE cells were dissociated with TVP (0.025% trypsin, 1 mM EDTA, 1% chick serum in phosphate-buffered saline, lacking Ca^{2+} and Mg^{2+}) (Bernstine *et al.*, 1973), and plated on gelatin-coated coverslips.

Cell lines and culture

The parietal yolk sac endoderm line PYS-2 (Lehman *et al.*, 1974) and the visceral yolk sac endoderm line PSA5-E (Adamson *et al.*, 1977) were provided by C.F. Graham (Department of Zoology, University of Oxford, UK). The cells were maintained in MEM, supplemented with 10% FCS. The cells were subcultured using TVP and plated in gelatin-coated plastic tissue culture dishes or glass coverslips.

Antibodies

Cytokeratin antibodies. Cytokeratin polypeptides were purified from human plantar callus. Antiserum, raised in rabbits, was affinity-purified as described elsewhere (Virtanen *et al.*, 1981).

Vimentin antibodies. Vimentin was isolated from cultured human fibroblasts. Antiserum was raised in rabbits and was affinity-purified as described elsewhere (Virtanen *et al.*, 1981).

Monoclonal vimentin antibodies. Monoclonal antibodies were raised against cytoskeletal proteins of a pig kidney epithelial cell line (LLC-PK, American Type Culture Collection, Rockville, MD). The cytoskeletal proteins were first enriched by extracting the cells with Triton X-100 and the low and high ionic salt buffers and then purified by a repeated polymerization/depolymerization procedure (cf., Lehto, 1983). Approximately 100 μg of cytoskeletal proteins, containing both cytokeratins and vimentin, were used to immunize BALB/c mice at 2 week intervals. After the second immunization, the spleens were dissociated and the cells were fused with the myeloma cell line P3/NS-1/1AG 4-1 according to Köhler *et al.* (1976). Antibody-producing hybrids were screened for activity against LLC-PK cytoskeletal polypeptides in enzyme-linked immunosorbent assay (ELISA) and by indirect immunofluorescence (IIF) with different types of cells. For the present study, one of the antibody-producing clones was selected, giving in IIF a typical vimentin-type of staining with cultured fibroblasts. These antibodies reacted with only

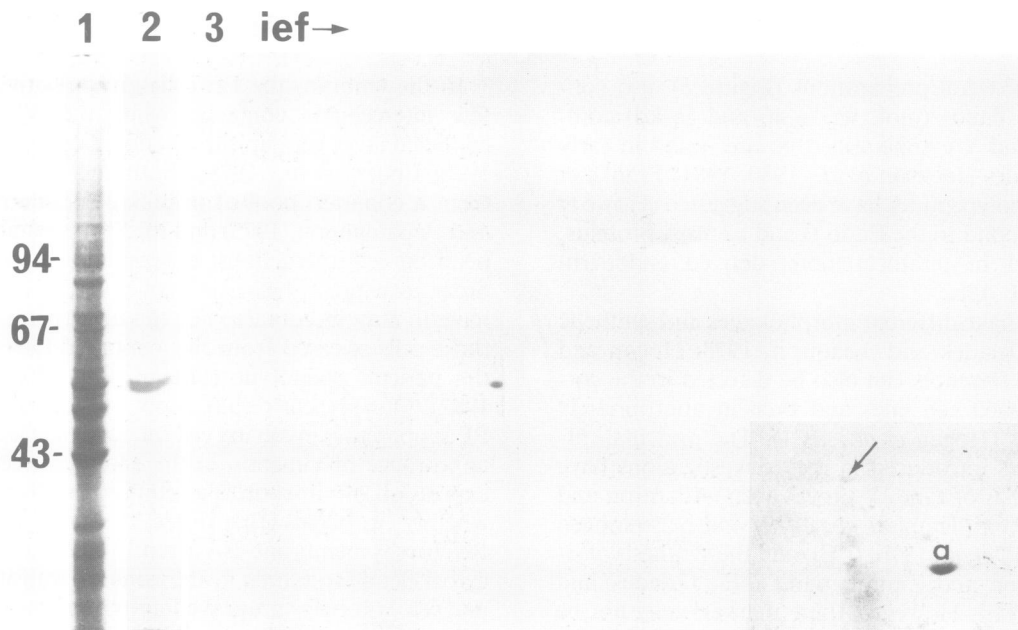


Fig. 6. Electrophoretically separated polypeptides of detergent-extracted 3T3 cells and cytoskeletal preparations from PE, transferred to nitrocellulose paper. After protein staining, several polypeptide bands are visible in the 3T3 cells (**lane 1**); in immunostaining of an identical run, only one polypeptide band (mol. wt. 57 kD) reacts with monoclonal vimentin antibodies (**lane 2**). In immunostaining of a blot of a two-dimensional run (isoelectric focusing followed by SDS-polyacrylamide gel electrophoresis) of PE cells, only one polypeptide spot reacts with the vimentin antibodies (run 3). Protein staining of an identical run (insert) shows that the mobility of the spot (arrow) corresponds to that of vimentin. Actin (a) is indicated for comparison. The mol. wt. markers are indicated ($\times 10^3$): 43, ovalbumin; 67, human serum albumin; 94, phosphorylase B.

