

## FIBRONECTIN IS PRODUCED BY HUMAN MACROPHAGES\*

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Mononuclear phagocytes are commonly considered to have important functions in the regulation of specific immune responses as well as in scavenging tissue and body fluids (1-3). The molecular mechanisms of these phenomena are not fully understood, but on the other hand, cultured macrophages are known to produce a number of defined substances that may be involved in macrophage functions in vivo. These substances include proteolytic enzymes, as well as both activators and inhibitors of specific proteases (4-9).

Clearance of body fluids of microparticulate fibrin and denatured collagen (gelatin) has been recently shown to be promoted by a high molecular weight plasma protein (10, 11), fibronectin, that is also present in connective tissues (12). We now show that cultured macrophages, derived from human blood monocytes, synthesize and secrete fibronectin, but do not deposit a pericellular fibronectin matrix as do various connective tissue cells in culture.

### Materials and Methods

*Cell Cultures.* Human monocytes were prepared from the buffy-coat fractions of citrated blood from healthy donors, supplied by the Finnish Red Cross Blood Transfusion Service, Helsinki, Finland. Mononuclear leukocytes were isolated as described before (13), suspended in warm serum-free medium 199 (Gibco-Biocult Ltd., Glasgow, Scotland) supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin, and seeded at  $1.0-2.0 \times 10^6$  cells/cm<sup>2</sup> on glass coverslips or directly on plastic Petri dishes (A/S Nunc, Roskilde, Denmark). After 90 min at 37°C, loosely attached cells were removed by four successive washes with warm phosphate-buffered saline (PBS)<sup>1</sup> and the adherent cells were fed with 5 ml of serum-free medium consisting of one part medium 199 and one part RPMI-1640 (Gibco-Biocult Ltd.). After overnight incubation at 37°C, which resulted in some more cell detachment, the cultures were refed with medium containing 10% fetal calf serum. Later on, fresh serum-containing medium was changed in the cultures every 2-3 d.

More than 95% of the cells remaining in the culture dishes after overnight incubation in the serum-containing medium were monocytes as judged by morphology, staining for the nonspecific esterase (14), and phagocytosis of latex particles (Dow-Latex, 1.1-µm, diameter; Serva Feinbiochemica, Heidelberg, Federal Republic of Germany) (9). Definite differentiation into macrophage-like cells normally required 4-7 d in culture. With a longer incubation time, the cell size further increased, but no mitoses were seen. The level of [<sup>3</sup>H]thymidine incorporation into DNA (13) was minimal in these cultures. Two main morphological types of cells could be seen in the differentiated cultures. First, large bipolar or triangular macrophages, and secondly, rounded or multiangular, tightly adherent cells with flat, barely visible cytoplasm (Fig. 3 B, C). These types of differentiated cells will be later referred to as fibroblastoid and epithelioid cells, respectively. More detailed description of the characteristics of the differentiated cells will be reported elsewhere.

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<sup>1</sup> Abbreviations used in this paper: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

For metabolic labeling of macrophage proteins, 10  $\mu\text{Ci/ml}$  of both L-[5- $^3\text{H}$ ]proline and L-[2- $^3\text{H}$ ]glycine (Radiochemical Centre, Amersham, England) were used in serum-free Eagle's minimal essential medium containing 0.05% bovine serum albumin. Harvested culture media were freed from detached cells by a brief centrifugation and stored at  $-20^\circ\text{C}$  if not immediately analyzed. Human adult skin fibroblasts used as control cells were cultured as described (15).

*Immunological Techniques.* Anti-human fibronectin antisera were prepared as described (15) and absorbed before use with agarose-conjugated fetal calf serum to block cross-reactivity with bovine fibronectin present in culture medium.

Fibronectin was immunoprecipitated from the radiolabeled culture medium by the double-antibody method as described (16). The specificity of the precipitation was controlled by using preimmune sera, as well as by a blocking reaction with the aid of nonlabeled antigen. Affinity-purified antibodies to the interstitial procollagens type I and type III and to basement membrane collagen type IV were kindly provided by Dr. R. Timpl, Max-Planck-Institut für Biochemie, Martinsried, Federal Republic of Germany and were used as described (15, 17).

Immunofluorescence methods followed previously published procedures (15). For selective staining of extracellular antigens, the cell layers were rinsed with cold ( $4^\circ\text{C}$ ) PBS, overlaid with the antisera or antibodies in the cold, fixed with 3% paraformaldehyde at room temperature, and incubated with fluorescein-conjugated anti-rabbit antiserum. For visualization of both intra- and extracellular antigens, the cell layers were fixed with paraformaldehyde and ice-cold acetone before staining.

