Studies on Fibronectin in the Skin

VII. Production in Cell Cultures from Normal Human Skin

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In the present study, cell cultures of fibroblasts from normal skin have been investigated regarding the production of fibronectin. The development of multimeric insoluble fibronectin is demonstrated as small dots at the cell surface, developing into a branched meshwork of fibrous structures in parallel arrays. Soluble dimeric fibronectin is also found in the culture medium. *Key words: Cell cultures; Human skin; Fibronectin.* (Received January 28, 1983.)

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Fibronectin are glycoproteins of the human organism. A soluble form circulates in plasma (12, 6, 14), while in the tissue, multimeric fibrous fibronectin is found in the intercellular matrix system (10).

The biological function of fibronectin is still debated, but it is known to posses adhesional properties, binding to cells and fibres. In the tissue, contact is established between fibroblasts and collagen. By binding to other cell surfaces, collagen, fibrin, mucopolysaccharides and bacteria, it stimulates phagocytosis of unwanted material (1).

As a product of fibroblasts and other cells lines, fibronectin is a component of normal skin (7) and, due to its affinity to fibrous material, fibronectin forms part of the intercellular matrix system, adding to the stability of these structures.

In the present study, in vitro production of soluble and fibrous fibronectin from normal skin cells has been investigated.

MATERIAL AND METHODS

Cell cultures

Biopsy specimens were taken from unaffected human skin of 10 adult outpatients at the surgical department. In Hanks' balanced salt solution (BBS) the biopsy material was minced and dissociated enzymatically in 0.25% trypsin in BBS with added antibiotics. After stirring at 37°C for 10 min, the solution was pipetted off into calf serum and centrifuged at 1 500 rpm for 10 min. The supernatant was discarded and the cell pellets resuspended in culture medium (Eagle's medium with Earle's salts and L-glutamine added to 20% fetal calf serum with antibiotics). The suspension was seeded in plastic bottles, Falcon type 3013, in CO_2-enriched air to pH 7.4.

After a few passages the cells demonstrated a typical fibroblast growth pattern, and were investigated before the 20th passage. For the present studies, cells were inoculated in an atmosphere of 5% Co_2 in air and the medium was renewed weekly. All cell samples inoculated in these studies were taken from the same cell pool after the cell population was determined by duplicate counts.

Indirect immunofluorescence studies (IIF)

Fibroblasts were cultured in Falcon Multi-Well type 3008 plastic trays with 14 mm diameter wells and a circular glass coverslip at the bottom of each chamber. Each well held 20000 cells. The culture medium was changed every second day. The monolayers on the coverslips were fixed for 10 min in 2% formaldehyde, incubated with absorbed monspecific antihuman plasma fibronectin rabbit antiserum (7), diluted 1 : 32 with phosphate-buffred saline solution (PBS), pH 7.3, added 4% bovine serum albumin. The coverslips were then washed in PBS, incubated 10 min with FITC-conjugated goat–antirabbit gammaglobulin (F/P ratio 2.2) diluted 1 : 20. Finally the coverslips were washed in PBS and

