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PRODUCTION OF FIBRONECTIN BY HUH6 CL5 CELL LINE ESTABLISHED FROM A HUMAN HEPATOBLASTOMA

Masanobu Tanaka[1], Kenichi Kawamura[1], Meiqi Fang[1], Kazuya Higashino[1], Susumu Kishimoto[1], Hidekazu Nakabayashi[2], Jiro Sato[2]

[1]The Third Department of Internal Medicine, Osaka University School of Medicine, Osaka 553, Japan and [2]Division of Pathology, Cancer Institute, Okayama University School of Medicine, Okayama 700, Japan

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Summary: Fibronectin was detected by indirect immunofluorescence on the cell surfaces of HUH6 C15 cells, established from a human hepatoblastoma and maintained with serum-free RPMI 1640 medium. Fibronectin synthesized by HUH6 C15 was purified by gelatin-Sepharose affinity chromatography and compared with human plasma fibronectin in respect to molecular weight, electrophoretic mobility and antigenicity. Fibronectin synthesized by this cell line was proved to be identical with human plasma fibronectin.

Introduction: Fibronectin is a high molecular weight glycoprotein present in the plasma, connective tissue and on cell surfaces. It is synthesized by many types of differentiated cells and is believed to be involved in the attachment of cells to the surrounding extracellular matrix. Fibronectin enhances the attachment of various types of cells to a plastic surface (for review see ref. 1-4).

Several investigators have reported that cellular fibronectin is consistently reduced or absent in numerous transformed cell lines (5-8).

HUH6 C15 cells were established from a human hepatoblastoma of a Japanese man and were grown in serum-free RPMI 1640 supplemented with 3×10^{-8}M sodium selenite. The present report describes that fibronectin was detected on the surfaces of these cells by indirect immunofluorescence technique and fibronectin was released into the medium by these cells.

Materials and Methods: Cell: The HUH6 C15 cells were kindly donated by Dr. Jiro Sato (Tissue Culture Laboratory, Division of Pathology Cancer Institute, Okayama University Medical School)(9). Those cells were routinely grown in RPMI 1640 medium supplemented with 3×10^{-8}M sodium selenite in a humidified atmosphere of 5% CO_2 in air at 37°C, the cells were removed from the surface of the Corning

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tissue culture flask with 0.1% Trypsin and 0.005% EDTA, washed with fresh culture medium and subcultured into two culture flasks.

Purification of fibronectin from the spent medium and plasma: Purification of fibronectin was carried out according to the method of Zardi (10). Briefly, spent medium or plasma, containing 0.1% NaN_3 and 0.2mM PMSF (phenylmethylsulfonyl fluoride), were passed through Sepharose CL-4B (Pharmacia, Uppsala, Sweden) chromatography column. All the fractions having an $A_{280} > 0.5$ were pooled and recycled through gelatin-Sepharose CL-4B chromatography column. Fibronectin was eluted with 3M urea in PBS (20mM phosphate, 0.15M NaCl, pH 7) containing 0.1% NaN_3.

Antisera: Purified human plasma fibronectin was homogenized in the same volume of complete Freund's adjuvant (Difco, Detroit, MI, U.S.A.). Five injections, each containing 300 μg of purified fibronectin, were given intradermally to rabbits every week. The antisera were collected seven days after the last injection and the γ-globulin fractions were obtained by fractionation with ammonium sulfate. Those fractions were absorbed with fibronectin-free serum proteins.

Immunofluorescence: For immunofluorescence studies, cells were cultured on a slide glass in Falcon tissue culture dishes and used 10 days after plating. After rinsing with PBS, the first antibody 1:10 in PBS was added and the cells were incubated for 30 min in a moist chamber at 37°C. The cells were then thoroughly washed with PBS and incubated for 60 min with fluorescein isothiocyanate conjugated goat anti-rabbit IgG (1:10 in PBS)(Cappel, PA, U.S.A.). The washed slide glass was then observed with phase contrast and epifluorescent illumination in a Zeiss 18FL microscope. Non-specific antibodies from preimmune rabbits were routinely used as controls.

