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Hyaluronan and Hyaluronectin in the Extracellular Matrix of Human Brain Tumour Stroma

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Hyaluronan (HA) and the hyaluronan-binding glycoprotein hyaluronectin (HN) were measured in 23 gliomas and 8 meningiomas and their location was revisited in 35 tumours. A clear-cut difference was found in the HN/HA ratio values of glioblastomas (below 0.5) and that of astrocytomas (above 0.5 $P < 0.001$). Besides their location in the intercellular part of gliomas, HA and HN displayed a perivascular location in 1/3 astrocytomas, 17/24 glioblastomas, and 3/7 meningiomas, suggesting they could be produced also by the vascular stroma of tumours and that they would characterise the neoangiogenesis. All cultivated glioma cells tested produced HA *in vitro*, whereas only 1/11 cell lines produced HN, at a low level. The results obtained suggest that glioma HA and HN are produced by both cancer cells and vascular stroma cells, which contribute to the edification of the extracellular matrix. In meningiomas only the stroma would be responsible for HA and HN production.
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INTRODUCTION

THE TUMOUR stroma is developed from normal tissues which grow along with and support cancer cells. The tumour stroma comprises new blood vessels, desmoplasia whose extracellular matrix is a major part, and inflammatory cells. The stroma is particularly important in tumour development. The nutritive blood supply and signalling components (hormones, growth factors) are brought to or emitted by cancer cells through the extracellular matrix. Among the numerous extracellular matrix components, hyaluronan (hyaluronic acid, HA[1]) is the object of an increasing interest in relation to its role in cancer cell development and invasion. HA is a highly polymerised saccha-

ride which has been isolated from different sources. In human tissues it has been found in very large amount in the umbilical cord and the vitreous body and, in lower concentration, in the extracellular matrix of soft connective tissues [1].

HA is thought to serve a number of functions during development and differentiation, migration and aggregation of cells, but its experimental effects may be different depending on its degree of polymerisation, the type of cells studied and the concentration used [2].

Many tumours are reported to be hyaluronan rich in man and other animals [3] but there are very few results for brain tumours. *In vitro* studies of glioma cells have shown they contain a higher glycosaminoglycan (GAG) concentration than normal nervous tissue [4]. In culture, cancer cells are sometimes surrounded by a HA-positive coat [5], and the HA coat of glioma cells plays a protective role against cytotoxic lymphocytes [6]. Questions which remain to be answered concern the actual concentration of HA in brain tumours and its relationship to tumour grade.

Hyaluronectin (HN) is a glycoprotein which was discovered in human brain and shown to have a specific affinity for HA [7]. It is antigenically different from the HA binding protein from cartilage [7, 8]. Immunohistochemical methods have shown that during fetal nervous system development, HN is associated with areas where cells migrate, and then with areas where neurites develop [7]. In normal nervous tissue, HN concentration increases during maturation of the nervous system whereas HA concentration in adult brain was found to be about 1/10 that in the fetal brain [7].

This work was designed to determine whether, in brain tumours, HA and HN were located in cells or, as is the case in other tumours, in the extracellular matrix, to quantitate HA and HN in human gliomas and in meningiomas, and to investigate whether or not they could be related to tumour type and grading. We found that HA and HN expression, although variable in gliomas, is present in a fetal pattern, that the HN/HA ratio is correlated with the tumour grade and that HA and HN concentrations expressed per gram of wet tissue are correlated with each other, suggesting that HA and HN expressions are mutually dependent.

MATERIALS AND METHODS

Tumour fragments (0.2–2 g) were selected from a non-necrotic area. A sample of the fragment to be extracted (0.1–0.2 g) was fixed in liquid nitrogen for histological examination and an adjacent fragment was fixed in formalin for neuropathological examination. These samples were graded by the criteria of Kernohan and Sayre [9] and compared with that of the whole tumour.