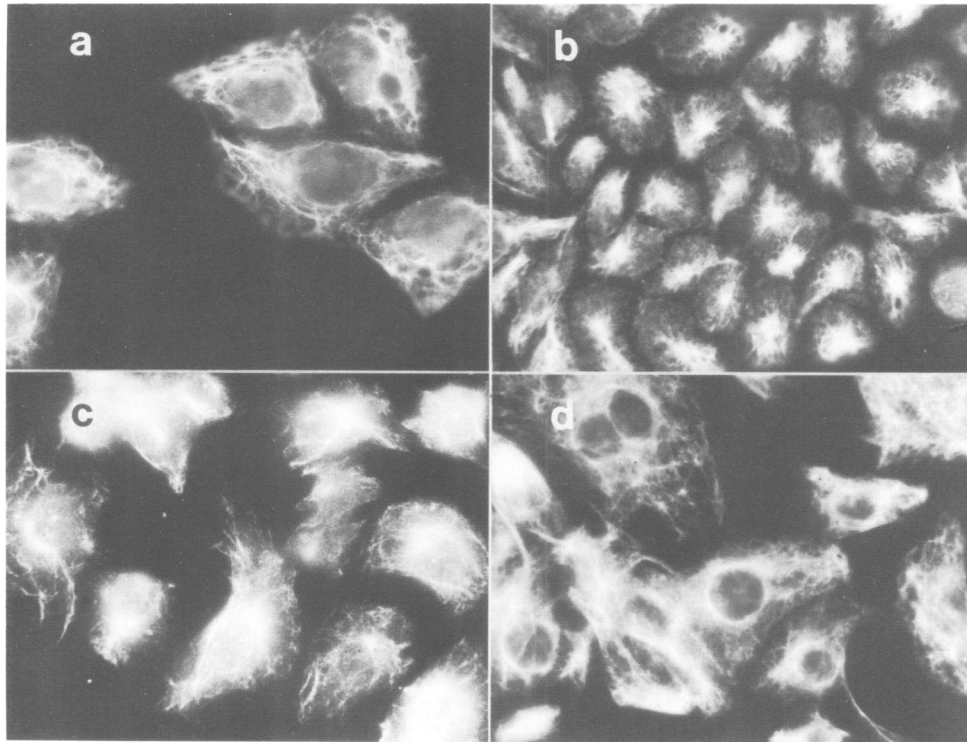


Fig. 7. The expression of IMFs in teratocarcinoma-derived endoderm cell lines. PYS-2 parietal yolk sac endoderm cells show fibrillar organization of cytokeratin (a) and vimentin (b). Similarly, PSA5-E visceral yolk sac endoderm cells express both cytokeratin (c) and vimentin (d). a, c–d, x 600; b, x 380.

vimentin in immunoblotting of 3T3 cells (Figure 6, lane 1,2), cultured human fibroblasts, and a variety of epithelial and non-epithelial cell lines (Virtanen *et al.*, in preparation). The antibodies (PK V1) are now commercially available from Labsystems OY (Helsinki, Finland).

Cytoskeletal preparations, gel electrophoresis and immunoblotting

To produce cytoskeletal preparations, the PE and VE cells, isolated from 10–15 embryos, were treated with 0.5% Triton X-100 in 50 mM Tris-HCl, pH 7.2, followed by treatment with low ionic, actomyosin extraction buffer, as described (Lehto *et al.*, 1978). SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970) and two-dimensional gel electrophoresis according to O'Farrell (1975). Immunoblotting experiments (Towbin *et al.*, 1979), using monoclonal vimentin antibodies, followed by peroxidase-conjugated rabbit anti-mouse IgG (Dakopats, Copenhagen, Denmark), were performed as described elsewhere (Lehtonen *et al.*, 1983). Amido black (0.1%) was used for protein staining of the polypeptide bands transferred to the nitrocellulose paper.

Immunofluorescence microscopy

Immunofluorescence studies were performed with two types of material. First, frozen sections were prepared from whole conceptuses or from the isolated membranes. Second, cells dissociated from the membranes were studied after 18–48 h in culture. For indirect immunofluorescence microscopy, the samples were fixed in -20°C methanol for 10 min and washed in NaCl-P buffer. The specimens were first exposed to rabbit antibodies against IMF proteins followed by tetramethyl rhodamine isothiocyanate (TRITC)-coupled swine anti-rabbit IgG (Dakopats, Copenhagen, Denmark). In double fluorescence experiments, the specimens were first exposed to rabbit cytokeratin antibodies followed by TRITC-coupled swine anti-rabbit IgG and then to monoclonal mouse vimentin antibodies followed by FITC-coupled rabbit anti-mouse IgG (Dakopats). The specimens were studied in a Zeiss Universal microscope equipped with an epi-illuminator III RS and filters for TRITC- and FITC-fluorescence. Control stainings with preimmune sera and with sera preabsorbed with the corresponding antigens consistently gave negative results (Figure 3a,b).

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Note added in proof

After the completion of this work, we learnt that Brigid Hogan *et al.* (ICRF, London) have also observed keratin and vimentin in PE cells (B.Hogan, personal communication).