Differentiation in human amniotic fluid cell cultures

II: Secretion of an epithelial basement membrane glycoprotein¹

J. M. MEGAW, J. H. PRIEST, R. E. PRIEST, AND L. D. JOHNSON

From the Department of Pathology and Laboratory Medicine, and Department of Pediatrics, Division of Medical Genetics, Emory University School of Medicine, Atlanta, Georgia 30322, U.S.A.

SUMMARY Cells obtained by amniocentesis for prenatal diagnosis were grown *in vitro* and examined for the presence of a glycoprotein component of epithelial basement membrane. Isolated colonies or clones of amniotic fluid-type cells secrete the glycoprotein, which was identified in association with the cells using indirect immunofluorescent antibody techniques. In addition, the glycoprotein was isolated from tissue culture medium and identified as a component of epithelial basement membranes by passive haemagglutination (PHA) and immunodiffusion assays. Fibroblast-type cells do not secrete the glycoprotein. These results correlate well with the synthesis of type IV collagen by amniotic fluid cells reported in the accompanying paper (Priest *et al.*, 1977) and indicate that amniotic fluid cells are epithelial in origin.

At the present time, cells cultured from human amniotic fluid are classified according to their growth and morphological characteristics as fibroblast-like, epithelial-like, and amniotic fluid. In order for these cells to be used effectively for prenatal diagnosis of inborn errors of metabolism, or for any other line of research, more specific criteria for distinguishing these cell types must be established. In conjunction with the studies of collagen reported in the preceding paper, cultured cells were examined for the presence of a glycoprotein component of epithelial basement membrane, which, if present in amniotic fluid cell cultures, could be used as a marker for defining cells of epithelial origin. The following studies were carried out on mass cultures as well as colony and cloned cultures classified as fibroblast- or amniotic fluid-type on the basis of their morphology.

Methods

CELL CULTURES

Cell cultures were derived and maintained as described in the preceding paper (Priest *et al.*, 1977). In addition, cultures for fluorescent antibody studies were grown in tissue culture slide chambers (Lab-Tek Products).

PROTEIN EXTRACTIONS

Growth medium from fibroblast or amniotic fluid mass cultures was harvested, and soluble epithelial basement membrane glycoprotein was extracted by the procedure of Johnson and Starcher (1972). The method was modified by using controlled pore glass (CPG-10-350) as a molecular sieve rather than agarose, and the column was eluted with 0.05 M phosphate buffer pH 7.2. Protein which eluted in the void volume was used to sensitize sheep erythrocytes for passive haemagglutination assays, or was dissolved in phosphate buffered saline and used in immunodiffusion assays.

IMMUNOLOGICAL METHODS

Antisera

Antisera used in the following assays were produced in New Zealand white rabbits against an epithelial basement membrane glycoprotein isolated from a murine teratocarcinoma cell line according to the method of Johnson and Starcher (1972), or extracted from murine kidney according to the method of Johnson and Warfel (1976). These antisera were shown to cross-react specifically with epithelial basement membrane of human origin (Johnson *et al.*, 1974).

(a) *Indirect fluorescent antibody techniques*, Cultures of human dermal fibroblasts, fibroblast-like-mass,

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colony, clone, amniotic fluid-mass, colony, and one primary amniotic fluid culture established from fetal membranes of a 14-week human spontaneous abortus were stained with fluorescein-labelled goat anti-rabbit serum after incubation with either non-immune rabbit serum, or antiserum to murine teratocarcinoma glycoprotein according to the procedure of Coons (1958). The antiserum to epithelial basement membrane glycoprotein was absorbed with fetal calf serum in order to decrease nonspecific background fluorescence. Each of the investigators examined each culture for the presence or absence of epithelial basement membrane glycoprotein. Presence of the glycoprotein was indicated by staining of extracellular material. Cultures were numerically coded before staining to prevent investigators from knowing the morphological classification of the cell types before examination of the slides.