Fibronectin in culture media was quantitated using anti-human fibronectin in a double antibody radioimmunoassay as described (18). Fetal calf serum was negative for fibronectin in this radioimmunoassay.

*Analysis of Radiolabeled Proteins.* Proteins were precipitated from cell-free culture media with 176 mg/ml of  $(\text{NH}_4)_2\text{SO}_4$  in the presence of proteinase inhibitors (19) and 50  $\mu\text{g/ml}$  gelatin (Sigma Chemical Co., St. Louis, Mo; type I) as carrier. For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the samples were dissolved in a sample buffer containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 50 mM Tris-HCl, pH 6.8, and trace amounts of bromophenol blue by heating the samples and the buffer in a boiling water bath for 3 min. The samples were analyzed in a 5% or 12% polyacrylamide gel according to Laemmli (20). After electrophoresis, the gels were impregnated with PPO (21), dried onto filter paper, and exposed to a pre-flashed X-Omat film (Eastman Kodak Co., Rochester, N. Y.) (22).

For peptide mapping, radiolabeled fibronectin synthesized by macrophage cultures was isolated from the culture medium by adsorption to gelatin-Sepharose (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) beads (23) and subsequent purification by electrophoresis in a 5% polyacrylamide gel (24). Digestion with *Staphylococcus aureus* protease V8 (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ill.) was performed in conditions described by Cleveland et al. (24), at enzyme concentrations of 0.1–1.0  $\mu\text{g/ml}$ . The substrates, the radiolabeled polypeptide bands, located with the aid of autoradiograms of dried gels, were cut out from the gel and minced into the digestion buffer containing the enzyme.

Digestions of the proteins with pepsin and collagenase was as described (25). Cell surface iodination followed the procedures that have been described (26).

## Results

*Detection of Newly Synthesized Fibronectin in the Medium of Macrophage Cultures.* 1- to 2-wk-old cultures of human monocytes that were well-differentiated into macrophages, were labeled overnight with tritiated glycine and proline. Labeled polypeptides from the culture medium were precipitated with ammonium sulphate and analyzed by electrophoresis in polyacrylamide gels followed by scintillation autoradiography. Several large molecular weight polypeptides were seen: at 220,000 dalton, at 165,000 dalton, at 130,000 dalton; a heavily labeled band at 100,000 dalton and smaller-sized polypeptides were also seen (Fig. 1, lane 4). The 220,000-dalton polypeptide frequently resolved as a closely spaced doublet and migrated slightly slower than the subunit of plasma fibronectin (Fig. 1, lane 8).

