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Differentiation in human amniotic fluid cell cultures:

I: Collagen production[1]

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SUMMARY The collagen produced by differentiated cells cultured from human amniotic fluid was characterized in two ways. By chain composition and by 4-hydroxyproline:3-hydroxyproline isomer ratio, the collagen synthesized by F-type (fibroblast) cells was indistinguishable from that made by cultured fetal dermal fibroblasts. The predominant cells in young amniotic fluid cultures, termed AF-type, produced collagen with a lower isomer ratio, resembling that of basement membrane collagen. The chain composition, as determined by chromatography on carboxymethyl cellulose, varied for different cultures of the AF-type, but the major pattern was consistent with that of basement membrane collagen. On the basis of these characteristics, F cells are of fibroblast origin, whereas most AF cells are of a different origin either endothelial or epithelial. Other evidence (Megaw *et al.*, 1977) suggests an epithelial origin for AF cells.

Studies using human amniotic fluid cell culture as an aid to prenatal diagnosis are increasing in complexity. We began to investigate collagen synthesized by these cell cultures because of the need for prenatal diagnosis of inherited diseases affecting collagen. Initial studies in this laboratory had shown increased solubility of collagen produced by cultured dermal fibroblasts from Marfan syndrome compared with controls (Priest *et al.*, 1973), and the question arose whether this or other disorders of connective tissue could be diagnosed in cultured amniotic fluid cells. Therefore, further studies were performed on collagen laid down in the cell layer and extracted by acid or neutral salt, or on collagen released into culture medium by two types of fetal cells cultured from amniotic fluid, F (fibroblast) and AF (amniotic fluid) types (Hoehn *et al.*, 1974).

We report here that the F and AF cells, distinguished from each other originally on the basis of morphology and growth characteristics, also differ biochemically in the types of collagen formed in culture. We also report another biochemical difference (Megaw *et al.*, 1977), the synthesis and extracellular release of specific epithelial basement membrane glycoprotein by AF but not by F cells in culture.

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Materials and methods

L-proline-(^{3}H) (G), specific activities ranging from 500 to 1000 mCi/mmol, was obtained from Amersham/Searle Corp.; β-aminopropionitrile fumarate (βAPN) from Abbott Laboratories and Sigma Chemical Co.; N-ethyl maleimide (NEM) and phenylmethylsulfonyl fluoride (PMSF) from Sigma; and carboxymethyl cellulose microgranular CM32 from Whatman. *Trans*-4-hydroxy-L-proline was obtained from British Drug Houses, and *trans*-3-hydroxy-L-proline was a gift from Dr E. Adams, Department of Biological Chemistry, University of Maryland.

CELL CULTURES

Human dermal fibroblast cultures previously established in our laboratory from skin biopsies were retrieved from storage in liquid nitrogen.

Amniotic fluid obtained by amniocentesis was placed in primary culture (Priest, 1977) in Dulbecco and Vogt's modification of Eagle basal medium (D & V) with 25% fetal bovine serum (Flow Laboratories). After diagnostic studies of chromosomes were performed, cultures with no chromosomal abnormalities were used for the present study. These were designated as mass cultures of either F or AF type by their morphology and culture characteristics.

Other cultures of F or AF types were kindly supplied by Drs H. Hoehn and G. M. Martin (Department of Pathology, University of Washington, Seattle). These had been selected by isolation from a single cell or isolation as colonies after dilute plating. Cultures grown from an isolated single cell or subcloned are designated as cloned cultures in the present report, whereas Hoehn and Martin's other isolations, as well as colonies isolated by dilute plating in our laboratory, are called colony cultures.

PREPARATION OF RADIOACTIVELY-LABELLED COLLAGEN

After cells reached confluency in Bellco roller bottles (690 cm^2 surface area) or in Falcon flasks (75 cm^2) in D & V medium supplemented with 15% fetal bovine serum, new medium was added. This contained 1 or 5 μCi/ml ^{3}H-proline, $1{\cdot}25 \times 10^{-4}$ M sodium ascorbate to promote collagen synthesis, and 1×10^{-4} M βAPN to inhibit crosslinking of collagen, thus making it more extractable. Cells were labelled for one or two weeks.