Immunodiffusion and immunoelectrophoresis: The immunodiffusion and immunoelectrophoresis were carried out in 1.2% agar (Difco, Detroit, MI, U.S.A.)(11, 12).

Sodium dodecyl sulphate(SDS) polyacrylamide gel electrophoresis: SDS polyacrylamide gel electrophoresis was carried out using 10% gels after the method of Weber and Osborn (13).

Results: Immunofluorescence: Hepatoblastoma cells examined 10 days after plating showed staining of fibrillar matrix around the cell surfaces and between the cells by indirect immunofluorescence (Fig.1).

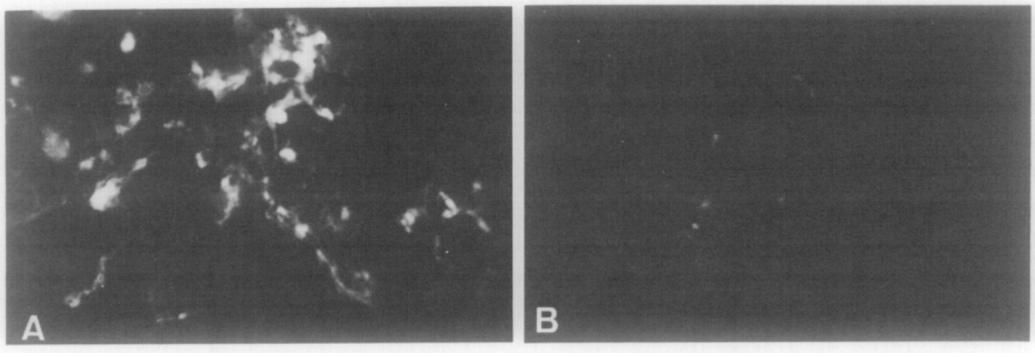

Fig. 1: Indirect immunofluorescence localization of cell surface attached fibronectin.

(A) Staining for fibronectin
(B) Control

Magnification: 208-fold for A and B.

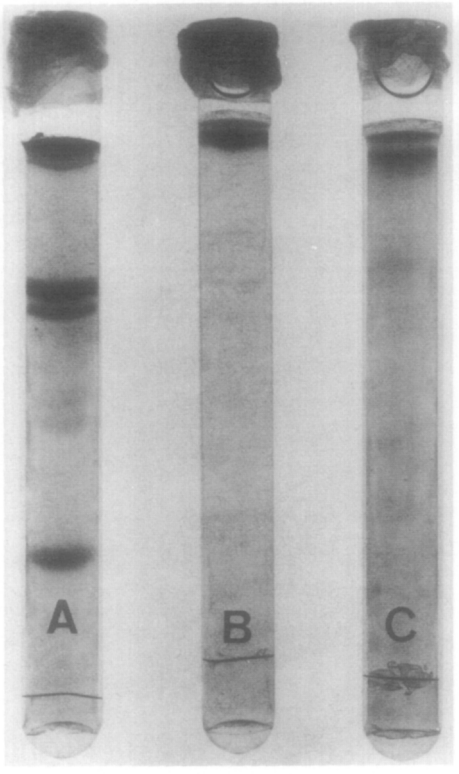

Fig. 2: SDS-polyacrylamide gel electrophoresis

(A) Protein markers; thyroglobulin (330K dalton), albumin (67K dalton), catalase (60K dalton), LDH (36K dalton), ferritin (18.5K dalton)
(B) Purified human plasma fibronectin
(C) Purified hepatoblastoma fibronectin

Release of fibronectin into medium: HUH6 C15 cells were grown in serum-free medium. So the fibronectin in the spent medium was newly synthesized by this cell line. This fibronectin in the spent medium was purified as described in Materials and Methods. SDS polyacrylamide gel electrophoresis showed that the molecular weight of this purified fibronectin was the same as the purified human plasma fibronectin and found to be 220K dalton (Fig.2). Immunochemical studies using Ouchterlony double diffusion experiments were carried out. Figure 3 shows clearly that the precipitation lines of the purified hepatoblastoma fibronectin and the purified human plasma fibronectin produced by the antibody to human plasma fibronectin fused completely. Figure 4 shows the result of immunoelectrophoresis that the mobility of the purified hepatoblastoma fibronectin and the purified human plasma fibronectin was the same.