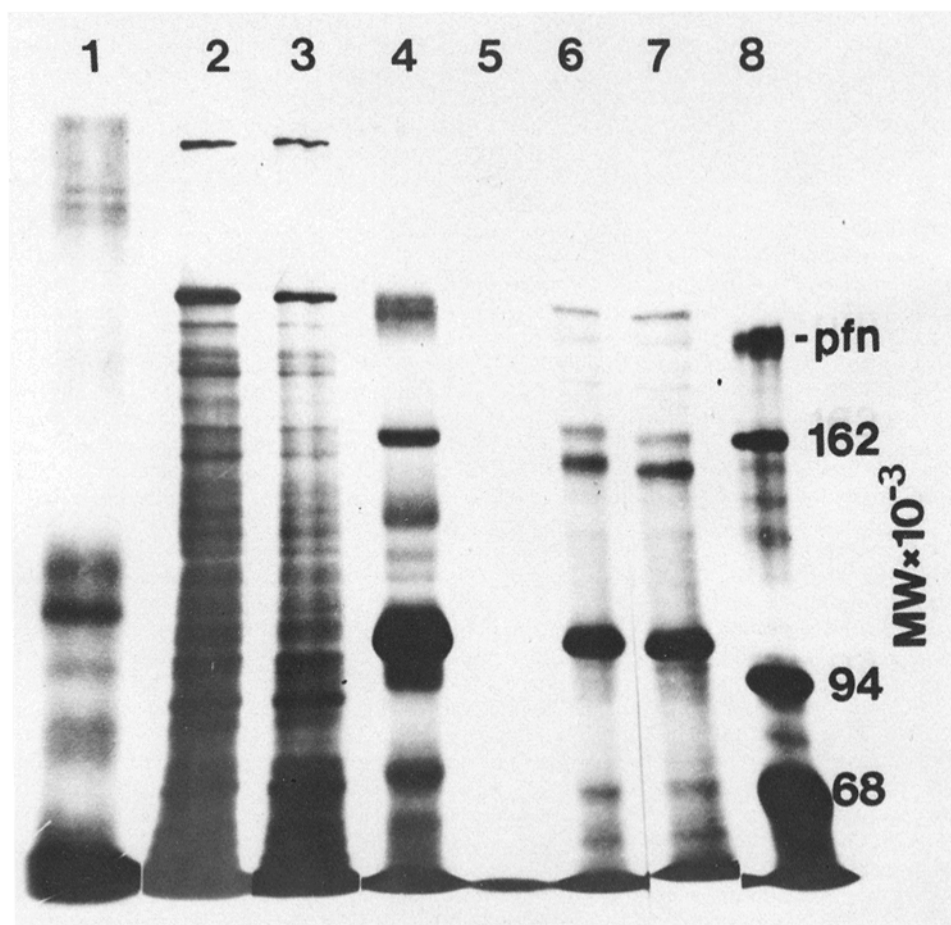


FIG. 1. SDS-polyacrylamide gel electrophoresis of labeled polypeptides in macrophage cultures. Lane 1: Surface-iodinated polypeptides of cell layers of 10-d-old macrophage cultures. Lanes 2 and 3: Cell layers of macrophage cultures labeled for 12 h with tritiated glycine and proline without (lane 2) or with (lane 3) subsequent trypsin treatment (0.25%, 5 min at 37°C). Lane 4: Polypeptides precipitated with ammonium sulphate from medium of macrophages metabolically labeled for 12 h with the above amino acids. Lane 5: Labeled culture medium treated with pepsin. Lane 6: Labeled culture medium treated with collagenase. Lane 7: Control incubation without collagenase and with buffer only. Note processing, particularly in the 160,000-dalton region. Lane 8: Marker polypeptides labeled with [ $^{14}$ C]formaldehyde according to Rice and Means (34): reduced subunits of plasma fibronectin,  $\alpha_2$ -macroglobulin (162,000 dalton), phosphorylase A (94,000 dalton), and human serum albumin (68,000 dalton).

The 165,000-dalton polypeptide had a mobility similar to that of the subunit of  $\alpha_2$ -macroglobulin, previously shown to be a product of human macrophages in culture (9).

No bands comigrating with fibroblast procollagens were seen on autofluorograms of the secreted proteins of macrophage cultures, even after ascorbate (50  $\mu$ g/ml) was added to the culture medium. Pretreatment of the labeled supernates with pepsin resulted in the complete disappearance of radioactive bands in the gels (Fig. 1, lane 5). No bands specifically sensitive to activity-controlled exogenous collagenase could

