POLYMERIZATION OF A MAJOR SURFACE-ASSOCIATED GLYCOPROTEIN, FIBRONECTIN, IN CULTURED FIBROBLASTS

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Received 18 October 1976

1. Introduction

Fibronectin [1] is a major fibroblast surface-associated glycoprotein, subunit molecular weight 220 000, which was originally described in our laboratory as a fibroblast surface antigen [2]. The same protein has independently been detected by several groups using surface labelling and is known to be absent from the surface of cells transformed by viruses [3–6]. The protein has been called fibronectin to emphasize its connections to fibrin and the fibrillar structures to which it is closely located on fibroblastic cells [7]. The circulating form of fibronectin in plasma has been known as cold-insoluble globulin [8]. The cellular and plasma forms show immunological identity [9] and both are known to be composed of two disulfide bonded polypeptide chains [10–12].

We reported earlier that a very high molecular weight protein complex is accessible to lactoperoxidase catalyzed iodination on the surface of cultured fibroblasts and, like fibronectin [13], it is present in normal but absent from transformed cells [14]. Other studies have shown that plasma fibronectin can be cross-linked by plasma transglutaminase (thrombin activated coagulation factor XIII) to fibrin and to itself [11] and that exposure of surface labelled cellular fibronectin to activated factor XIII resulted in cross-linking of fibronectin into a very high molecular weight protein complex [15]. These data suggested that the very high molecular weight material on the cell surface might represent fibronectin in a polymerized state. Furthermore the fibrillar distribution of surface fibronectin, as shown by immunofluorescence [16], implied that the molecules might be in a position favourable for polymer formation.

In the present experiments I demonstrate that monomeric surface-associated fibronectin molecules are converted to polymeric complexes spontaneously under conventional cell culture conditions. These polymeric complexes, like monomeric fibronectin cross-linked experimentally by transglutaminase, show sensitivity to degradation by trypsin.

2. Materials and methods

2.1. *Reagents*

Reagents were purchased from the following suppliers: glucose oxidase, Worthington Biochem. Corp., Freehold, N.J., USA; lactoperoxidase and trypsin TPCK, Sigma, St. Louis, Mo., USA; carrier-free $Na^{125}I$ Radiochemical Centre, Amersham, England; RP Royal X'Omat X-ray film, Kodak, Rochester, N.Y., USA. Bovine thrombin, Parke Davis, Detroit, Mich., USA, was purified further by chromatography as described [11]. Blood coagulation factor XIII was purified from human plasma [19].

2.2. *Cell cultures*

Secondary chick embryo fibroblasts were prepared as described earlier [17] and grown at +39°C in medium 199 supplemented with 10% tryptose phosphate broth and 5% calf serum.

2.3. *Surface labelling*

The cell cultures were washed quickly three times

with warm serum-free medium. Lactoperoxidase-catalyzed iodination was performed in phosphate buffered saline containing glucose (5 mM), carrier-free $Na^{125}I$ (100 μCi/ml), and lactoperoxidase (10 μg/ml) as described [15]. After 15 min new culture medium was added and the cells were incubated at +39°C until analysis. Cells were extracted in a small volume (250 μl/2 × 10^6 cells) of Tris-buffered 4% sodium dodecyl suphate containing 10% 2-mercaptoethanol, pH 6.8, and treated for 3 min in a boiling water bath to complete dissociation.

2.4. *Polyacrylamide gel electrophoresis in sodium dodecyl sulphate*

Vertical slab gels and a discontinuous buffer system were used [18]. The acrylamide concentrations of the stacking and running gels were 3.3% and 5.0%, respectively. Following electrophoresis the gels were dried and subjected to autoradiography.

2.5. *Cross-linking by activated blood coagulation factor XIII*

Surface labelled fibroblast cultures were exposed to factor XIII (30 μg/ml) in medium 199, and thrombin (1.6 NIH units/ml) was added to activate factor XIII, as previously described [15].

