Differences in Glycosylation State of Fibronectin from Two Rat Colon Carcinoma Cell Lines in Relation to Tumoral Progressiveness1

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ABSTRACT

We have investigated the biosynthesis and carbohydrate structure of fibronectin secreted by two rat colon carcinoma cell lines. The cell line Prob yields progressive tumors after s.c. injection in syngeneic BD IX rats while tumors developed by Regb cells disappear after 20 days. No difference was observed in the fibronectin biosynthesis from both cell lines; however, the glycosylation degree was higher in Regb than in Prob cells indicating probable differences in the posttranscriptional process. The analysis of the glycosylation nature shows that fibronectin doesn't bear O-linked carbohydrate chains. The fibronectin of progressive Prob cells is more sialylated than that of the regressive Regb ones. In addition, the tri- and tetraantennary glycans are more important in Prob, while the fucosylated triantennary glycans are three times higher in Regb cells.

These differences in the glycosylation state of the fibronectins could explain their differential susceptibility to the proteases treatment. In fact, the low glycosylated fibronectin from the progressive Prob cells was more rapidly degraded by several proteases than that of regressive Regb cells. The identification of the specific sites of proteolytic cleavage by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the cell attachment domain as well as the collagen binding domain of Regb cell fibronectin are particularly protected against proteolytic degradation.

INTRODUCTION

Fibronectin constitutes a group of glycoproteins, found in plasma and the extracellular matrix of connective tissues, which promote the attachment and spreading of cells (1–3). The insoluble variant of fibronectins which belongs to the cell surface binds collagen, heparin/heparan, hyaluronic acid, and fibrin (4, 5). The fibronectin cell attachment domain has recently been shown to bind to a receptor complex with a molecular weight of 140,000 that contributes to microfilament bundles at cell substrate adhesion sites (6–9). Through the use of small synthetic peptides deduced from the primary structure of the fibronectin cell attachment domain, the tetrapeptide Arg-Gly-Asp-Ser has been found to be the major fibronectin sequence which confers cell attachment activity (10).

Several studies report that the cellular form of fibronectin has a much higher biological role in cell spreading (11). Decreasing and disappearance of fibronectin, observed in cancer tissues, have been related to the metastatic potential of tumoral cells. Thus, the lack of pericellular fibronectin has been considered as one of the most distinct phenotypic alterations of cultured malignant mesenchymal cells (2, 12–15).

Tissue and plasma fibronectins exhibit differences in their carbohydrate structures in several species. These differences are essentially due to fucosylation and sialylation degrees of N-linked carbohydrate chains (16). Recently, Virtranen et al. (17) established significant differences in the distribution of biotinylated glycan chains between fibronectin from normal and trans-
Purification of Fibronectin

\[^{3}H\]-, \[^{125}I\]-, or \[^{35}S\]-labeled fibronectin was purified according to Engvall and Ruoslahti (27). The cell supernatant was harvested and protease inhibitors were added (1 mM EDTA, 1 mM aprotinin, 0.5 mg/ml benzamidine, 0.5 mg/ml trypsin inhibitor). This protease inhibitor mixture was added to all the buffers used in the purification procedure to prevent proteolytic degradation of fibronectin. The medium was then applied onto a gelatin-Sepharose column, equilibrated with 40 mM Tris-HCl buffer containing 140 mM NaCl, pH 7.4 at room temperature. After extensive washing with a 4 M NaCl, 1 M urea buffer, the bound fibronectin was eluted with 4 M urea. The elution profile was monitored by radioactivity counting. The fractions containing fibronectin were pooled then desalted on a Trisacryl GF 05 column equilibrated with distilled water containing protease inhibitors. They were frozen at \(-70^\circ\)C until use.

Fibronectin Biosynthesis

Prob and Regb cells (2.5 x 10^7) were grown in complete medium in presence of 50 \(\mu\)Ci [\(^{1}H\)]glucosamine (4.2 Ci/mmol) and 10 \(\mu\)Ci of [\(^{14}C\)]glucose (342 mCi/mmol). The incubation was conducted for 48 h at 37°C in 5% CO2/95% air humidified atmosphere incubator. The fibronectin was then purified as described before, and the double labeling was estimated by counting with a Beckman \(\beta\) scintillator counter.

