Fibronectin observed in the nuclear matrix of HeLa tumour cells

GEROLD ZERLAUTH, JÓZEF WESIERSKA-GADEK and GEORG SAUERMANN

Institute of Tumourbiology-Cancer Research of the University of Vienna, A-1090 Vienna, Austria

* Author for correspondence

Summary

We have investigated the intracellular distribution of insoluble fibronectin in HeLa tumour cells. By indirect immunofluorescence microscopy fibronectin was detected in the nuclear region, but not in the region of the cell surface. Isolated nuclei and isolated nuclear matrices also stained for fibronectin. By quantitative enzyme-linked immunosorbent assay (ELISA), fibronectin was found almost exclusively in the subcellular fraction of isolated nuclear matrices. Using immunoblotting techniques fibronectin was detected in nuclear matrices isolated from cells grown in standard and in fibronectin-depleted medium. The data demonstrate that fibronectin in HeLa tumour cells is preferentially associated with the nuclear matrix.

Key words: fibronectin, nuclear matrix, HeLa tumour cells.

Introduction

Fibronectins are large glycoproteins which are considered to play a major role in cell-substratum and cell-cell interaction. Insoluble fibronectin has been found on the surface of most untransformed cells and in basement membranes. Alterations in the synthesis and localization of fibronectin have, however, been observed to result from the malignant transformation of cells. Though capable of synthesizing the glycoprotein, different types of tumour cells have been reported to be depleted of cell surface fibronectin (for reviews, see Hynes & Yamada, 1982; Morgan & Garrod, 1984; Vaheri & Mosher, 1978). There are also indications of an alternative splicing of fibronectin mRNA (Paul et al. 1986) and of structural differences at the protein level in transformed cells (Borsi et al. 1985; Matsuura & Hakomori, 1985). Furthermore, the presence of nuclear fibronectin in autopsy specimens of human liver tumours has been reported (Jagirdar et al. 1985).

In the present study the intracellular distribution of insoluble fibronectin in HeLa tumour cells has been investigated by immunofluorescence microscopy, immunosorbent assays and immunoblotting. The data demonstrate that the fibronectin of the tumour cells is found in association with the nuclear matrix, the skeletal framework of the interphase nucleus (Berezney & Coffey, 1977).

Materials and methods

Cell culture

HeLa S3 cells were grown at 37°C in monolayer cultures in Eagle's Minimal Essential Medium supplemented with 10% foetal calf serum in an atmosphere of 5% CO₂ in air. In some experiments fibronectin-depleted foetal calf serum was used. The fibronectin was removed by affinity binding to gelatin-Sepharose.

Isolation of nuclei and nuclear matrices

The nuclei were isolated by variations of the methods of Hodge et al. (1977) and Capco et al. (1982) and the nuclear matrices were isolated following the method of Van Eckelen & Van Venrooij (1981) as described previously (Wesierska-Gadek & Sauermann, 1985). Briefly, washed HeLa cells were homogenized in hypotonic buffer in the presence of 0.5% Nonidet P-40. After sucrose step centrifugation, the nuclei were washed in 1% Nonidet P-40, 0.5% sodium deoxycholate in low-salt buffer, and subsequently digested with 500 μg ml⁻¹ DNase I and 100 μg ml⁻¹ pancreatic RNase. After a sucrose step centrifugation the nuclease-treated nuclei were twice extracted with 0.4 M-ammonium sulphate and washed. All solutions except that used for nuclease digestion contained the protease inhibitors N-α-tosyl-L-lysine chloromethyl ketone, N-α-tosyl-L-phenylalanine chloromethyl
ketone, and aprotonin, at concentrations of 1 μg ml⁻¹ each, plus 1 mM-pepsatin and 1 mM-phenylmethylsulphonyl fluoride.

