Immunocytochemical Characterization of Extracellular Matrix Proteins Expressed by Cultured Glioma Cells

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ABSTRACT

The immunolocalization of type I, III and IV collagens and fibronectin in two rat glioma cell lines in vitro (BT4C and BT4Cn) is described. In addition, antibodies against denatured type I and III collagens were used to study breakdown products of native type I and III collagens. For the BT4C cells, the extracellular matrix expression in monolayer cultures and in multicellular tumor spheroids was compared. Type IV collagen was strongly expressed in BT4C tumor spheroids but was negative in the corresponding monolayer cultures. Denatured type I collagen was found both in monolayers and in spheroids of BT4C, suggesting either a rapid turnover (i.e., synthesis and immediate breakdown) of type I collagen or an altered collagen gene transcription. Both cell lines were negative for native type I and III and denatured type III collagen.

Fibronectin was strongly expressed in both cell lines. Supporting the immunofluorescence data, the hydroxyproline content in the tumor spheroids was twice the amount found in monolayer cultures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis combined with immunoblotting also verified the immunostaining experiments, showing that glioma spheroids and injected tumor cells have the potential for fibronectin and collagen production, given the appropriate growth conditions.

INTRODUCTION

The pathogenesis of tumor growth and invasion depends both on host factors and on properties of the tumor cells. These entities are involved during tumor progression. However, it is difficult to know what contribution the tumor cells or the host tissue have in the invasive process (1). In a previous paper (2), we reported the distribution of ECM components in brain tumors in vivo but could not identify the origin of the proteins synthesized de novo. Human glioma cells have the ability to synthesize ECM components in vitro grown both as spheroids and as monolayers (3, 4). However, the production of such components in monolayers has not been directly compared to the production of ECM components in three-dimensional cultures. Furthermore, no comparison to the in vivo situation has been made.

In this paper, we have examined the expression of different collagen types and fibronectin by two rat glioma cell lines in monolayer and spheroid cultures, and these results are compared with our results reported in vivo (2).

MATERIALS AND METHODS

Spheroid Cultures. Multicellular tumor spheroids were prepared by incubating BT4C cells in medium-agar overlay cultures on a non-adherent medium-agar substrate as has been described earlier (5). The cells were grown in Dulbecco’s modification of Eagle’s medium (Flow Laboratories, Glasgow, Scotland), supplemented with 10% heat inactivated newborn calf serum, 4 times the prescribed concentration of nonessential amino acids, l-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). They were kept in a standard tissue culture incubator (100% relative humidity, 95% air, and 5% CO2). After 7 days, individual spheroids with diameters of 300 μm were frozen in isopentane, cooled with liquid N2, and embedded in Tissue-Tek (Miles Laboratories, Inc., Naperville, IL). Cryostat sections (5 μm) were cut using a Leitz micromtome (Leitz, Wetzlar, Federal Republic of Germany). The sections for immunostaining were prepared as has been described previously (2). Some spheroids were washed in PBS and freeze dried for SDS-PAGE analysis or hydroxyproline estimation. The BT4Cn tumor cell line is unable to form spheroids in culture (6).

BT4C spheroids, between 300 and 600 μm in diameter, were also prepared for light microscopy (for details, see Ref. 2). Semithin 1.5-μm-thick sections were cut on a Reichert-Jung microtome 2040 (Reichert, Nussloch, Federal Republic of Germany) and stained with toluidine blue.

Monolayer Cultures. Monolayer cultures of BT4C and BT4Cn were propagated on sterile coverslips in 10-cm Petri tissue culture dishes (Nunc, Roskilde, Denmark), using the same culture medium as described above. Cells were analyzed for ECM components both in the experiment and at confluence. The coverslips were rinsed in PBS, fixed for 2 min in ice-cold acetone, and air dried. The cells were also grown in 250-cm² tissue culture flasks (Nunc). Monolayers in confluent culture (3 × 10⁶ cells/flask) were scraped off the vessels with a rubber policeman, washed in PBS, and freeze dried for hydroxyproline determination, electron microscopy, and immunoblotting.

Hydroxyproline Estimation. For hydroxyproline estimation, freeze dried BT4Cn cells and BT4C spheroids were hydrolyzed in 5.7 m HCl at 180°C for 20 h. The 3-hydroxyproline content was then measured colorimetrically (7). The hydroxyproline content in the BT4A and BT4An tumor in vivo was also measured using the same tumor material as described previously (2). In addition, the hydroxyproline content in normal rat brain tissue was determined. This estimate neither differentiates between collagen localized in blood vessels and interstitial collagen nor does it distinguish between different collagen types.

Immunofluorescence Procedures. The antibodies directed against ECM proteins were described previously (type I, III, and IV collagens, fibronectin, and denatured forms of type I and III collagens; for details, see Refs. 2 and 8). Air dried, acetone fixed monolayer cultures of BT4C and BT4Cn and acetone fixed cryostat sections of BT4C tumor spheroids were immunostained as described previously (2).