EXTRACTION OF COLLAGEN FROM CELL LAYERS

Cells were rinsed and scraped into 20 ml of 0·5 M acetic acid or 1·0 M NaCl. Collagen was extracted by reciprocal shaking at 60 cycles/min for 1 to 2 days at 4°C. Acetic acid extracts were centrifuged at 10 000 $\times$ g for 30 min and neutral salt extracts at 38 000 $\times$ g for 2 hours; supernatant solutions were stored at 4°C for analysis. Neutral salt solutions used for extraction and storage contained protease inhibitors (0·5 mM PMSF, 10 mM NEM) as well as 20 mM EDTA and were buffered at pH 7·5 with 0·05 M tris-HCl.

PREPARATION OF COLLAGEN FROM MEDIUM

Solid ammonium sulphate (20 g/100 ml medium) was added slowly with stirring. The precipitate after standing overnight was collected by centrifugation at 20 000 $\times$ g for 30 minutes, then dissolved in a volume of 0·5 M acetic acid $\frac{1}{10}$ that of original medium. The retentate obtained after dialysis against 0·5 M acetic acid was clarified by centrifugation at 38 000 $\times$ g for 2 hours and saved. All procedures were done at 4°C. In some later experiments, protease inhibitors were included, and the ammonium sulphate precipitate was dissolved in and dialysed against the neutral salt solution already described.

CHROMATOGRAPHY ON CARBOXYMETHYL CELLULOSE (CMC)

Non-radioactive carrier collagen (20 to 30 mg) was added to the acetic acid extract of the cell layer from one roller bottle. Collagen in the mixture was precipitated by adding 1 g solid sodium chloride to each 10 ml extract and allowing the mixture to stand for 2 to 3 hours at 4°C. The precipitate was collected by centrifugation at 10 000 $\times$ g for 25 minutes at 4°C, redissolved in 20 ml of 0·5 M acetic acid, and dialysed overnight at 4°C against one litre of start buffer, 0·04 M (Na^+) sodium acetate, pH 4·8, containing 1·0 M urea. The retentate was warmed to 43°C and centrifuged at low speed to remove any undissolved material. The supernatant solution, warmed to 43°C to denature the collagen, was chromatographed according to Miller (1971). Absorbance at 225 nm of the carrier collagen was monitored as reference for the radioactive peaks of collagen produced by the cultures. A 0·5 ml aliquot of each of the 5 ml fractions collected from the column was counted in the scintillation mixture of Prockop and Ebert (1963). Proportions of the radioactivity in alpha-1 and alpha-2 collagen chain regions were determined by plotting the peaks on heavy paper of uniform thickness, cutting out the peaks, and weighing them.

DETERMINATION OF RADIOACTIVE 4-HYDROXYPROLINE AND 3-HYDROXYPROLINE

A preparation of cell layer or medium collagen was rotary-evaporated to dryness and transferred to a hydrolysis tube with 10·0 ml of 6 N hydrochloric acid. The sample tube was flushed with nitrogen, sealed, and the sample was hydrolysed at 108°C for 24 hours. The hydrolysate was cooled, rotary-evaporated to dryness, and taken up in 0·01 N HCl. Isomer separation was achieved at 56°C with 0·35 N sodium citrate buffer (pH 2·400 $\pm$ 0·002) on LCR-2 resin in the 55 cm column of a J.E.O.L. 6AH amino acid analyser. Fractions were collected (using a stream splitter) at 2-minute intervals, and a 0·5 ml aliquot of each fraction was counted in the scintillation mixture previously described. Radioactivity patterns were plotted on charts of absorptivity (570 and 440 nm) of ninhydrin-reacted column effluent. Elution positions of 4- and 3-hydroxyproline were established by co-chromatography with authentic standards. Areas of the radioactive 4- and 3-isomer peaks were determined by triangulation after replotting on graph paper, and the ratio of 4- to 3-hydroxyproline in the collagen was calculated.