**Indirect immunofluorescence microscopy**

Cells grown on glass coverslips were fixed in 3-7 % formaldehyde at room temperature for 20 min, then permeabilized with 0.2 % Triton X-100 in phosphate-buffered saline for 1 min (Beyth & Culp, 1984). Isolated nuclei and nuclear matrices were applied directly to glass coverslips and fixed. The washed samples were incubated with rabbit anti-human fibronectin antibody (Cappel Lab., Cochranville, PA) in 1:40 dilution at 37°C for 30 min, then washed and incubated with anti-rabbit immunoglobulin G (IgG) coupled to rhodamine (Dakopatts, Glostrup, Denmark). In control experiments with human fibroblasts and HeLa tumour cells the anti-human fibronectin antibody was either omitted or pre-absorbed with fibronectin. These specimens did not stain.

**SDS–polyacrylamide gel electrophoresis and immunodetection of fibronectin**

Proteins were separated by electrophoresis in 7.5 % and 12 % polyacrylamide gels as described by Laemmli (1970), except that 7 M-urea was included in the gel buffer. Routinely, 5 % β-mercaptoethanol was included in the sample buffer. The separated proteins were electrophoretically transferred to nitrocellulose sheets as described by Towbin et al. (1979) with the addition of 0.01 % SDS in the blotting buffer. A blotting time of 40 h ensured quantitative transfer of high molecular weight proteins (Lee et al. 1984). To detect fibronectin, the blots were incubated with horseradish peroxidase-conjugated anti-human fibronectin antibody (Dakopatts) in 1:200 dilution (Figs 3–5). Colour formation by horseradish peroxidase was induced by incubation with chloronaphthol and hydrogen peroxide (Ruoslahti et al. 1982). Alternatively, the blots were incubated with a monoclonal anti-human fibronectin antibody (IST-1) specified as nonreacting with bovine fibronectin (Sera-Lab), and then exposed to biotinylated anti-mouse IgG and, finally, to [125I]streptavidin (Fig. 6). The immune complexes were detected by autoradiography.

**Enzyme-linked immunosorbent assay (ELISA) for fibronectin**

Cells and subcellular fractions were extracted with 6 M-urea, 1 % Triton X-100 and 1 mM-phenylmethylsulphonyl fluoride in phosphate-buffered saline (Mosher & Vaheri, 1978). The fibronectin concentration was determined by an ELISA method described previously (Zerlauth & Wolf, 1984). In brief, samples were placed in microtitre plates coated with gelatin (Type I; Sigma Chemical Co., St Louis, MO). The gelatin-bound fibronectin was quantified by the use of horseradish peroxidase-conjugated anti-fibronectin IgG (Dakopatts) as antibody and of o-phenylenediamine as enzyme substrate. In each assay a standard curve of human plasma fibronectin was made. The curve was linear between 4 and 40 ng of fibronectin and values obtained for identical samples assayed in parallel varied less than 5 %. The protein content was measured according to Bradford et al. (1976).

**Results**

**Immunofluorescence microscopy for fibronectin**

Indirect immunofluorescence studies were performed to localize fibronectin in cultured HeLa tumour cells. Cells grown on glass coverslips were fixed in situ and the glycoprotein identified by the use of anti-human fibronectin antibody. Bound antibody was then stained with rhodamine-conjugated second antibody.

The unpermeabilized cells did not stain in the cellular or pericellular region (not shown), indicating the absence of extracellular fibronectin. To permit the passage of the antibodies through the plasma membrane, cells were permeabilized with Triton X-100. The permeabilized cells stained intensely and uniformly for fibronectin in the nuclear region (Fig. 1A). The experiments, thus, indicated the existence of fibronectin in the tumour cell nuclei.

Fig. 1B shows that isolated nuclei also stained for fibronectin. Apparently, fibronectin is tightly associated with the nucleus, since the nuclei had been extensively washed with ionic and non-ionic detergents during the isolation procedure.