3. Results

3.1. *Appearance of surface-iodinatable proteins after subculture*

Chick fibroblasts were iodinated by the lactoperoxidase catalyzed iodination method. Shortly after subculture, about one hour, the cells exhibited a labelled band at the top of the gel (fig.1, gel 1). The possibility that some of the radiolabelled polypeptides of very high molecular weight might originate from the culture dish or culture medium was studied by incubating a dish in culture medium. The labelling pattern of this plate did not show bands of high molecular weight (fig.1, gels 6 and 7). In cells labelled two hours after subculture some fibronectin monomer was detectable (fig.1, gel 2). The characteristic heavily labelled fibronectin band was found five hours after subculture and reached maximal intensity within two days (fig.1, gel 5).

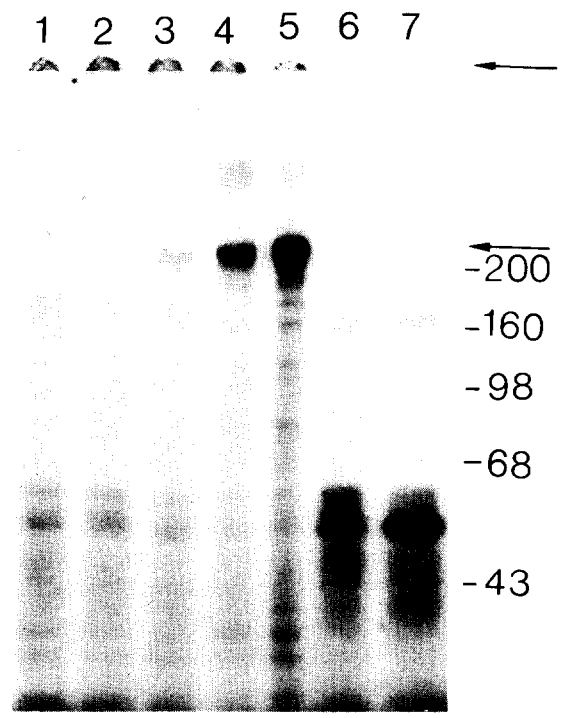

Fig.1. Autoradiogram of electropherogram of surface-iodinated chick fibroblasts. The cells were iodinated after subculture as follows: 1 one hour, 2 two hours, 3 five hours, 4. 21 hours, 5 two days. 6. Culture dish incubated in growth medium without cells for one hour. 7. Culture dish incubated in growth medium without cells for five hours. Approximately the same amount of protein (20 μg) determined by the method of Lowry et al. [20] was placed on slots 1–5. A sample from a place incubated without cells corresponding to cells labelled one and five hours after subculture was placed on slots 6 and 7. Arrows indicate the positions of fibronectin and of the very high molecular weight material. The following proteins were used as molecular weight markers: ovalbumin (43 × 10^3); human serum albumin (68 × 10^3); phosphorylase A (98 × 10^3); human $alpha_2$-macroglobulin (160 × 10^3); and reduced human plasma fibronectin (200 × 10^3).

3.2. *Fate of prelabelled fibronectin in cell culture conditions*

The cultures were surface iodinated 18 h after subculture and then incubated ('chased') in cell culture conditions in serum containing medium until analysis. Only a small amount of label was detectable on top of the gel at the beginning of the incubation (fig.2, gel 1). During the incubation the label in the position of fibronectin was decreased in quantity and an increase