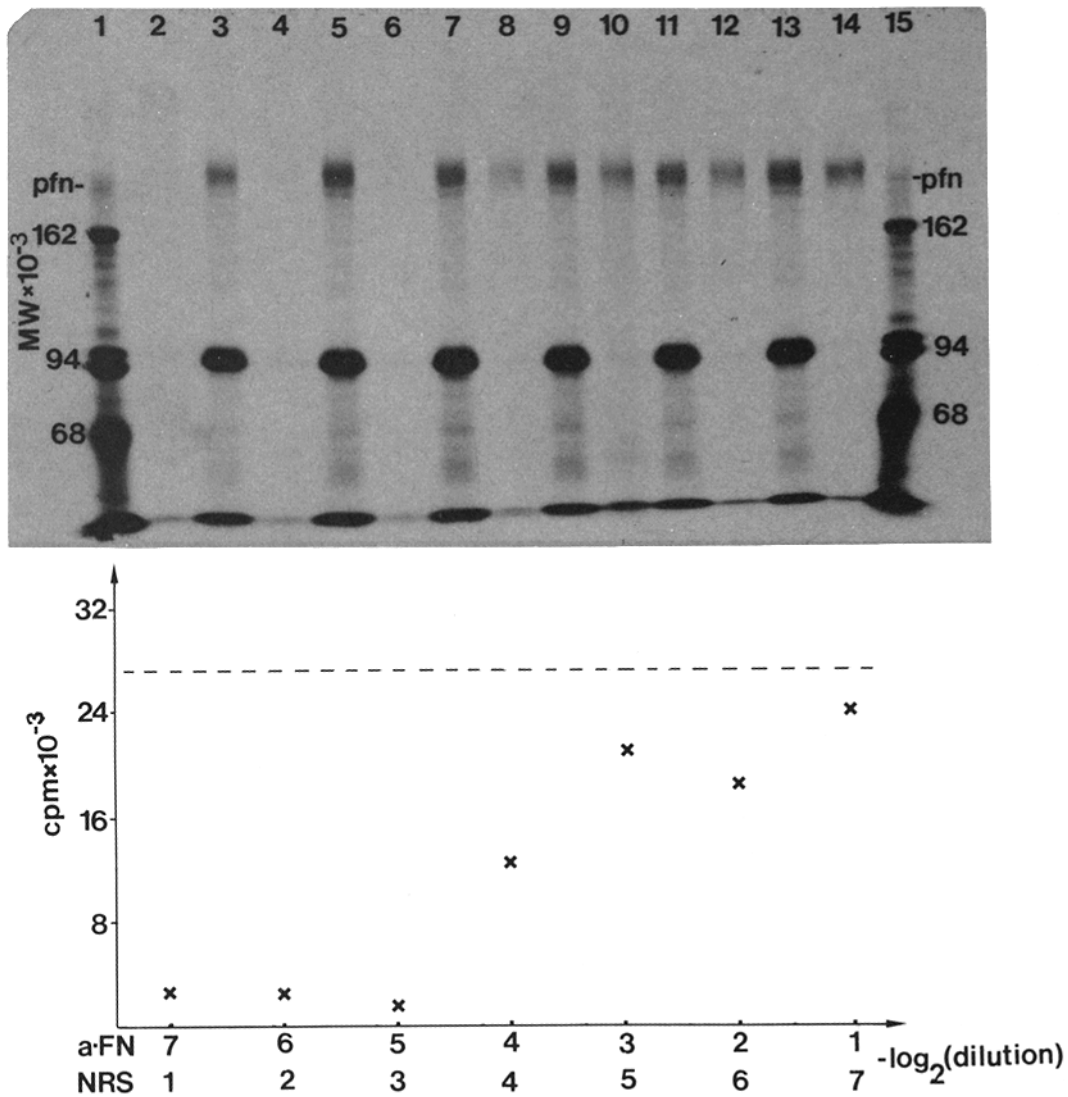


FIG. 2. SDS-polyacrylamide gel (5%) electrophoretic analysis of immunoprecipitates of labeled medium from 10-d-old macrophage cultures. Lanes 1 and 15 show molecular weight (MW) markers (see Fig. 1). Lanes with odd numbers show ammonium sulphate precipitates of aliquots of culture medium. Lanes with even numbers show precipitates obtained with the combination of different dilutions of anti-fibronectin (a-FN) and preimmune normal rabbit serum (NRS) as specified in the lower panel of the figure. The starting dilution of the sera was 1:10. The dotted line shows the mean radioactivity in the 220,000-dalton (fibronectin) position of the ammonium sulphate precipitates and the crosses depict the radioactivities recovered from the 220,000-dalton region of the gels of the immunoprecipitates. pfn, plasma fibronectin.

be identified (Fig. 1, lanes 6 and 7). The two amino acids used for labeling are known to be enriched in collagenous sequences.

Surface iodination of cultured macrophages labeled several polypeptides but no label was detected in the position of the 220,000-dalton polypeptide (Fig. 1, lane 1).