To investigate whether the nuclear fibronectin was associated with the chromatin or with the nuclear matrix, the nuclei were digested with DNase I and the chromatin constituents extracted with high-salt buffer. To ensure the removal of ribonucleoproteins from the nuclear matrix, the nuclei were also treated with ribonuclease A. SDS–gel electrophoresis revealed that the chromatin constituents were effectively separated from the nuclear matrix (Fig. 2). Comparison of the 10–20 K (K = 10 3Mr) zones of lanes D and E demonstrates that the bulk of the core histones was released by high-salt treatment of the nuclease-treated nuclei. As regards the DNA, previously published experiments have shown that less than 0.1 % of the nuclear DNA remained in the isolated nuclear matrix prepared as above (Wesierska-Gadek & Sauermann, 1985). The isolated nuclear matrix shown to be free of chromatin constituents also stained for fibronectin in immunofluorescence microscopy (Fig. 1C). This suggests that fibronectin in HeLa tumour cells occurs in association with the nuclear matrix.

To quantify the fibronectin in the different subcellular fractions an ELISA was used. As the assay used is based on two selection criteria for fibronectin, i.e. gelatin binding plus interaction with a specific antibody, it is a reliable method for the analysis of small amounts of fibronectin in a complex mixture of proteins (Zerlauth & Wolf, 1984). Table I shows that fibronectin was present in the isolated nuclei, while it was not detectable in the postnuclear supernatant of the cell homogenate. Furthermore, fibronectin could not be detected in the combined supernatants resulting from nuclease and high-salt treatment of isolated
nuclei. Apparently, the fibronectin content of the chromatin-derived fraction is very low. Significant amounts of fibronectin were, on the other hand, observed in the isolated nuclear matrix. Approximately 90% of the nuclear fibronectin was present in the nuclear matrix.

**Characterization of fibronectin by immunoblotting**

To characterize further the fibronectin detected in the isolated nuclear matrix, matrix samples were electrophoresed in SDS–polyacrylamide gels. The separated proteins were electrophoretically transferred to nitrocellulose membranes and the blotted fibronectin was identified by reaction with anti-human fibronectin antibody. Coomassie Blue staining revealed a number of protein bands in the nuclear matrix (Fig. 3, lane 1). The anti-human fibronectin antibody recognized a double band characteristic of the fibronectin subunit at approximately 220 K (Fig. 3, lane 2). This was observed after electrophoresis under reducing conditions. When, however, the routinely employed β-mercaptoethanol was omitted from the sample buffer, the antibody detected the 440 K fibronectin dimer as an additional band (Fig. 4B lanes 1, 3). These results lend further support to the identification of fibronectin in the isolated nuclear matrix.

To ensure that the fibronectin originated from the HeLa tumour cells and not from the calf serum present in the cell culture medium, control experiments were performed. First, fibronectin was removed from calf serum by affinity binding. Nuclear matrices were prepared from HeLa tumour cells grown in fibronectin-depleted medium. Fig. 5 shows a blot of the

*Fig. 1. Immunofluorescence detection of fibronectin.*

A, permeabilized cell; B, isolated nuclei; C, isolated nuclear matrix. Staining with rhodamine-conjugated anti-human fibronectin as in Materials and methods. D, isolated nuclei; E, isolated nuclear matrices in phase contrast. ×700.
gelatin-bound fraction of the nuclear matrix sample. It can be seen that the polyclonal antibody still recognized the characteristic fibronectin double band at 220 K.
Second, experiments were performed with anti-human fibronectin monoclonal antibody, IST-1, which does not cross-react with bovine fibronectin (Zardi et al. 1980). The antibody has been described to be specific for the Hep-2 domain of human fibronectin (Sekiguchi et al. 1985). Fig. 6 shows that the antibody recognized

Table 1. Distribution of fibronectin in subcellular fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein per 2×10^8 cells (mg)</th>
<th>Fibronectin (ng)</th>
<th>Amount of fibronectin (ng per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postnuclear supernatant of cell homogenate</td>
<td>90.2</td>
<td>•</td>
<td></td>
</tr>
<tr>
<td>Nuclei</td>
<td>20.9</td>
<td>157</td>
<td>7.5</td>
</tr>
<tr>
<td>DNase- , RNase-, (NH₄)₂SO₄-released</td>
<td>16.7</td>
<td>•</td>
<td></td>
</tr>
<tr>
<td>Nuclear matrix</td>
<td>2.1</td>
<td>150</td>
<td>71</td>
</tr>
</tbody>
</table>

Isolation of subcellular fractions and assays as in Materials and methods. • Below the limit of detection.