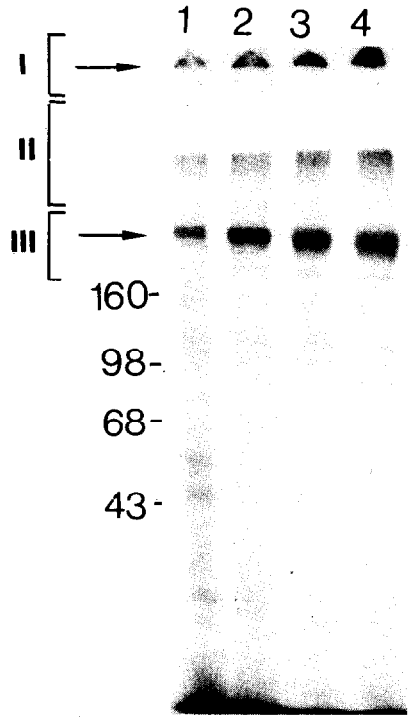

Fig.2. Autoradiograms of electropherograms of chick fibroblasts iodinated 18 h after subculture. The cells were incubated at +39°C until analysis. Approximately the same amount of acid insoluble radioactivity was placed on each slot. 1. Cells iodinated 18 h after subculture, no chase. 2. One day's chase. 3. Two days' chase. 4. Three days' chase. For the bars see caption of table 1. For other symbols see the caption to fig.1.

in the label of the very high molecular weight region was seen (fig.2, gels 3 and 4; table 1). Some accumulation of label at the interphase of the gels was also detected (table 1). During the 'chase' the 220 000 mol. wt fibronectin band became broader, towards small molecular weight and a faster moving polypeptide band was seen (fig.2).

3.3. *Trypsin-sensitivity*

Like monomeric fibronectin, the very high molecular weight material was found to be very sensitive to trypsin (ref. [14] and fig.3, gels 1 and 2). Fibronectin that is cross-linked to a very high molecular weight complex by factor XIII is also sensitive (fig.3, gels 3 and 4). In cell cultures incubated

Table 1
Redistribution of radioactivity during incubation of surface-iodinated fibroblasts[a]

		Days after subculture			
		0	1	2	3
Part of gel	I	555	849	842	973
	II	1494	1640	1758	1923
	III	6493	6010	5794	5689
Total		8452	8499	8394	8585

[a] Averages of three experiments

The tops of the dried gels were cut in three pieces as follows and their radioactivity determined in a gammacounter (counts per minute): I. Top region of the gel. II. Interphase region including the supposed oligomer region in 5% gel. III. Fibronectin subunit and polypeptides 170 000–250 000 daltons. The roman numbers correspond to the bars in fig.2. (The lowest part of the gel was not counted because the amount of free iodine varied greatly.)

('chased') for three days after surface iodination, small concentrations of trypsin abolished the label both in monomeric fibronectin position and in the very high molecular weight region (fig.3, gels 5 and 6). Also the labelled band near the interphase of the gels was lost by trypsin treatment.

4. Discussion

These studies show that a portion of monomeric surface-associated fibronectin is converted to high molecular weight form during conventional cell culture conditions where 5% calf serum is used. This conclusion is based on the following observations: cells surface-iodinated 18 h after subculture, showed some protein accessible to labelling in the fibronectin monomer position. During subsequent incubation the intensity of this label decreased and increased simultaneously at the top of the gel. Monomeric fibronectin, the very high molecular weight complexes and fibronectin cross-linked by factor XIII treatment are sensitive to mild treatment with trypsin. In addition some high molecular weight proteins also accessible to surface labelling, are cleaved in this treatment. This finding along with the increase of label at the interphase of the