Trypsinization of metabolically labeled macrophages also did not reveal external

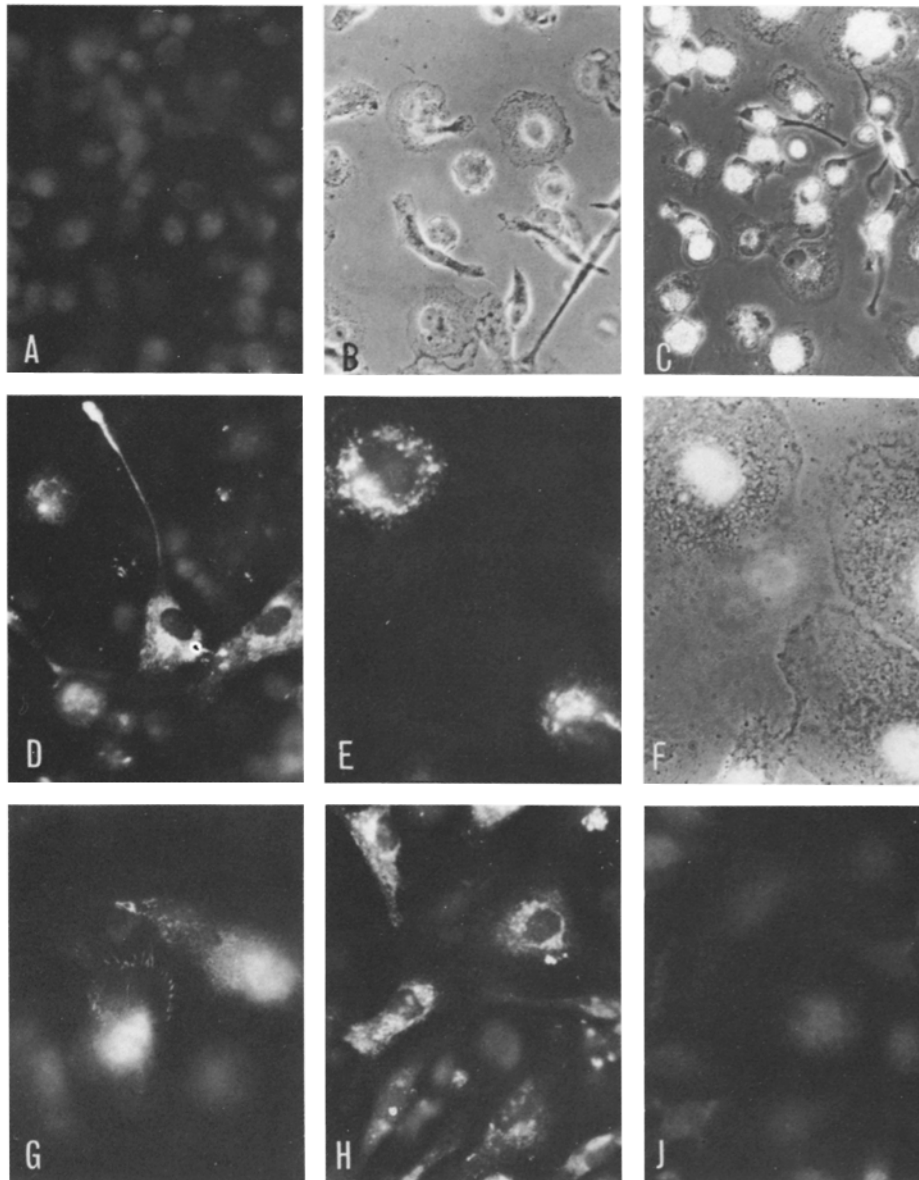


FIG. 3. Indirect immunofluorescence and phase-contrast microscopy of human monocyte-macrophage cultures. The fixation was performed with paraformaldehyde and acetone. (A) 1-d-old monocyte-enriched cultures stained with anti-fibronectin.  $\times 320$ . (B) and (C) Phase-contrast micrographs of 5-d-old macrophage cultures before (B) and after (C) incubation with latex particles (Diam:  $0.01 \mu\text{m}$ ).  $\times 250$ . (D) 5-d-old macrophage cultures stained with anti-fibronectin.  $\times 320$ . Note strong staining in the cytoplasm of the fibroblastoid macrophages. (E) and (F) Anti-fibronectin immunofluorescence (E) and phase-contrast micrograph (F) of a 10-d-old macrophage culture showing cytoplasmic fibronectin staining in some of the large epitheloid cells. Staining of DNA with the benzimidazole derivative Hoechst 33258 dye (Hoechst AG, Frankfurt, Federal Republic of Germany) according to Russell et al. (35) has been used to localize nuclei.  $\times 650$ . (G) Fibronectin staining on the growth substratum in the periphery of macrophages in 10-d-old culture. The microscope was focused to the surface of the coverslip and accordingly the prominent cytoplasmic staining is out of focus.  $\times 400$ . (H) and (J) 10-d-old macrophage cultures stained with anti-fibronectin serum before (H) and after (J) blocking with purified plasma fibronectin. As shown in (H), up to one-fourth of the cells were fibronectin-positive in old macrophage cultures.  $\times 320$ .