SDS-PAGE

Cells (2.5 x 10^9) were grown in complete medium containing [\(^{35}S\)]methionine (50 \(\mu\)Ci/ml, 800 Ci/mmol) during 48 h at 37°C. Fibronectin was then purified and submitted to SDS-PAGE (6% acrylamide) according to Laemmli (28). After migration, the gel was impregnated with Amplify and fluorographed. The proteins used for calibration were: catalase (M, 232,000), ferritin (M, 220,000), phosphorylase b (M, 94,000), bovine serum albumin (M, 67,000), ovalbumin (M, 43,000), and carbonic anhydrase (M, 30,000).

Analysis of Fibronectin Carbohydrate Structures

Prob and Regb were metabolically labeled with [\(^{3}H\)]glucosamine and [\(^{14}C\)]glucosamine, respectively. The two conditioned media were pooled and fibronectin was then purified as described above. The glycopeptides were obtained by extensive pronase digestion (2 \(\mu\)g/ml) for 24 h at 60°C in a 0.2 M ammonium acetate buffer, pH 6.5 (29). After desalting on a Trisacryl GF 05 column equilibrated in 40 mM Tris, 110 mM NaCl buffer, pH 7.40. After washing, the elution was performed with the same buffer containing 300 mM of the competitive sugar: galactose, \(\alpha\)-methyl-D-mannoside, and \(\alpha\)-acetylneuraminic acid for RCA, Con A, and LPA, respectively.

Specific Deglycosylation Procedures

Neuraminidase Treatment. [\(^{35}S\)]methionine-labeled fibronectin was incubated with 0.3 U/ml of neuraminidase at 37°C for 8 h (30). After treatment, the sample was submitted to affinity chromatography on LPA and RCA ultrigel columns, successively.

Alkali-sensitive O-linked Carbohydrate Chains. The O-glycan structures were released from the polypeptide backbone by alkaline treatment (31). [\(^{14}C\)]Glucosamine-labeled fibronectin was incubated in a freshly prepared solution of 0.05 M NaOH, 1 M NaBH\(_4\) for 15 h at 45°C. NaBH\(_4\) was then destroyed by adding progressively a 50% acetic acid solution till pH 5. The reaction mixture was then fractionated by gel filtration on a Trisacryl GF O5 column equilibrated with 40 mM Tris-HCl, 110 mM NaCl buffer, pH 7.4. The efficiency of this treatment was checked by detecting the residual O-glycan structures by dot blot with an anti-T-antibody (clone 49 H8 kindly provided by Chembiomed Ltd., Edmonton, Canada). Briefly, after alkaline treatment, fibronectin was incubated with 0.3 unit/ml of neuraminidase at 37°C for 8 h (30). The mixture was dotted on nitrocellulose sheet (32). The sheet was saturated with Blotto (5% w/v nonfat dry milk) in 1 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl buffer, pH 7.4 (33). The sheet was then successively incubated with anti-T-antibody and anti-mouse IgG (H + L) antibodies conjugated with peroxidase. The immune complexes were reacted with 0.1% 3,3'-diaminobenzidine plus 0.01% H\(_2\)O\(_2\) in 50 mM Tris-HCl buffer, pH 7.6. A positive control was obtained with mucins prepared from saliva heated at 100°C during 5 min. Mucins were enriched in the supernatant obtained after centrifugation and are treated with sialidase as for fibronectin.

Endoglycosidase F Treatment. Cells were grown in complete medium containing [\(^{35}S\)]methionine (10 \(\mu\)Ci/ml, 800 Ci/mmol) and [\(^{14}C\)]glucosamine (50 \(\mu\)Ci/ml, 17.5 Ci/mmol) during 48 h at 37°C. After fibronectin purification, the asparagine N-linked carbohydrates were released by incubating the labeled fibronectin with Endo F as described by Elder and Alexander (34). The samples (100 \(\mu\)l) were incubated in a 0.1 mM sodium phosphate buffer, pH 6.1, containing 50 mM EDTA, 0.5% Nonidet P 40. Twenty \(\mu\)l of Endo F (40 milliunits) were added and the incubation was conducted for 24 h at 37°C. The interaction of the fibronectin with Con A-Sepharose column was studied.

Digestion of Fibronectin by Proteinases

Cells were grown in complete medium supplemented with 10% fetal calf serum. When cells were confluent, the medium was changed to, and the cells were maintained in the same medium without fetal calf serum for 24 h. The medium was harvested, protease inhibitors were added, and fibronectin purified as described above.

The purified fibronectin from two clones was labeled with \(N\)-succinimidyld-\(2,3,3\)'-propionate according to the method of Bolton and Hunter (35). Protein (50–100 \(\mu\)g) were incubated for 60 min at room temperature in the presence of 1 mM of \(N\)-succinimidyld-\(2,3,3\)'-propionate. The samples were then chromatographed on a GF O5 column equilibrated with 40 mM Tris-HCl, 110 mM NaCl buffer, pH 7.4. The \(^{3}H\)-labeled protein, excluded from the column, was then submitted to proteolytic digestion.