Results and discussion

CELL MORPHOLOGY

We identified our cell cultures as F or AF types using the terminology of Hoehn *et al.* (1974). Several other classifications have been made (Uhlendorf, 1970; Schmid, 1972; Sutherland *et al.*, 1974) and, in general, most of the cell types described by these

workers can be identified with the types described by Hoehn *et al.* The F cells are of spindle shape and form a swirled pattern of parallel arrays when confluent, resembling cultured dermal fibroblasts. The AF cells predominate in most diagnostic amniotic fluid cultures. These cells are less spindle-shaped, are more pleomorphic, and have different primary colony morphology. The tissue of origin is not known. Typical epithelioid (E-type) cells also grow from amniotic fluid, but cannot be subcultured readily using trypsin, and we have not studied them.

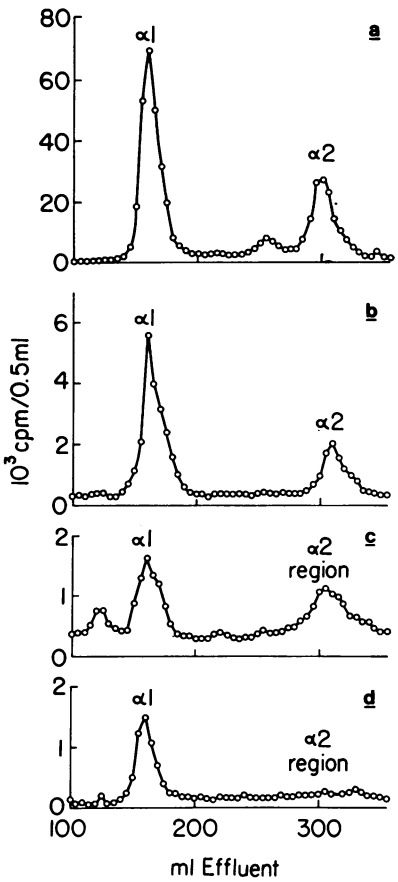

Fig. 1 *Patterns of elution from CMC of collagen extracted by acid from cell layers of three types of cultures: (a) fetal dermal fibroblast, (b) F-type amniotic fluid cell culture, (c) and (d) AF-type amniotic fluid cell cultures, showing two types of patterns obtained—(c) broad peak with shoulders in alpha-2 region; (d) very small amount of radioactivity in the alpha-2 region. Cultures were labelled with ^{3}H-L-proline (1 μCi/ml) in the presence of a lathyrogen. The collagen was denatured at 43°C for chromatography.*

CHAIN COMPOSITION OF COLLAGENS

When labelled alpha chains of cell layer collagen were separated by CMC chromatography, cultures in which F cells predominated produced collagen with alpha-1 to alpha-2 chain ratios between 2·5 and 3·0 to 1. These ratios resembled those of collagen from dermal fibroblasts, and results were consistent whether mass cultures or clones were examined. Typical chromatograms from a culture of F-type cells and from fetal dermal fibroblasts are compared in Fig. 1b and 1a. The consistency of these findings contrasted with results obtained using preparations from cultures with AF-type cells predominating. The chromatographic tracings varied widely among cultures. Some yielded chromatograms with a radioactive peak in the position of alpha-1, but little or no radioactivity in the position of alpha-2 (Fig. 1d). More often a small, broad peak was present in the region of alpha-2, which was even quite large in some cultures (Fig. 1c). This variability might be expected in mass cultures if they contained small, but variable proportions of F-type cells. However, as seen from Fig. 2, colonies and clones of different AF-type cultures showed even greater variability than mass cultures. This result cannot be explained