Fig. 2. Protein patterns of the subnuclear fractions. Isolation of subnuclear fractions and 12 % polyacrylamide gel electrophoresis as in Materials and methods. Coomassie Blue staining. A. Nuclei; B, proteins released from nuclei by DNase I digestion; C, proteins released by RNase A digestion; D, proteins extracted with 0.4 M-ammonium sulphate; E, nuclear matrix.

Fig. 3. Identification of fibronectin by immunoblotting. Electrophoretic separation of nuclear matrix proteins in 7.5 % polyacrylamide gel and immunological identification of blotted fibronectin as in Materials and methods. Lane 1, Coomassie Blue staining; lane 2, immunostaining.
Fig. 4. Electrophoretic separation of fibronectin under different reducing conditions. Electrophoresis and immunostaining as in Fig. 3.
A. Coomassie Blue staining; B, immunostaining. Lanes 1, 2, human plasma fibronectin; lanes 3, 4, nuclear matrix proteins; lanes 1, 3, samples dissolved in the absence of β-mercaptoethanol; lanes 2, 4, samples dissolved in the presence of 5% β-mercaptoethanol.

Fig. 5. Fibronectin in nuclear matrices of cells grown in fibronectin-depleted medium. Cell growth in fibronectin-depleted medium as in Materials and methods. The fibronectin of isolated nuclear matrices was bound to and eluted from gelatin-Sepharose. Immunostaining as in Fig. 3.

Thus, in addition to immunofluorescence microscopy and quantitative analysis by ELISA, electrophoretic analysis and immunoblotting with mono- and polyclonal antibodies demonstrate the presence of fibronectin in the nuclear matrix of HeLa tumour cells.

Discussion

The present data show that the intracellular insoluble fibronectin in HeLa tumour cells is localized in the cell nucleus. Interestingly, the major part of the nuclear fibronectin was found in tight association with the isolated nuclear matrix and not in association with the chromatin. These conclusions result from concordant data obtained by such different experimental approaches as immunofluorescence microscopy, immunoblotting and quantitative analysis of subcellular fractions.

The nuclear matrix is operationally defined as the insoluble structure remaining after DNase and high-salt treatment of isolated nuclei (Berezney & Coffey, 1977). It is considered to represent the main structural
framework of the interphase nucleus (for review, see Barrack & Coffey, 1982).

Our conclusions regarding the intranuclear localization of fibronectin are in contrast to those of Zardi et al. (1979) who reported the existence of chromatin-associated fibronectin. However, this discrepancy can be explained by differences in the procedures for subcellular fractionation. From the experimental protocol of Zardi et al. (1979) it can be deduced that their 'chromatin' fraction contained, among other cellular components, also the nuclear matrix. In our present experiments the chromatin constituents were clearly separated from the residual nuclear matrix by nuclease digestion and high-salt treatment of isolated nuclei. This is demonstrated by electrophoretic analysis of the proteins in the individual subnuclear fractions (Fig. 2) and by quantitative analysis of the DNA-derived material (Wesierska-Gadek & Sauermann, 1985).

The question is open as to whether the association of fibronectin with the nuclear matrix in the HeLa tumour cells is due to alterations of the fibronectin molecule or to specific properties of the nuclear matrix. As regards the properties of the nuclear matrix of neoplastic cells, knowledge is still scarce (for review, see Berezney, 1979). On the other hand, the formation of fibronectin variants with differing peptide domains (Borsi et al. 1985; Castellani et al. 1986; Sekiguchi et al. 1985) and differing binding behaviour (Borsi et al. 1985) has been observed in tumour cells. Apparently, alternative splicing of the primary transcript of the fibronectin gene occurs (Colombi et al. 1986; Paul et al. 1986). Furthermore, transformation-dependent differences in the degree of branching, internal fucosylation, and terminal sialylation of fibronectin have been reported (Nichols et al. 1986).

We are indebted to Mrs E. Kainzbauer and Mr D. Printz for their excellent technical assistance.

References


(Received 10 August 1987 – Accepted, in revised form, 27 November 1987)