About 1 \(\mu\)g of fibronectin was incubated at 37°C in 50 mM Tris-HCl, 10 mM CaCl\(_2\), buffer, pH 8.5, with thermolysin (100 ng) or elastase (1 \(\mu\)g). In the case of cathepsin D, the fibronectin was incubated with 400 ng of this enzyme in 50 mM acetate buffer, pH 3.5, at 37°C. The reactions were stopped at several times using protease inhibitors: 10 mM phenylmethylsulfonyl fluoride for elastase, 10 mM EDTA for thermolysin, and 10 \(\mu\)g/ml pepstatin A for cathepsin D. The products of protease digestion were, on one hand, precipitated with 10% trichloroacetic acid and the radioactivity determined in the soluble fraction (19), or subjected to analysis by SDS-PAGE (5–15% acrylamide) according to Laemmli (28) and fluorographed.

RESULTS

Fibronectin Purification and Biosynthesis. The method used for fibronectin purification allowed us to obtain a highly purified preparation. No contaminant molecule was observed.
Glycosylation Nature of Tumoral Fibronectins. In a preliminary experiment, we have checked the presence of O-linked carbohydrates on fibronectin molecule. The labeling of fibronectin from two clones by [14C]glucosamine, followed by a β-elimination treatment, indicated that no O-linked carbohydrate chains were detected. This observation was confirmed by an immunological study with the anti-T-antibody after sulfidase treatment.

Any positive immunoreaction in dot blot was observed with both fibronectins before alkaline release as well as after this chemical procedure. In order to confirm the presence of only N-linked carbohydrates, we have treated by endoglycosidase F the two fibronectins labeled to both [35S]methionine and [3H]-glucose and we have studied the modifications induced in the Con A-fibronectin interactions. Table 2 shows the percentage of 3H and 35S radioactivity bound to Con A before and after endoglycosidase F treatment. A similar amount of 3H and 35S radioactivity was bound to Con A column before treatment in each cell type. Endoglycosidase F completely abolished the interactions between the polypeptide backbone and Con A; all radioactivity was recovered in the carbohydrate chains proving that they are N-linked type. The higher percentage of glycans bound to Con A after endo F treatment could be explained by the denaturation of the molecule inducing more exposure of the carbohydrate chains to Con A.

In addition, the binding of fibronectin on LPA-ultrogel column is higher in progressive Prob cells (68%) as compared to regressive Regb cells (38%). This recognition was completely abolished after neuraminidase treatment, indicating a better exposure of sialic acids on oligosaccharide structures of fibronectin secreted by the progressive Prob cells. When the LPA-unbound fibronectin fraction was applied on a RCA-ultrogel column, only a part of fibronectin was found to be retained by the column (19 and 15% for Prob and Regb fibronectin cells respectively), indicating that a minor fraction of fibronectin molecules bears accessible galactose residues.

Fibronectin Oligosaccharide Structures. Fig. 2 and Table 3 show that a first group of glycopeptides, unbound on the two lectins (Con A−, LCA−) and corresponding to tri- and tetra-antennary glycans, is more represented in progressive Prob (37%) than in regressive Regb (24%) cells. A second group (Con A+, LCA−), corresponding to fucosylated triantennary glycans, is three times higher in regressive Regb than in progressive Prob cells. These differences are less important with the other glycan structures, i.e., biantennary (Con A−, LCA−), corresponding to fucosylated biantennary (Con A+, LCA−) and in the group of high mannose or hybrid type glycans (18, 29).

Proteolytic Digestion of Fibronectins. To determine whether the carbohydrate moieties of fibronectin play a role in its susceptibility to protease degradation, we attempted first a kinetic study on fibronectin hydrolysis by several proteases. Fig. 3 shows the rate of conversion of both fibronectins to trichloroacetic acid-soluble products. All of the proteases digest the low glycosylated fibronectin of Prob cells more rapidly than the high glycosylated fibronectin of Regb cells. The cathepsin D treatment degraded almost all the Prob fibronectin after 60 min, but only 15% of Regb fibronectin were hydrolyzed in the same conditions. A similar phenomenon was observed with thermolysin or elastase, but to a lesser degree. In addition, the rates of proteolysis of both fibronectins are biphasic; rates are more rapid during the first 10 min of incubation with all proteases.