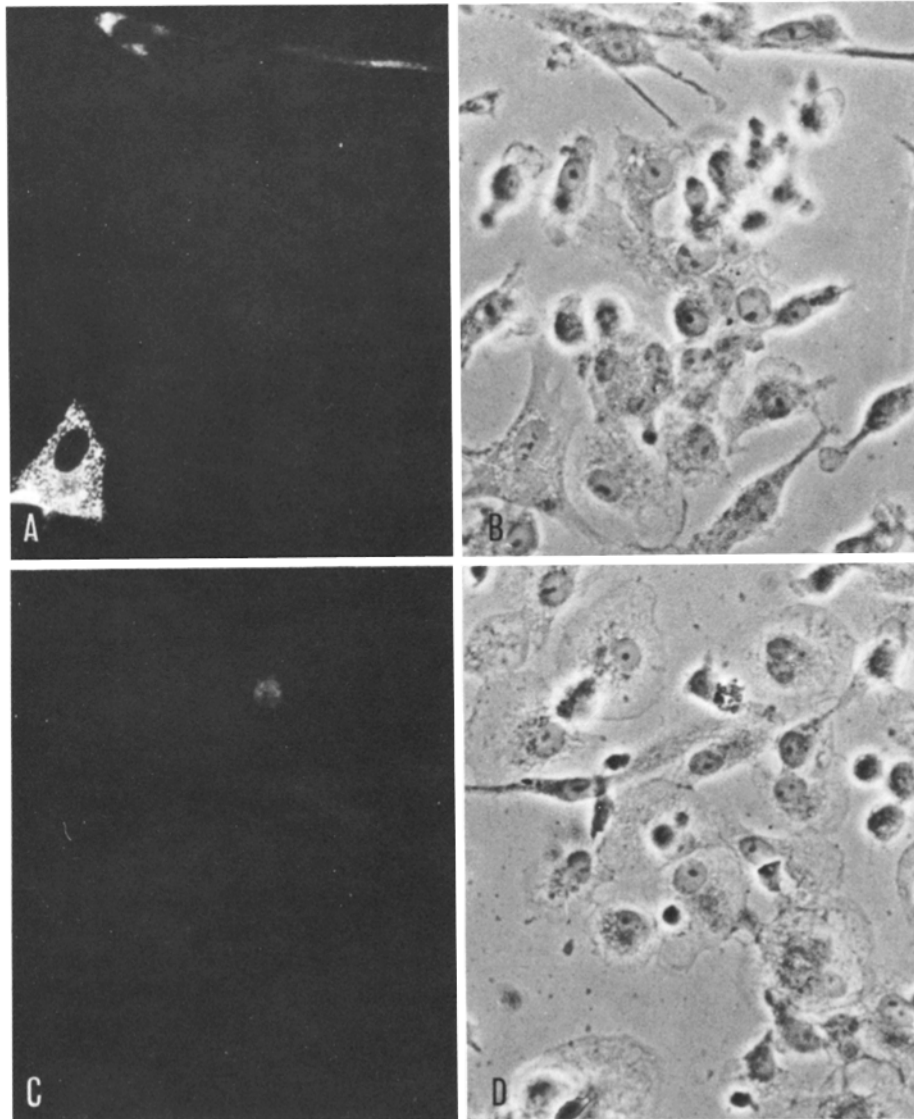


FIG. 4. Indirect immunofluorescence for procollagen type I and phase-contrast microscopy of macrophage cultures contaminated on purpose with human fibroblasts.  $10^3$  low-passage human adult skin fibroblasts were seeded on 7-d-old macrophage cultures, fixed with paraformaldehyde and acetone, and stained for procollagen type I and examined by immunofluorescence (A) or phase-contrast microscopy (B). Macrophage control cultures without fibroblasts stained for procollagen type I and examined by immunofluorescence (C) or phase-contrast microscopy (D).  $\times 320$ .

trypsin-sensitive polypeptides in this position (Fig. 1, lanes 2 and 3).

The 220,000-dalton polypeptide in the labeled culture medium was identified as fibronectin by immunoprecipitation (Fig. 2). The 220,000-dalton polypeptide was the only polypeptide precipitated by anti-fibronectin antibodies, the precipitation was dependent on the concentration of the antibodies and was nearly quantitative. This is illustrated in the lower panel of Fig. 2, where the dotted line indicates the amount