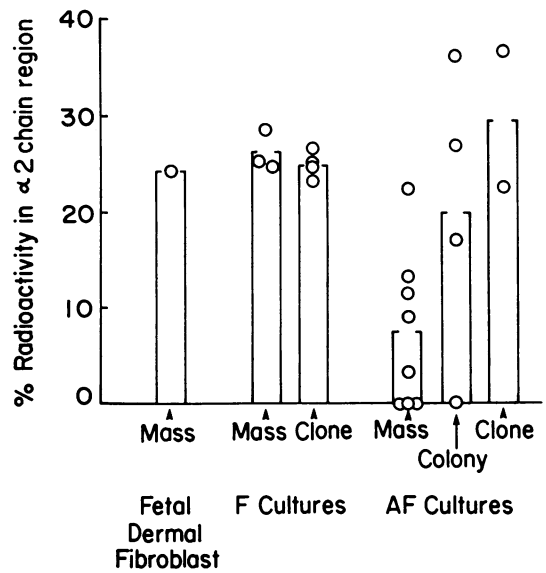

Fig. 2 *The alpha-2 region radioactivity (expressed as a percentage of the total alpha-1 plus alpha-2 chain region radioactivities in chromatograms like those of Fig. 1) in the acid-extractable collagen from the cell layer of fetal dermal fibroblast and various types of amniotic fluid cell cultures. Each point is for a different culture. Tops of bars represent mean values.*

by the presence of F-type cells. Other explanations could account for the variability. For instance, there may be more than one type of AF cell. Alternatively, differences in culture conditions could be an explanation since it has been shown that chondrocytes, as they become older in culture, may produce a collagen like that of dermal fibroblasts (Mayne *et al.*, 1975). It will be necessary to establish the identity of these collagen chains by other means, such as peptide patterns after cleavage with cyanogen bromide, in order to explain the observed variability among cultures of AF-type cells. However, it is clear that the collagens produced by F cells and by fetal dermal fibroblasts are similar in chain composition and most closely resemble type I collagen (Trelstad, 1974). It is also clear that collagen produced by most AF cells is different in chain composition, resembling that of the type IV collagen of epithelial and endothelial basement membranes described by Kefalides (1971).

4-HYDROXYPROLINE:3-HYDROXYPROLINE ISOMER RATIO

In order to classify this collagen further, the content of radioactive 4-hydroxyproline and 3-hydroxyproline in collagen preparations from cell layer and medium of several kinds of cells was determined. The 4-hydroxy isomer predominates in all types of collagen, but the degree of preponderance varies with different collagen types. Fig. 3 shows the results for cell layer collagen, and Fig. 4 presents similar results for collagen in the culture medium, in both cases expressed as a ratio of 4-hydroxyproline to 3-hydroxyproline. The collagen made by F cells is similar to that synthesized by fetal and other dermal fibroblasts in that the ratio is about 60:1 for cell layer and 84:1 for medium. These values are comparable to those of collagen of human dermis (Pinnell *et al.*, 1972). By contrast, average ratios of 13:1 and 14:1, respectively, were obtained for cell layer and medium collagen made by mass and colony cultures of AF cells. These values are comparable to those found for collagen of basement membranes, 11:1 to 19·5:1 (Kefalides, 1971). Also, our lowest values for AF cultures were roughly comparable to those found for basement membrane collagen produced during short-term radioactive labelling *in vitro* of a number of epithelial and endothelial cell systems, 5:1 to 8:1 (Clark *et al.*, 1975; Grant *et al.*, 1972; Grant *et al.*, 1975; Kefalides *et al.*, 1976). One cloned and one colony AF culture had a 4:3 ratio for cell layer collagen which was relatively high

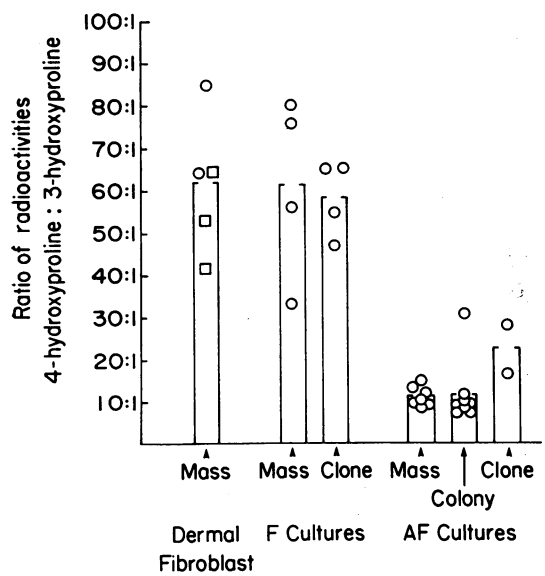

Fig. 3 *The proportions of radioactive 4- and 3-hydroxyproline in collagen extracted by acid or neutral salt solution from the cell layers of various types of cultures. Each point is for a different culture.* □ *denotes non-fetal cultures, i.e. from newborn or older children. Tops of bars represent mean values.*