In order to determine the specific sites of proteolytic cleavage, the peptide products of all proteases after 30-min incubation were analyzed by SDS-PAGE. The proteolytic treatment analysis showed that fibronectin of progressive Prob cells was more

Table 1 Incorporation of [14C]leucine and [3H]glucosamine into fibronectin secreted by Prob and Regb cells

| Cells | [14C]Leucine (dpm/mg protein) | [3H]Glucosamine (dpm/mg protein) | Ratio H/C
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Prob</td>
<td>8,975 ± 50</td>
<td>8,350 ± 300</td>
<td>0.93</td>
</tr>
<tr>
<td>Regb</td>
<td>9,150 ± 100</td>
<td>15,250 ± 700</td>
<td>1.66</td>
</tr>
</tbody>
</table>

Table 2 Effect of endoglycosidase F on the binding of fibronectins to Con A-ultrogel

<table>
<thead>
<tr>
<th>Cells</th>
<th>Isotope</th>
<th>Binding (%)</th>
<th>-Endo F</th>
<th>+Endo F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reg</td>
<td>H</td>
<td>51 ± 3</td>
<td>75 ± 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>50 ± 2</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>H</td>
<td>43 ± 2</td>
<td>80 ± 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>36 ± 4</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* ND, not detected.
OLIGOSACCHARIDIC STRUCTURE OF TUMORAL FIBRONECTINS

Fig. 2. Sequential affinity chromatography on immobilized lectins. The pronase digest of fibronectins is applied onto a Con A Sepharose column (A) as described under "Materials and Methods." The unretained fraction (peak I) and the 10 mM α-methyl-D-glucoside eluate (peak II) were subsequently submitted to LCA-Ultrogel chromatography in B and C, respectively. Arrows, the 150 mM α-methyl-D-glucoside addition. X, Regb; *, Prob.

Table 3 Glycopeptide distribution in fibronectin secreted by Prob and Regb cells
Labeled fibronectin with [3H]glucosamine (Prob) or [14C]glucosamine (Regb) was extensively treated by pronase and submitted to sequential chromatography on immobilized lectins as described in "Materials and Methods." The values were expressed in percentage of total dpm.

<table>
<thead>
<tr>
<th>Lectins binding</th>
<th>Carbohydrate structures</th>
<th>Prob (%)</th>
<th>Regb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A α, LCA α</td>
<td>Tri and tetraantennary</td>
<td>37</td>
<td>24.8</td>
</tr>
<tr>
<td>Con A α, LCA α</td>
<td>Fucosylated triantennary</td>
<td>3.5</td>
<td>10</td>
</tr>
<tr>
<td>Con A α, LCA α</td>
<td>Biantennary</td>
<td>42</td>
<td>44.5</td>
</tr>
<tr>
<td>Con A α, LCA α</td>
<td>Fucosylated biantennary</td>
<td>11.3</td>
<td>14.9</td>
</tr>
<tr>
<td>Con A α, LCA α</td>
<td>High mannose and hybrid types</td>
<td>5.6</td>
<td>5.56</td>
</tr>
<tr>
<td>Con A α, LCA α</td>
<td>High mannose and hybrid types</td>
<td>5.6</td>
<td>5.56</td>
</tr>
</tbody>
</table>

* Fraction eluted by 10 mM α-methyl-D-glucoside.
* Fraction eluted by 300 mM α-methyl-D-glucoside.

often converted to small peptide fragments than that of regressive Regb cells (Fig. 4). Thermolysin cleaved the high glycosylated Regb cell fibronectin to three major polypeptides at M, 160,000, 68,000, and the collagen binding domain M, 40,000 (19). In the case of low glycosylated Prob cell fibronectin, these polypeptides are significantly degraded to more small polypeptides and are present at a trace amount after protease treatment. The cathepsin D completely cleaved the progressive Prob fibronectin generating, among others, a polypeptide (M, 130,000) corresponding to the cell attachment domain (36). This degradation is very limited in the case of high glycosylated Regb cell fibronectin since a high amount of this protein remains intact. The same phenomenon was obtained after elastase degradation which cleaves the fibronectin in major polypeptides at M, 190,000, 175,000, 150,000, 76,000, 67,000, and 33,000. These

Fig. 3. Protease digestion of Prob and Regb fibronectin. Fibronectins are incubated with different proteases as described under "Materials and Methods." At the indicated times, reactions were stopped and trichloroacetic acid was added at a final concentration of 10%. Radioactivity was determined in both soluble and precipitable fractions. □, Prob; *, Regb.