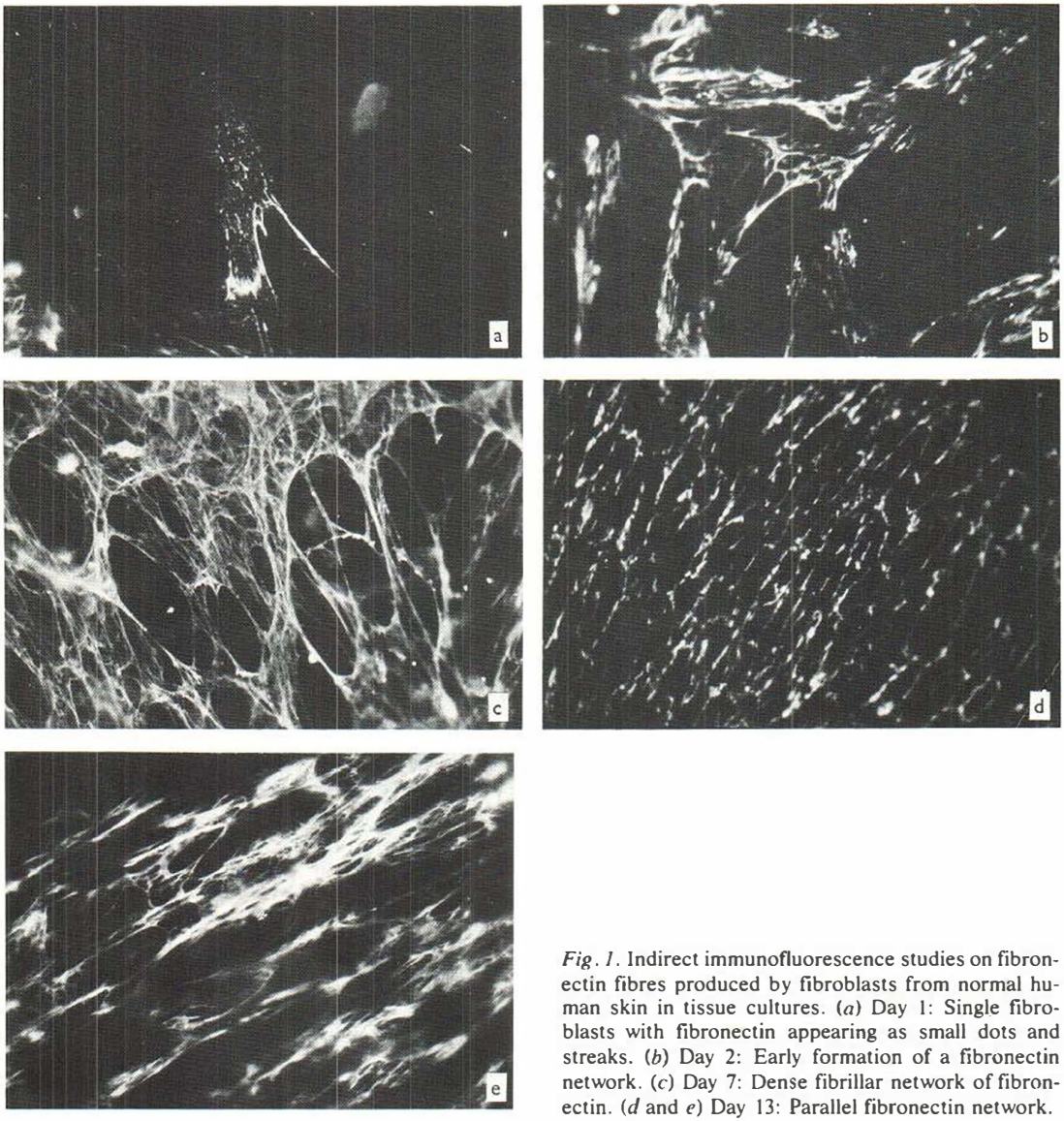

Fig. 1. Indirect immunofluorescence studies on fibronectin fibres produced by fibroblasts from normal human skin in tissue cultures. (*a*) Day 1: Single fibroblasts with fibronectin appearing as small dots and streaks. (*b*) Day 2: Early formation of a fibronectin network. (*c*) Day 7: Dense fibrillar network of fibronectin. (*d* and *e*) Day 13: Parallel fibronectin network.

mounted in PBS-glycerol. The microscope and the photogrpahic equipment used were as previously described (7), in this study using epi-illuminatoin of the samples.

The cultures were run for 13 days. Day 1 of the culture started after 24 hours.

Quantitative electroimmunoassay

Cells from the same cell pool used for IIF studies were cultured in 30/3 Falcon plastic bottles in 5 ml culture medium as described above. From the culture, 100-µl samples were taken every second day and the same amount of fresh medium added. These samples were then investigated by quantitative electroimmunoassay for soluble fibronectin, as reported elsewhere (6). In the gel, 2% absorbed monospecific anti-fibronectin antiserum was used on 10×20 cm glass plates. Duplicate runs on 10 µl undiluted samples were tested. As the cells were grown in a culture medium with fetal calf serum, small amounts of bovine fibronectin were present in the medium. With the present concentration of antiserum in the gels and with negative controls of fresh culture medium in each run, bovine fibronectin in the medium never affected the amount of human fibronectin produced by the cells. As a

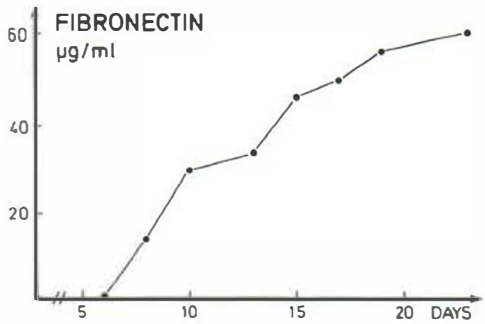

Fig. 2. Introduction of soluble fibronectin, produced by human skin fibroblasts, into the culture medium.

standard in the assay, fibronectin from human plasma isolated on gelatin–sepharose affinity chromatography was used as previously reported (7). The cell cultures were run for 23 days.