The extraction procedure is described for 0.1 g samples. The tissue was ground in 9.9 ml of phosphate buffered saline (0.15 mol/l NaCl buffered with 0.01 mol/l sodium phosphate at pH 7.4) containing 0.25 g/l sodium azide, with an Ultra-Turrax for 30–40 s to achieve maximum disruption of the tissue. The suspension was then divided into three parts. The first aliquot was stored at −80°C. The second was digested with protease for HA assay. The 50 μl sample was supplemented with 450 μl of 1 mg/ml protease from *Streptomyces griseus* type XIV (Sigma, L'Isle d'Abeau-Chesnes, France) dissolved in the same buffer, for 18 h at 37°C. Protease activity was destroyed by heating at 100°C for 20 min in an oil-bath, and the sample was spun (13 000 *g*, 10 min) in a Sigma 201 M centrifuge (Bioblock Strasbourg, France). The third aliquot was hyaluronidase digested for HN assay by supplementing the 300 μl sample with 3 μl of 100 TRU/ml solution of *Streptomyces hyaluronidase* (Calbiochem) and incubated at 37°C for 18 h, and spun as above.

HA assay was performed using an enzymoimmunological assay on the protease digested sample as described previously [10]. Briefly, 0.3 ml of the sample (diluted 10^{-1}–10^{-4}) was supplemented with 0.3 ml of a 60 μg/l solution of HN isolated from sheep brain [11], and incubated for 4 h at 4°C in an HA-coated ELISA plastic microtest plate to allow free HN to bind to insolubilised HA. A standard was established with purified, protease-digested HA, whose concentration was measured by the carbazole technique [12]. After washing, the plate was incubated overnight at 37°C, with rabbit anti-sheep brain HN antibodies (IgG fraction diluted 1/40 000 in PBS with sodium azide and 1g/l bovine serum albumin) supplemented with donkey alkaline phosphatase conjugated anti-rabbit IgG antibodies (Jackson Immunoresearch, Interchim, Montluçon, France) diluted 1/12 000 in the same buffer. The colour reaction used 1 mg/ml paranitrophenyl phosphate in 1 mol/l diethanolamine at pH 9.8 and was read on a Titertek Multiskan (Flow laboratories). On-line calculations were made with a Victor computer.

HN assays were performed with the enzyme linked immunosorbent assay (ELISA) method on hyaluronidase-digested samples. The digested sample, neat or diluted 1/10 (i.e. 10^{-3} and 10^{-4} final dilution of the tumour sample), was incubated on anti-HN antibody coated plates. A standard was made with human brain HN prepared on insolubilised HA and purified by high performance liquid chromatography (HPLC) to get rid of residual HA traces as previously described [7]. Samples were incubated for 4 h at 37°C. The detection method used anti-human brain HN antibodies conjugated to alkaline phosphatase [10].

Western blotting [13] of tumour HN was carried out as previously described [11] using alkaline phosphatase conjugated anti-human brain HN antibodies as a probe. The detection method employed paranitro blue tetrazolium and 5-bromo 4-chloro 3-indolyl phosphate (NBT + BCIP procedure [14]).

HPLC was performed on a Superose 6 column (Pharmacia, Saint Quentin en Yvelines, France) driven by Beckman equipment.

Cell cultures were carried out in RPMI 1640 medium (Eurobio, Paris, France) supplemented with glutamine and 10% fetal calf serum.

Histological detection of HA was carried out on 4 μm cryostat sections, fixed in 95% ethanol, with 1 μg/ml biotinylated sheep brain HN, prepared with the Amersham kit, as a probe. Biotinylated HN was detected with alkaline phosphatase conjugated streptavidin (Biogenex, Eurobio, Paris, France) diluted 1/2 and incubated for 30 min at room temperature. The staining procedure used Naphthol As-Mx and Fast Red (Sigma, La Verpillière, France), in the presence of 1 mmol/l Levamisole to inhibit tissue alkaline phosphatase activity. Nuclear counterstaining was performed with haematoxylin (Shandon, Paris, France).

Immunohistological detection of HN was performed on serial sections, with rabbit polyclonal or with monoclonal antibodies

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[15] to HN. The second antibody was alkaline phosphatase conjugated (Jackson Immunoresearch) anti-rabbit IgG or anti-mouse IgG. Staining was carried out as described above.

RESULTS

The results in Table 1 show clearly that HA concentration expressed per gram tumour (wet weight) is in most cases (15 out of 23 glial tumours) similar to that of fetal brain and higher than the HA concentration in adult brain tissue (37–108 μg/g [7]) regardless of the area tested.