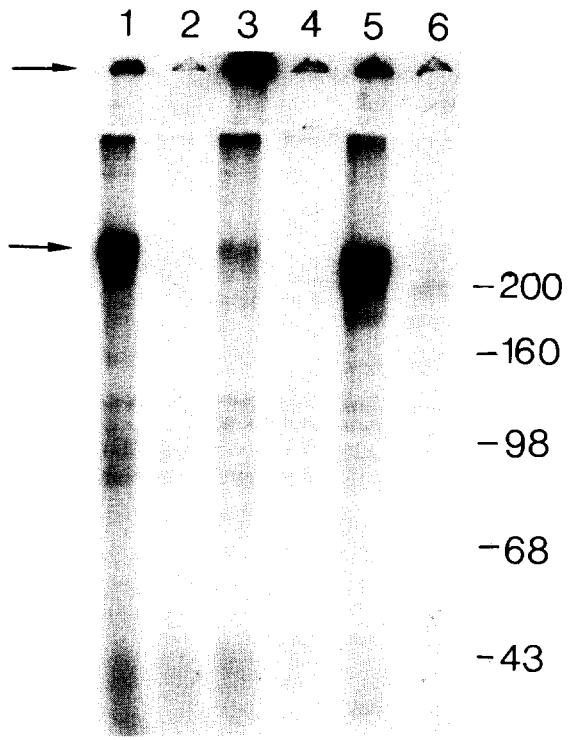

Fig.3. Sensitivity of the very high molecular weight protein complexes to trypsin. Surface labelled cultures of chick fibroblasts were incubated in medium 199 in the presence or absence of trypsin (5 μg/ml) for 15 min and analyzed in gels followed by autoradiography. 1. Cells iodinated three days after subculture. 2. Cells iodinated three days after subculture and treated with trypsin. 3. Cells iodinated three days after subculture and cross-linked by factor XIII. 4. Cells iodinated three days after subculture, cross-linked by factor XIII and treated with trypsin. 5. Cells iodinated one day after subculture, three days' chase. 6. Cells iodinated one day after subculture, three days' chase, treated with trypsin. For other symbols see the caption of fig.1..

gel during the chase, suggests that these proteins may be oligomers of fibronectin.

Previous studies on the restoration of cell surface fibronectin have shown that it is gradual, requiring more than 24 h and is affected by cell density [21,22] which are in accordance with the present results. The extent of fibronectin polymerization is apparently limited. Polymerization was difficult to demonstrate if the cells were labelled later than 24 h after subculture. It seems that most polymerization of fibronectin takes place when the cell density is low and when cells are growing fast.

The material at the top of the gels is relatively sensitive to manipulation and much of the radioactivity was lost when tube gels were processed for slicing and counting. The analysis of the extent of spontaneous polymerization, therefore, was done by taking the counts from the sections of the high molecular weight area of dried slab-gels as shown in table 1. This observation was reproducible whereas radioactivity in the lower part of the gels showed no reproducible differences. The radiolabelled bands from empty dishes (fig.1, gels 6 and 7) apparently do not originate from serum. The same bands were seen in plates incubated in the presence of medium 199 without serum (data not shown). Lactoperoxidase (mol. wt 78 000) and glucose oxidase (mol. wt 153 000) do not comigrate with these bands.

The mechanism of spontaneous complex formation is not known. It is possible that factor XIII present in calf serum is responsible for cross-linking in culture. By immunofluorescence we have found that both chick and human fibroblasts stain with antibodies raised against subunits of factor XIII (in preparation). Cellular transglutaminases could play a role in cell to cell interactions in vivo and attachment of cells to the plate in vitro as polymerizer of fibronectin on the surface and between cells.

Complexing of fibronectin to high molecular weight form as shown here, by covalent disulfide bonding [12] and/or transglutaminase mediated cross-linking [15] suggests a structural role for the protein. In immunofluorescence studies, fibronectin antigenicity is localized not only on the cell surface, but also to intercellular fibrillar structures [16] and also, in tissues, to various basement membrane structures separating groups of different cell types [23]. These immunochemical studies, however, cannot differentiate monomeric fibronectin from that in the polymeric cross-linked state.

Malignantly transformed cells, such as chick fibroblasts transformed by Rous sarcoma virus, activate circulating plasminogen to plasmin [24] and, at least in cell culture conditions, lack surface-associated fibronectin [3–6, 13]. As shown here, fibronectin complexed to polymeric form either 'spontaneously' or by blood coagulation factor XIII shows a similarly

high degree of sensitivity to cleavage by serine proteases as does monomeric fibronectin. The characteristic tissue invasiveness of malignant cells may in part be based on their ability to pervade structures containing monomeric or polymeric fibronectin assumed to be important in formation of intercellular matrix [23].

Acknowledgements

I thank Drs Antti Vaheri and Deane Mosher for discussions and Ms Anja Virtanen for technical assistance. This work was supported by grants from the Finnish Cancer Foundation and a grant, number CA 17373–01, awarded by the National Cancer Institute, DHEW.

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