Biocult Ltd, Paisley, Scotland: Foetal calf serum, Antibiotic–antimycotic, Hanks' balanced salt solution, Eagle's medium, Earle's salt, L-glutamine. Difco Lab., Detroit, USA: Trypsin Behringwerke, W-Germany: FITC conjugated goat-antirabbit gammaglobulin.

RESULTS

Indirect immunofluorescence studies for fibrous fibronectin

Cellular growth pattern. After day 1 the fibroblasts had attached to the surface of the coverslip as single separate cells. On days 2 and 3, contacts were established between the cells and after days 4–5 a confluent monolayer was established.

At first, the growth pattern was irregular, with scattered cells extending in all directions, gradually forming clusters of cells. From days 4 and 5 the fibroblasts developed a longitudinal cell shape, with a parallel orientation from days 7–10.

Immunofluorescence pattern. The changing morphological pattern of the cell picture is reflected in the IF pattern of fibronectin (Fig. 1 *a–e*). From day 1, fibronectin was found in the form of small spots on the cell surface, mainly facing the coversip (Fig. 1 *a*). More abundantly from day 2, this was also seen on other cell surface areas (Fig. 1 *b*), and also as fibrous structures along cell extensions. With prolonged culturing, the IF intensity and the number of fibres increased. In some areas, fibres extended from one cell to the next. From day 3, a dense fibrillar network of fibronectin developed, mainly at the cell junctions. With increasing number of cells, the dense fibrillar network was more pronounced (Fig. 1 *c*). At the moment of parallel orientation of the spindle-shaped fibroblasts, parallel orientation of a branched network of fibrillar material was found, with long fibres extending parallel to the cellular axis (Fig. 1 *d*, *e*), concentrated mainly at the polar ends of the cells. At the end of culturing, the monolayer of confluent parallel fibroblasts formed itself into a dense network with intense fluorescence of fibronectin fibres arranged into parallel fibrillar arrays.

Production of soluble fibronectin

With the present technique, fibronectin could not be found in the culture medium between day 1 and day 6. From day 7, a gradual increase in fibronectin was found, with the highest concentration of 60 µg/ml on day 23 (Fig. 2).

DISCUSSION

Fibronectin is found in the cell, at the cell surface, and in the extracellular matrix. Intracellular fibronectin is found mainly in mitotic cells (16) in the form of monomeric

chains. These dimerize inside the cell on the way to the cell surface and into the culture medium (2).

At the cell surface, fibronectin is located too far from the plasma membrane to possess the membrane-embedded segment characteristic of integral membrane proteins (11). Associated with the intracellular cytoskeleton, such as actin (8), a contiguous connection has been found (15) between intracellular microfilament bundles and fibronectin fibrils outside the cells.

Cultured fibroblasts have fibronectin at all surfaces. At first, fibronectin is concentrated at the sites of cellular contact with the substratum. Detachment of cells leaves material behind, containing fibronectin and proteoglycans (4). This was observed in the present study, with fibronectin appearing as small dots at the substratum-facing side of the cultured cells. In the phase of cellular migration, fibronectin was found as fibrillar structures along the cellular extensions, forming a fibrillar meshwork. Gradually, fibronectin becomes reoriented into fibrillar parallel arrays in the direction of the axis of the cultured fibroblasts. At the end of the culture, the cells are embedded in a dense network of fibronectin structures.

A number of cells, such as fibroblasts, introduce soluble fibronectin into the culture medium. In the present investigation the amount of soluble fibronectin increased gradually to 60 μg/ml from day 6 to day 20. This tallies with another study reporting 50 μl/ml fibronectin at the stage of confluence (13).

In vivo, fibronectin is found in connective tissues, also in the skin especially at the dermo-epidermal junction (7, 3). Under normal conditions, fibronectin is not found in the epidermis. The IIF pattern corresponds to the distribution of collagen as found at the cell surface of cultured fibroblasts (16). The ability of fibronectin to react with macromolecules such as collagen is also seen with proteoglycans and fibrin. Such reactions are based upon specific binding sites in the fibronectin structure, linking fibronectin to other molecules. Structural domains have been mapped by enzymatic dissection of the fibronectin structure into subfragments demonstrating different binding sites (9). This indicates an ability to link extracellular matrix structures to each other and to the surface of cellular elements where receptors of fibronectin are demonstrated.

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