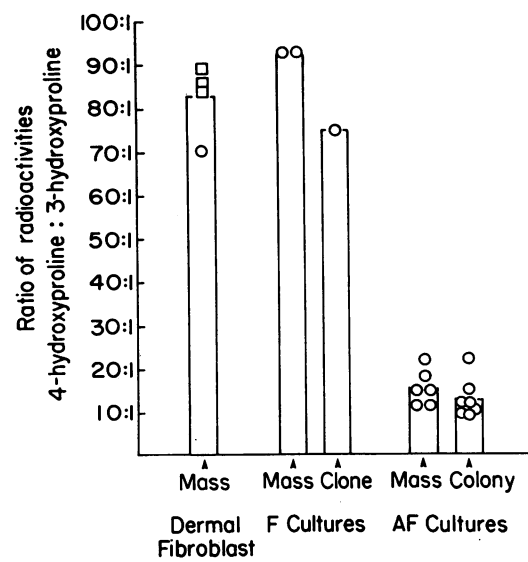

Fig. 4 *The proportions of radioactive 4- and 3-hydroxyproline in collagen prepared from the culture medium of various types of cultures. Each point is for a different culture, most of which are also represented in Fig. 3.* □ *denotes non-fetal cultures. Tops of bars represent mean values.*

Again, these selected cultures may reflect heterogeneity of AF cell type. However, collagen made by mass AF cultures is clearly different from that made by early mass F cultures, not only in the lower 4:3-hydroxyproline isomer ratio, but also in the lower or absent alpha-2 chain region radioactivity already discussed. Thus, it more closely resembles basement membrane (type IV) collagen than it does type I collagen. Though the fetal tissue of origin of AF cells is not known, the foregoing results suggest either an endothelial or an epithelial origin. Other evidence (Megaw *et al.*, 1977) supports the view that AF cells are of an epithelial type, since they produce epithelial basement membrane glycoprotein.

COLLAGEN PRODUCTION AND CULTURE AGE

Several unsuccessful attempts were made to determine collagen characteristics of a mass AF culture as it aged, to see if changes occurred. In one case the culture was overgrown quickly by F cells, and in other attempts the culture selected reached senescence before successive tests could be done. Several F-type lines were tested at different culture ages: no consistent trend of differences was found in the chain composition, but the 4:3 isomer ratios declined as the cultures aged. This finding was true also of the one fetal dermal fibroblast culture tested. Therefore, the data presented in Fig. 3 and 4 are from the earliest tests done on each culture. We do not know if this decline in ratio was truly an effect of culture ageing or if it was the result of an undetermined change in culture or experimental conditions. In any case, it seems important to perform collagen studies as early as possible in the culture life of amniotic fluid cells when the 4:3 ratio appears to be a suitable marker for distinguishing biochemically F and AF cells.

PRENATAL DIAGNOSIS OF COLLAGEN DISORDERS

Usefulness of these findings for prenatal diagnosis of collagen diseases may be limited in specific cases by the type of cell predominating in a particular culture of amniotic fluid. When F cells predominate, they might be suitable for diagnosis of diseases affecting collagen of dermis and other tissues from which cultures of fibroblast-like cells can be grown. But AF cells usually predominate in early cultures used for prenatal monitoring. They make a different type of collagen resembling that of basement membrane. It is as yet unclear how this type of cell may be useful in the investigation of collagen disorders. Nevertheless, for correct prenatal diagnosis of connective tissue disorders, the 'normal' amniotic fluid cells chosen as controls should be of the same culture type as the unknown cells to be tested.

Addendum

During preparation of this manuscript, there appeared a report (Hurych *et al.*, 1976) presenting studies performed on one amniotic fluid culture 'after 19 weeks of cultivation'. From a determination of the chain ratio, the authors concluded that this culture synthesized type I collagen, and they then discussed possible applications to prenatal investigation of inherited disorders of collagen. We believe the problems inherent in prenatal diagnosis of collagen disorders using amniotic fluid cultures are more complex than suggested by this report.

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