Fig. 4. Proteolytic cleavage products of Prob and Regb fibronectin. After treatment of both fibronectins with proteases during 30 min, samples were electrophoresed by SDS-PAGE as described under "Materials and Methods." Odd numbers, Prob fibronectin; even numbers, Regb fibronectin. 1 and 2, fibronectin control; 3 and 4, fibronectin treated with thermolysin; 5 and 6, fibronectin treated with cathepsin D; 7 and 8, fibronectin treated with elastase.
polypeptides are accumulated after hydrolysis of high glycosylated Regb cell fibronectin and conversely very degraded in the case of the Prob cell one.

DISCUSSION

In this report, we have compared structural properties of fibronectin secreted by two rat colon tumoral cell lines which differ in their metastatic capacities. Our results show that no difference in fibronectin biosynthesis can be correlated to the progressive ability of each cell clone. However, the glycosylation degree of fibronectin is higher in regressive Regb cells than in progressive Prob ones. This can be related to the differentiated state of the cell. In fact, two recent observations have shown that on one hand, the glycosylation state of fibronectin during the gestation increased with fetal development (37), and on the other hand, tumoral cells present dedifferentiated phenotypes (38). Thus, the lower glycosylation degree of fibronectin from Prob cells as compared to Regb could indicate that Prob are less mature. Such alteration of the glycosylation has also been observed in fibronectin receptors (integrins) between immature and mature human monocytic leukemia cells (39). In addition, our results suggest that the glycosylation degree of fibronectin does not alter its biosynthesis. This phenomenon is commonly observed in the secretion of fibronectin (40) in contrast with other glycoprotein biosynthesis. The nature of the glycosylation of the secreted fibronectin from the two cells seems to be only N-linked asparagine type as it was shown in other cases (41). However, other structures have been described as minor O-linked glycans borne by fibronectin originating from human amniotic fluid (42) as well as human oncofetal hepatomas (43).

The binding on a Con A-Sepharose column reveals differences in the carbohydrate structure between the two cell clones. In fact, the fibronectin fraction bound to this lectin is higher in Regb cells. The existence of unbound fibronectin on Con A in tumoral cells has been reported by Murayama et al. (44). This phenomenon can be explained by the high level of sulfation of fibronectin as it has been described in fibronectin from endothelial cells (45). On the contrary, Prob cell fibronectin is more sialylated than the one from Regb cells. This increase in the sialylation ratio of the fibronectin seems to characterize the metastatic phenomenon, since it has been observed in SV 40 virus-transformed WI 38/VA 13 cell fibronectin (44) and in BHK 21/C 13 cells (18).

The glycopeptide analysis of cell surface glycoproteins allows the differentiation of two clones since tri- and tetraantennary glycans are more abundant in progressive Prob cells while the fucosylated triantennary glycans are three times higher in Regb cells. The higher expression of tri- and tetraantennary glycans in transformed cells concomitant with an increase of the sialylation ratio of fibronectin has also been observed in the fibronectin from sarcoma-induced BHK 21/C 13 cells and could be associated to the neoplastic properties of tumoral tissue (18). Such highly branched and sialylated N-linked oligosaccharides are also found in malignant and transformed cell surface glycoproteins (46).

One of the sequential steps involved in the metastatic process is the disorganization of the extracellular matrix by its component hydrolysis (47, 48). Our results clearly show that proteases degrade more rapidly the fibronectin secreted by progressive Prob cells than that of regressive Regb ones. This is probably due to the low glycosylation degree of Prob cell fibronectin. Higher susceptibility to Pronase digest was observed in nonglycosylated than in native fibronectin (19, 49).

In addition, carbohydrates seem to selectively protect the glycosylated domains of fibronectin such as collagen binding fibronectin (thermolysin digest, M, 40,000) as has been described in the case of fibronectin from fibroblasts (19). Finally, higher susceptibility of the attachment cell domain of the Prob cell fibronectin to be released by cathepsin D (M, 130,000) could explain the metastatic properties of these cells. The molecular mechanism of protective effect of carbohydrates against proteolytic degradation remains unknown. The oligosaccharide moieties may impose a spatial conformation rendering the fibronectin a poor substrate for proteolytic enzymes (50). However, another explanation is that oligosaccharide chains may induce a steric hindrance which prevents access of proteases to potential proteolytic sites of fibronectin.

This relationship between the low glycosylation degree of Prob cell fibronectin and their susceptibility to exogenous protease degradation should be confirmed by the existence of endogenous proteases in cancer cells. Thus, experiments are underway in our laboratory to detect any protease activity on cancer cell surfaces or in the medium secreted by the two clones.

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