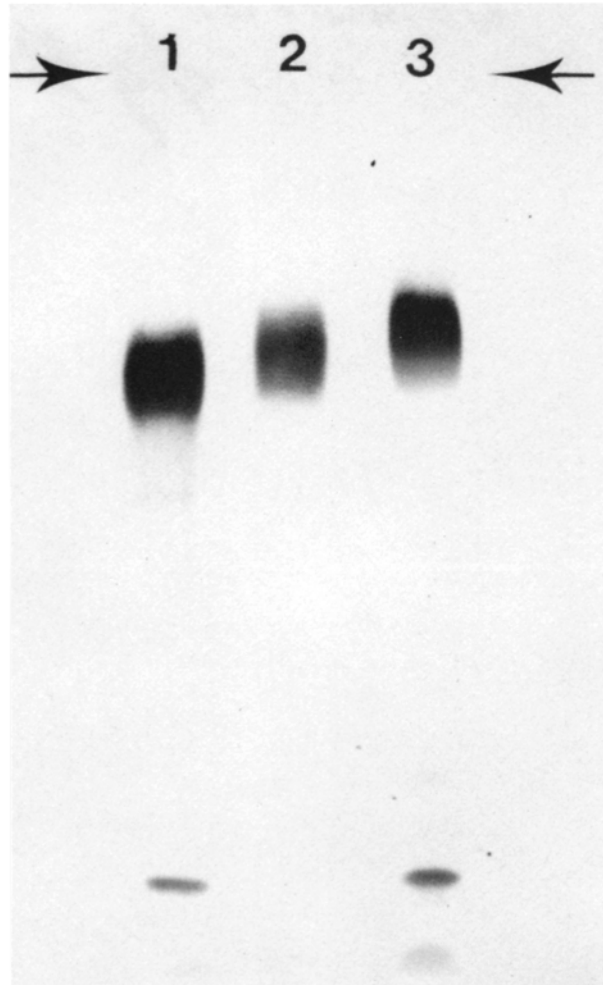


FIG. 5. SDS electrophoresis in a 5% polyacrylamide gel of metabolically labeled fibronectins immunoprecipitated from culture media of human adult skin fibroblasts (1), human adult macrophages (2), and human embryonic skin fibroblasts (3).

of radioactivity in the 220,000-dalton bands, and the crosses show the mean amount of radioactivity precipitated by different concentration of the antibody and detected at 220,000 dalton.

Fibronectin was not detected by radioimmunoassay in the supernates of young monocyte cultures (<10 ng/ml), whereas in well-differentiated macrophage cultures (containing ~250  $\mu$ g of cellular protein) fibronectin concentrations of 50–150 ng/ml were found in the medium after cultivation for 3–6 d. In samples of the culture media containing fibronectin, no procollagen type I or type III (<5 ng/ml) was detected in radioimmunoassays (kindly performed by Dr. R. Timpl).

*Identification of the Fibronectin-synthesizing Cells.* Fresh monocyte cultures grown overnight in serum-free conditions showed no specific staining for fibronectin in indirect immunofluorescence (Fig. 3 A). Considerable background staining was, how-

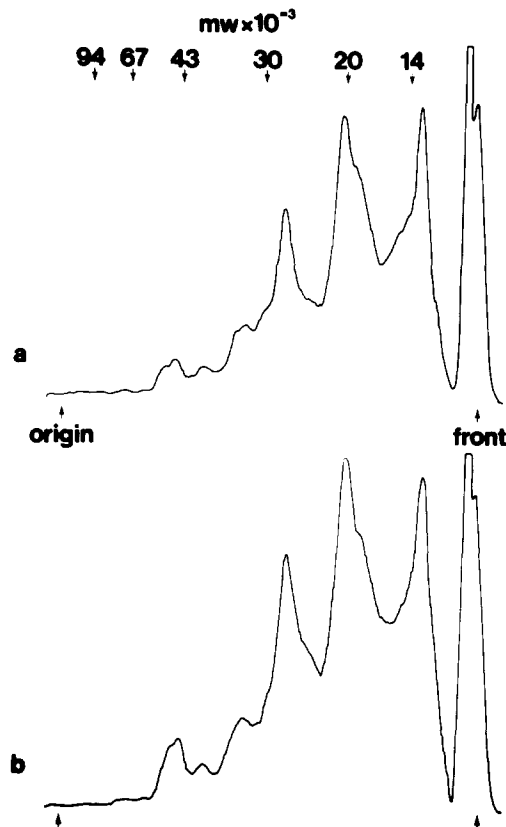


FIG. 6. Scanning densitometric tracings of proteolytic digest maps of fibronectins isolated from cultures of human macrophages (a) and of human adult skin fibroblasts (b). The metabolically labeled fibronectins were purified as described in Materials and Methods, subjected to cleavage by *S. aureus* protease V8 for 30 min at 37°C, and were analyzed by electrophoresis in a 12% polyacrylamide gel. Autoradiographic maps obtained were subjected to scanning densitometry. As marker polypeptides, commercially obtained (Pharmacia Fine Chemicals, Inc.) low-molecular-weight (mw) markers were used.

ever, seen both with anti-fibronectin and with control sera as well as with the conjugate alone, presumably because of the Fc-receptors of the cells. After cultivation of the cells for 3–5 d, when morphological differentiation had started (Fig. 3 B, C), specific staining for fibronectin was seen first in some of the elongated fibroblastoid cells (Fig. 3 D). After further incubation some of the epitheloid cells also became fibronectin positive (Fig. 3 E, F). In old cultures, where practically all cells were morphologically differentiated, approximately one-fourth of the cells were fibronectin positive (Fig. 3 H).

At all stages studied, the cellular fibronectin detected was localized to cytoplasm, and only traces of pericellular fibronectin, such as is characteristic of fibroblastic cells in culture, could be seen (Fig. 3 G).