Electrophoresis and Immunoblotting. Two mg of freeze dried cells were solubilized using 1 ml SDS-PAGE sample preparation buffer (Bio-Rad, Ltd., Watford, England) and reduced by addition of 10 μl 2-mercaptoethanol. Proteins were separated on 9% SDS-PAGE according to the method of Laemmli (9) and were then transferred to nitrocellulose paper according to Towbin et al. (10). The paper was blocked with 3% bovine serum albumin in PBS at 4°C for 16 h. Immundetection of type IV collagen, denatured type I collagen, and fibronectin was performed by incubation with primary antibody diluted in PBS-0.05% Tween 20. Detection was obtained by a biotinylated second antibody and streptavidin peroxidase using 4-chloronaphthol as a substrate.

RESULTS

Light Microscopy

The BT4C spheroids were densely packed with atypical cells. In the larger spheroids, a necrotic core was observed starting approximately 150 μm from the surface (Fig. 1).
EXPRESSION OF ECM PROTEINS BY GLIOMA CELLS

Fig. 1. Semithin toluidine stained section of a BT4C spheroid. Note the high density of cells in the spheroid and the necrotic core starting approximately 150 μm from the spheroid surface. ∗ 90.

Immunofluorescence

BT4C Spheroids. The native type I and III collagens were negative in BT4C spheroids (Fig. 2A). However, the denatured type I collagen showed an immunopositive fibrillary network, especially in the ECM (Fig. 2B). The denatured type III collagen was negative. Type IV collagen was strongly immunopositive (Fig. 2C), as was fibronectin (Fig. 2D). Both components were located in the ECM of the tumor spheroids.

Monolayers of BT4C and BT4Cn. Single BT4C cells in exponential growth were negative for all types of collagen. At this stage, fibronectin was expressed in a perinuclear position (Fig. 3A). The fibronectin expression changes to the cell membranes when the cells started to make cellular contacts (Fig. 3B). In confluent cultures, a strong immunopositive reaction was observed as a network between the cells (Fig. 3C). Confluent cultures were also slightly immunopositive for denatured type I collagen (Fig. 3D).

Exponential and confluent cultures of BT4Cn were negative for all antibodies tested, except for fibronectin, which showed an expression similar to that of BT4C cells (data not shown).

The immunolocalization of the ECM components of both tumors in vitro and in vivo (2) are summarized in Table 1.

Hydroxyproline Determinations

The hydroxyproline contents of BT4Cn and BT4C monolayers, BT4C spheroids, as well as the BT4A and BT4An tumors in vivo are shown in Table 2. The BT4A and BT4An tumors in vivo contained 3-4 times more hydroxyproline than normal rat brain. The amount of hydroxyproline found in vitro was for both cell lines less than the amount found in vivo. BT4C tumor spheroids had twice the amount of hydroxyproline as compared to the corresponding confluent monolayer cultures. The BT4Cn cells contained very little hydroxyproline (0.53 nmol/mg).

Electrophoresis

BT4C spheroids and BT4C and BT4Cn monolayers were subjected to SDS-PAGE on 9% gels and immunoblotted after transfer to nitrocellulose paper (Fig. 4). Immunodetection by antibodies against denatured type I collagen showed multiple immunopositive bands in BT4C spheroids. Monolayers of BT4C showed only one slightly stained band. These bands do not correspond to α1(I) or α2(I) intact collagen chains. The BT4Cn cell line was negative for this antibody.

Type IV collagen was not detected in monolayers of BT4C and BT4Cn. BT4C spheroids were, however, positive.

Fibronectin was detected in both BT4C spheroids and BT4Cn monolayers. The bands seen in these samples were of lower

Fig. 2. Immunostaining of native type I collagen (A), denatured type I collagen (B), type IV collagen (C), and fibronectin (D) in BT4C tumor spheroids. A–C, × 550; D, × 240.
**Table 1** Semiquantitative estimate of the immunolocalization of ECM components in rat gliomas in vitro and in vivo

<table>
<thead>
<tr>
<th>ECM components</th>
<th>In vitro</th>
<th>In vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BT4Cn</td>
<td>BT4C</td>
</tr>
<tr>
<td>Native type I collagen</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Denatured type I collagen</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Native type III collagen</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Denatured type III collagen</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Type IV collagen</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*Confluent cultures.

* -, negative; +, slightly positive; ++, moderately positive; ++++, strongly positive.

* Positive in the brain-tumor border zone.