In contrast, the HN concentration is often lower than in either fetal or normal adult neural tissue, particularly for the grade III/IV gliomas (glioblastomas). The HN/HA ratio is therefore much lower in gliomas than in normal tissues. In all gliomas of grade III and IV tested, it was less than 0.5. The 'normal' tissue taken in the vicinity of gliomas (cases 24, 25) had a 'normal' HA content and HN/HA ratio was greater than 1. In contrast to glioblastomas, the lower grade astrocytomas (cases 19–22) and an oligodendroglioma (case 23) had HN/HA ratio values close to that of normal adult tissue, and were significantly different from the HN/HA ratios of glioblastomas ($P < 0.001$).

Table 1. HA and HN in astrocytoma (grade I/II) and glioblastoma (grade III/IV) extracts

	Sex	Age	Grade	HA (μg/g)	HN (μg/g)	HN/HA	Survival (months)	Alive (months)
1	F	70	IV	810	24	0.03	8	
2	F	62	IV	360	8	0.02	6	
3	M	55	IV	52	1.5	0.03	20	
4	M	52	IV	164	3.2	0.02	13	
5	M	64	IV	490	14.7	0.03	14	
6	M	67	IV	84	5	0.06	12	
7	F	37	IV	410	4.8	0.01		36
8	M	58	IV	51	3.4	0.07	5	
9	M	64	IV	52	1.2	0.02	15	
10	M	66	IV	300	14	0.05	12	
11	M	40	IV	380	31	0.08	61	
12	M	60	IV	330	44	0.13	15	
13	M	25	III	26	8.3	0.32		
14	F	48	IV	135	67	0.50	8	
15	M	51	IV	190	54	0.28	13	
16	F	18	III	20	7	0.35		25
17	F	36	IV	728	146	0.20	10	
18	F	50	IV	208	79	0.38	11	
19	F	17	a.c.	900	540	0.60		44
20	M	42	II(III)	300	250	0.83		18
21	M	40	II(III)	3.3	8.5	2.6	27	
22	M	40	II(III)	31	132	4.3	21	
23	M	54	OL(III)	126	130	1.00	4	
24	M	42	N	37	78	2.1		
25	F	51	N	31	39	1.3		
A1	M	52	cortex	170	650	3.8		
A2	F	55	cortex	67	437	6.5		
A3	M	62	cortex	94	504	5.4		
FB1		13w		350	153	0.4		
FB2		15w		620	127	0.2		
FB3		27w		420	150	0.4		

HA and HN concentration in human gliomas (23 cases). The grade indicates that of the fragment extracted, which in some cases (19–23) was lower than that of the whole tumour (in brackets). a.c. : Astrocytoma of the cerebellum. OL : Oligodendroglioma. N : Normal fragment taken in the vicinity of a glioblastoma. A1 : Frontal fragment excised for traumatism. A 2–3 : Normal brain extracts were made within 16 h after death for a non-neurological disease or syndrome. A2 : Mean ± S.D. in six cortical areas (limit values HA : 31–105, HN : 260–700); A3 : mean ± S.D. in 12 cortical areas (limit values HA : 54–160, HN : 350–660). Fetal brain (FB) : from [7] with permission of the Ciba Foundation. Brains were taken at abortion before chemotherapy and totally extracted (w : age in weeks).

Interestingly, in some cases the HA content was either close to that of normal tissue (cases 3, 8, 9) or even lower (case 13, 16). In those cases the HN content was reduced by a similar proportion so that the HN/HA ratio conformed with that of the series.

Thirty-five tumour fragments were examined for HN/HA location (1 grade I glioma, 2 grade II glioma, 24 glioblastomas grade III/IV and 8 meningiomas).

HN and HA were found in all (27/27) gliomas tested. Their locations were superimposable. HA and HN were in all cases found in pericellular areas suggesting they are components of the extracellular matrix (Fig. 1a). A perivascular location (Fig. 1c,d) was seen in 18/27 gliomas (1 grade I and 17 glioblastomas). The staining involved the vascular wall and the endothelial capillary cells, whose cytoplasm contained some positively stained granules. When perivascular staining was present, it was predominant around small capillaries, the larger vessels being inconstantly stained.