Cultured monocytes and fibronectin-positive macrophages were examined for interstitial and basement membrane collagens using antibodies to procollagen type I and type III and to collagen type IV. At all stages of the culture, consistently negative



results were obtained (Fig. 4A and B). To ascertain that possibly contaminating fibroblasts could be detected in the macrophage cultures, various amounts of human adult skin fibroblast were added either to freshly prepared or differentiated macrophage cultures. As seen in Fig. 4C and D, fibroblasts could be readily distinguished from the macrophages by their intracellular interstitial procollagen. Even a small contamination could be detected. This was the case even when 100 fibroblasts were mixed with either  $10^7$  peripheral blood leukocytes before starting the cultures or with  $10^7$  differentiated macrophages.

*Comparison of Macrophage Fibronectin with Fibroblast Fibronectin.* After reduction of disulfide bonds, fibronectin isolated from human macrophage cultures had an electrophoretic mobility in between those of fibronectins isolated from adult and embryonic skin fibroblast cultures (Fig. 5). In nonreduced conditions, macrophage fibronectin, like fibronectins isolated from fibroblast cultures or from human plasma, migrated in the dimeric 440,000-dalton position (data not shown).

One-dimensional peptide maps of fibroblast and macrophage fibronectins, obtained by digestion with *S. aureus* protease V8, showed a closely similar pattern (Fig. 6).

### Discussion

This paper shows that fibronectin, a glycoprotein present in connective tissues and in various body fluids, is one of the major products of human macrophages in culture. In vitro, fibroblastic cells as well as several other adherent cell types—mostly those of mesenchymal origin—can synthesize fibronectin (27). It seems very unlikely that the fibronectin detected in macrophage cultures would be a result of contamination by other cell types, such as skin fibroblasts or venous endothelial cells. No collagenase-sensitive polypeptides were detected in the culture media. No procollagen type I or type III (<5 ng/ml) was detected by radioimmunoassays in the media. These values exclude contamination of fibroblasts of >0.1% (25). A hypothetical contamination by  $10^3$  fibroblasts among  $10^6$  macrophages would be able to produce ~6 ng fibronectin per culture, which is clearly less than the amounts detected in these cultures. Furthermore, we found no staining by immunofluorescence using antibodies to procollagen type I and type III or to collagen type IV, kindly provided by Dr. R. Timpl. The latter data also exclude the contamination by endothelial cells (28). It seems possible that phagocytic cells take up bovine fibronectin from their culture medium. As anti-human fibronectin cross-reacts with bovine fibronectin present in calf serum, it was important to deplete the antisera used in these experiments from cross-reacting antibodies by adsorption.

The distribution of endogenously produced fibronectin in macrophage cultures seems to differ in several aspects from that in cultures of fibroblastic cells. Unlike the situation in normal fibroblasts (27), macrophages were not found to have significant amounts of fibronectin at their cell surface, neither did they lay down an intercellular matrix containing a fibronectin network under the conditions used in our experiments. Our findings agree in this respect with those of Pearlstein et al. (29), who found no extracellular fibronectin by immunofluorescence or cell surface iodination in cultured mouse peritoneal macrophages. Pearlstein et al. (29) also did not find intracellular fibronectin in these cells. In contrast, Colvin et al. (30) have reported, in abstract form, on the detection of cell surface fibronectin in guinea pig peritoneal macrophages.

The possible physiological role of fibronectin production by activated macrophages

can only be speculated at the present state of knowledge. There is some evidence that fibronectin is involved in cell migration (31). Accordingly, it is possible that production of a major connective tissue matrix component, fibronectin, together with enzymes capable of degrading the matrix (32), including collagenase (4) and elastase (5), is necessary for the macrophages to control their migration through the tissues. In tissues where the circulating fibronectin may not be available in sufficient amounts, capability of endogenous fibronectin production might also provide macrophages a means for promoting phagocytosis of fibrin- or collagen-containing particles and thus supplement the armament of these cells in body defenses.

After this paper was submitted, Johansson et al. (33) reported on biosynthesis of fibronectin by mouse peritoneal macrophages.

### Summary

Monocyte-enriched cultures were prepared from human blood mononuclear leukocytes by adherence to growth substratum. Synthesis and secretion of fibronectin was detected in these cultures concomitantly with morphological differentiation, starting on day 3–5. Production of fibronectin by macrophages was documented by metabolic labeling followed by immunoprecipitation and gel electrophoresis, radioimmunoassay specific for human fibronectin, and by indirect immunofluorescence. Fibronectin was detected mainly intracellularly but was also detected pericellularly only in minute amounts. No production of collagenous proteins was seen in these cultures. Macrophage fibronectin might act *in vivo* as a nonspecific opsonin and promote cell adhesion during macrophage migration in tissues.

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