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**Table 2** Hydroxyproline content of rat glioma cells in vitro and in vivo

<table>
<thead>
<tr>
<th>Tissue</th>
<th>In vitro</th>
<th>In vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT4C (monolayers)</td>
<td>1.09</td>
<td>-</td>
</tr>
<tr>
<td>BT4C (spheroids)</td>
<td>2.06</td>
<td>5.50*</td>
</tr>
<tr>
<td>BT4Cn (monolayers)</td>
<td>0.53</td>
<td>6.50*</td>
</tr>
<tr>
<td>Normal rat brain</td>
<td>1.48</td>
<td>-</td>
</tr>
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</table>

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**DISCUSSION**

Tumors are generally known to produce ECM components similar to those produced by their tissue of origin. However, as a result of the transformation process, new gene products may well be observed in the tumors (11, 12). The present study supports earlier observations on the production of type IV collagen and fibronectin by human glioma cells *in vitro* (13, 14). These components are not generally found in their normal glia counterparts (15, 16). However, one might speculate that molecular weight than fibronectin in an undegraded form with reference to molecular weight markers. Control sera were negative for both cell lines tested.
astrocytes can be stimulated to synthesize basement membrane components by contact with mesenchymal tissue. A basal lamina formation by astrocytes has, for instance, been observed in organotypic cultures of mouse spinal cord tissue (17). A similar process may occur when glioma cells are grown in vitro. Type IV collagen expression was negative in confluent monolayer cultures of BT4C (not shown) but was strongly positive in the parenchyma of the BT4C tumor spheroids (Table 1; Fig. 2C). In contrast, in a previous paper, we demonstrated the expression of type IV collagen in the vasculature and mesenchymal components of the gliomas but not in the tumor parenchyma (2). This finding suggests that the synthesis of type IV collagen by glioma cells is an unstable property which may depend on the growth conditions. It is also in line with the earlier observations that tumor cells can stimulate matrix production by nonneoplastic host cells, either by direct cell to cell contact (18) or by the release of soluble factors (19). This indicates that collagen can be produced by both malignant cells and normal surrounding cells, depending upon their environment.

In view of the lack of native type I and III collagens in vitro, it was surprising to find denatured type I collagen in the parenchyma of BT4C spheroids and in confluent monolayer cultures (Table 1; Figs. 2 and 3). These components were confined to the brain-tumor border zone in vivo. This is possibly due to collagenase activity at the periphery of the tumor, as has previously been reported for other tumors (20). In vitro, the presence of denatured type I collagen may be explained by an altered collagen gene expression. Recent studies by Rupard et al. (21) have shown that tumor cells can synthesize α1(I) collagen chains but not α2(I) chains, leading to the production of a less stable α1(I) homotrimer. The cell lines described here may also synthesize such a molecule, which is not recognized in situ by the antibody to native type I collagen, as this antibody has a requirement for a conformation dependent epitope. This less stable collagen configuration may undergo helical to random coil transition, after which it is recognized by the antibody to denatured collagen (sequence dependent epitope). Furthermore, conditioned serum-free medium of these cells (BT4C) has been shown to be able to degrade thermally denatured type I and V collagens.4

Fibronectin was strongly expressed in both cell lines in vitro and in vivo, suggesting that the fibronectin expression in these continuous cell lines may be more stable regardless of changes in the environment, i.e., solid tumor versus cell culture. Fibronectin expression in normal brain tissue and in human gliomas is usually associated with capillary vessel walls in a pattern corresponding both to the vessel lumen and the basement membrane of the vessel wall (22). However, we also found strong interstitial fibronectin staining in vivo, similar to that observed for human glioma cell lines grown in nude mice (13). This apparent difference in fibronectin expression between primary tumors and by tumors grown in animals may be caused by several factors. The fibronectin expression has been shown in cell types other than glial cells to be transformation sensitive (23–25), cell cycle sensitive (26), and dependent on the growth conditions (27). SDS-PAGE combined with immunoblotting can be considered more sensitive for protein detection and identification than immunostaining due to the concentration effect inherent in the method. We have verified the immunostaining experiments, showing that tumor spheroids and injected tumor cells have the potential to synthesize collagens if appropriately stimulated (Tables 1 and 2). The use of a heterologous species tumor-host invasion model with double immunolabeling experiments may resolve the problem of the cellular origin of collagen synthesis.

We conclude that the environment may play an important role for the expression of connective tissue components in glioma cells. This might give the malignant cells an extra advantage in their adaptation to host tissue conditions during invasion and tumor progression.

REFERENCES

9. Laemmli, U. K. Cleavage of structural proteins during the assembly of the

R. Bjerkvig, O. D. Laerum, and G. J. Rucklidge, unpublished observations.

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Fig. 4. Immunoblots of tumor cells solubilized with SDS. Spheroids of BT4C (Lane 1), monolayers of BT4C (Lane 2), and monolayers of BT4Cn (Lane 3). A, denatured type I collagen expression; B, type IV collagen expression; C, fibronectin expression. The positions of proteins of known molecular weights are indicated. Phosphorylase b, M, 92,000; bovine serum albumin, M, 68,000.


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