(b) *Passive haemagglutination assays* Following standard tanning procedures (Stavitsky, 1954), aliquots of sheep erythrocytes were sensitized with the following antigens; human kidney glycoprotein, murine kidney epithelial basement membrane glycoprotein, murine teratocarcinoma epithelial basement membrane glycoprotein, and protein extracted from fibroblast-type or amniotic fluid-type cell culture medium. An aliquot of tanned cells not sensitized with any antigen was carried through the procedure as a control. Sera tested for reactivity with each class of sheep erythrocytes described above included: non-immune rabbit serum, antiserum to murine kidney epithelial basement membrane glycoprotein, unabsorbed or absorbed with protein extracted from the media of fibroblast-type cell cultures; and antiserum to murine teratocarcinoma epithelial basement membrane glycoprotein, unabsorbed or absorbed with fetal calf serum. Results were expressed as the final dilution at which haemagglutination occurred.

(c) *Immunodiffusion assays* Assays were performed according to the procedures of Ibrahim and Hammon (1968) and Megaw (1974). The antigens and antisera used were those described above for the passive haemagglutination assays. Absorbed or unabsorbed antisera were placed in the central wells of agar immunodiffusion slides. Test antigens were placed in the peripheral wells. After 24- or 48-hour incubations at 37°C in humid chambers, the slides were examined and photographed using an oblique lighting system.

Results

INDIRECT FLUORESCENT ANTIBODY STUDIES

The results of these studies are summarized in Table 1.

Table 1 *Immunofluorescence of human amniotic fluid cells*

Cell type	No. of cultures examined	No. of assays	No. of positive assays*
Dermal fibroblast	2	3	0
Fibroblast-mass	2	4	2
Fibroblast-colony or clone	4	7	0
Amniotic fluid-mass	3	5	5
Amniotic fluid-colony	5	11	11
Fetal amniotic membrane culture	1	1	1

*An assay was considered positive if specifically stained extracellular material was demonstrated.

Some cultures were assayed more than once. Amniotic fluid-type mass and colony cultures were positive for the presence of the specific extracellular material. Human dermal fibroblast and fibroblast-type colony and clone cultures failed to produce specifically staining material. Of two fibroblast-mass cultures examined, one produced specific extracellular material and one did not. A primary culture established from amniotic membranes of a human abortus was also positive for the presence of specifically staining extracellular material. When cultures were incubated with non-immune rabbit serum, no staining of extracellular material was shown.

Fluorescent micrographs of various cultures show the staining of extracellular epithelial basement membrane glycoprotein (Fig. 1). Fig. 1a shows an amniotic fluid-type colony stained with the specific antiserum. Specifically stained extracellular material appears as delicate bands around the cells. Fig. 1b shows the same culture stained with non-immune rabbit serum. No specifically stained extracellular material is present. Neither the dermal fibroblast culture (Fig. 1c) nor the fibroblast-type clone (Fig. 1d) contained epithelial basement membrane glycoprotein.

PASSIVE HAEMAGGLUTINATION ASSAYS

The results of the passive haemagglutination assays are summarized in Table 2. The unabsorbed antisera (columns 1 and 3) reacted with both the amniotic fluid and fibroblast-type proteins. However, when the antiserum to murine kidney glycoprotein was absorbed with the void volume protein isolated from growth medium in which fibroblast-mass cultures were grown, it continued to react with the amniotic fluid-type but no longer reacted with the fibroblast-type (column 2). Absorption of the high titre antiserum to murine teratocarcinoma epithelial basement membrane glycoprotein with fetal calf serum (column 4) reduced reactivity against the fibroblast-type protein to insignificant levels but did not significantly alter

Fig. 1 Immunofluorescent microscopy of cultured amniotic fluid cells. Strands and filaments of extracellular epithelial basement membrane glycoprotein surround amniotic fluid cells following incubation with antiserum specific for epithelial basement membrane glycoprotein (1a). Amniotic fluid cells incubated with nonimmune rabbit (1b); human dermal fibroblasts (1c) and fibroblast-type cells (1d), both incubated with specific antiserum, show no epithelial basement membrane glycoprotein. ($\times 210$.)

agglutination of cells sensitized with the amniotic fluid-type protein.