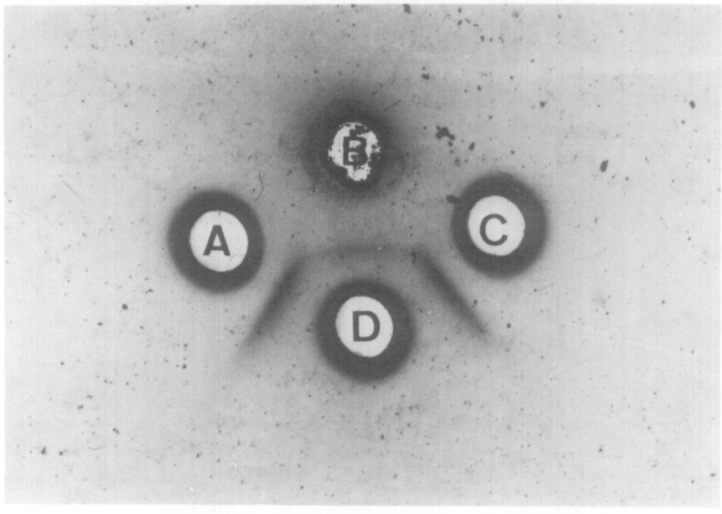

Fig. 3: Double immunodiffusion

(A, C) Purified human plasma fibronectin
(B) Purified hepatoblastoma fibronectin
(D) Anti-fibronectin antibody

Discussion: HUH6 C15 cells have been established from a human hepatoblastoma of a Japanese man and have been maintained with serum-free medium.

The products released into the medium by this cell line can be analyzed easily using the spent medium. This cell line synthesizes α-fetoprotein (9700 μg/ml medium) and bile acids (chenodeoxy cholic acid, cholic acid and litho-cholic acid)(14). Fibronectin is synthesized by primary cultures of rat hepato-

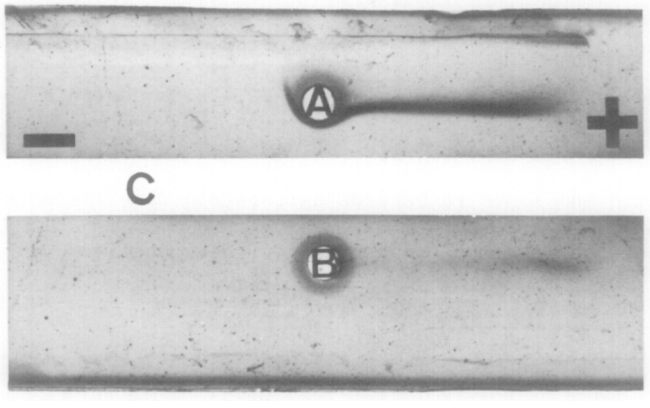

Fig. 4: Electroimmunophoresis

(A) Purified human plasma fibronectin
(B) Purified hepatoblastoma fibronectin
(C) Anti-fibronectin antibody

cytes and detected by indirect immunofluorescence on their cell surfaces (15). Because it is reported that fibronectin is consistently reduced or absent in numerous transformed cell lines, it is interesting whether HUH6 C15 cells synthesize fibronectin and retain it on their cell surfaces. HUH6 C15 cells retain fibronectin on their cell surfaces as shown by indirect immunofluorescence (Fig. 1). Purified fibronectin from the spent medium by gelatin affinity chromatography is identical with human plasma fibronectin in respect to molecular weight, electrophoretic mobility and antigenicity (Fig. 2,3,4). The fibronectin synthesized by this cell line seems to have normal function. It could conversely be said that this cell line is maintained in serum-free medium partially due to fibronectin synthesis.

The concentration of the fibronectin in the spent medium was about 3 μg/ml measured by our enzyme immunoassay system (unpublished data).

Further investigations have been carried out about products of this cell line.

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