All of these antisera, when reacted with sheep erythrocytes sensitized with their respective homologous antigens, continued to react at dilutions considerably higher than those shown in the Table. Un-

sensitized tanned sheep erythrocytes, when tested with the different antisera or with non-immune rabbit serum, showed no agglutination. Further, when the sheep cells sensitized with the different antigens were tested against non-immune rabbit serum, no agglutination was observed. All antisera used in this assay, when tested against sheep erythrocytes sensitized with human kidney glycoprotein, gave strongly positive results, confirming the specific cross-reactivity of the antisera mentioned earlier.

Table 2 *Passive haemagglutinin assay of sheep erythrocytes sensitized with protein extracted from media from amniotic fluid or fibroblast type mass cultures*

Antigens	Antisera A-MK*	A-MK/F†	A-MTC‡	A-MTC/FCS§
F	1:8	0	1:16	1:4
AF	1:32	1:16	1:1024	1:512

Results are expressed as the final dilution of antiserum at which haemagglutination was noted.

*Antiserum to murine kidney epithelial basement membrane glycoprotein.

†Antiserum to murine kidney glycoprotein absorbed with fibroblast-type antigen.

‡Antiserum to murine teratocarcinoma epithelial basement membrane glycoprotein.

§Antiserum to murine teratocarcinoma epithelial basement membrane glycoprotein absorbed with fetal calf serum (FCS).

IMMUNODIFFUSION ASSAYS

Results of the immunodiffusion experiments are shown in Fig. 2. Antiserum to murine teratocarcinoma epithelial basement membrane glycoprotein, absorbed with fetal calf serum, was placed in the central well. Lines of identity developed between homologous murine teratocarcinoma epithelial basement membrane glycoprotein antigen (well A); murine kidney glycoprotein (well B); and amniotic fluid-type protein (well C), but no precipitin line developed against the fibroblast-type protein (well D).

Fig. 2 Immunodiffusion of anti-epithelial basement membrane glycoprotein against glycoproteins isolated from cultures of amniotic fluid-type and fibroblast-type cells. The centre well contained antimurine teratocarcinoma epithelial basement membrane glycoprotein absorbed with fetal calf serum. Lines of identity are present between homologous glycoprotein (well A), murine kidney epithelial basement membrane glycoprotein (well B), and glycoprotein isolated from amniotic fluid-type cultures (well C). No precipitin line developed against glycoprotein isolated from fibroblast-type cells (well D).

Similar results were obtained when antiserum to murine kidney epithelial basement membrane glycoprotein, absorbed with fibroblast-type protein, was placed in the centre well. When unabsorbed antisera were used, reactivity was noted against both the amniotic fluid and fibroblast-type antigens.

Discussion

The results of these studies clearly indicate that fibroblast- and amniotic fluid-type cells obtained by amniocentesis are of different origin. Cells designated amniotic fluid are probably shed from surface or lining epithelial layers of the fetus since they secrete a component of epithelial basement membranes. Cells capable of secreting epithelial basement membrane glycoprotein are undoubtedly sloughed into the amniotic fluid from the skin, lower urinary tract, or gastrointestinal tract. Hoehn *et al.* (1975) have shown that fetal urine is a potential source of amniotic fluid-type cells. Potier *et al.* (1975) showed intestinal glycosidases in human amniotic fluid, indicating that desquamated intestinal cells are present.

Cultured cells can clearly be identified as either fibroblast-type or amniotic fluid-type only after

they have been subcultured from isolated colonies or cloned from single cells. Though mass cultures appear morphologically to be composed of only one cell type, examination by fluorescent immunohistochemistry indicated that this was not always the case. Occasional mass cultures morphologically designated as fibroblast-type contained small clusters of cells which were surrounded by epithelial basement membrane glycoprotein. The glycoprotein isolated from the medium of cultures was obtained from mass cultures, thus explaining the small amounts of epithelial basement membrane glycoprotein extracted from fibroblast-type cultures.

These studies, in conjunction with those described in the preceding paper, indicate the necessity of defining cells obtained by amniocentesis by measurements in addition to morphological and growth characteristics. Some defects in collagen metabolism cannot be studied in amniotic fluid-type cells nor can defects expressed only in epithelial cells be studied in fibroblast-type cells. The morphological characteristics of mass cultures appear to reflect the predominant cell type, but do not always indicate the presence of only one cell type.

Address reprint requests to L. D. Johnson, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia 30322, U.S.A.

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