HN was in all circumstances observed in HA positive areas, that is in the extracellular matrix positive areas and in perivascular HA positive areas, except in a unique glioblastoma case where astrocyte cell bodies were stained by anti-HN antibodies thus showing an intracellular location of HN (Fig. 1c) whereas no HA was detectable in the cell bodies. No clinical or pathological feature was associated with the intracellular location of HN.

Glioma HA was analysed by HPLC and found to have a high molecular mass. The search for small HA derived oligosaccharides was negative by this technique. Glioma HN was analysed by western blotting and compared with HN of normal neural tissue. Where possible the blotting did not show significant differences between HN from normal neural tissue and tumour HN (Fig. 2). We have established a series of human glioma-derived cell lines and found that they all produce high molecular weight HA (Fig. 3 and Table 2) which shows that cancer astrocytes could contribute along with other tumour cells to the edification of the extracellular matrix. Conversely, HN was only found in one glioma cell line and at a very low level (values in culture medium: HN = 9 μg/l vs. HA = 3 mg/l). The quantitative results obtained in a short series of eight benign meningiomas (Table 3) were clearly different from those obtained in gliomas. In 7 of the 8 cases studied, HA concentration was below 5 μg/g and HA was not detected in 2 cases. HN also was very low—less than 4 μg/g in all cases and not detected in 2 cases. A faint perivascular location was seen in 3/7 cases of another series of meningiomas, and no staining was seen in the extracellular matrix.

DISCUSSION

The extracellular matrix of gliomas has been studied by McKeever *et al.* [16] who showed that glioma cells release type IV collagen, fibronectin and laminin and they stated that 'the spectra of proteins released by glioma lines closely resembled fibroblast spectra of proteins'. It is also true for glial fibrillary acidic protein (GFAP)-positive cell lines [17] which shows that

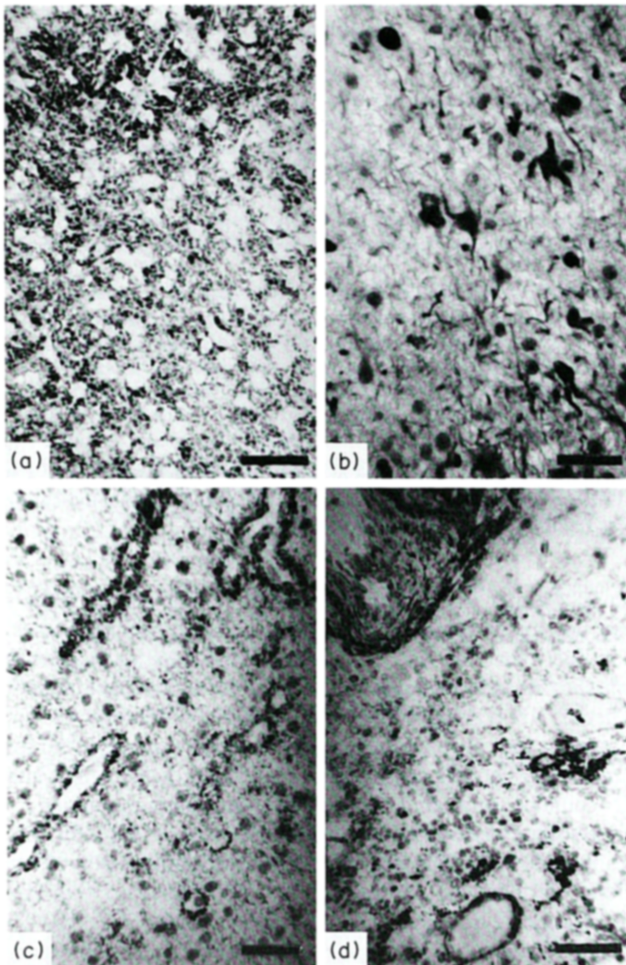

Fig. 1. Histochemical patterns of HA and HN in human gliomas. (a) The extracellular matrix was positive in all cases for both components whose locations were superimposable. Note the negativity of cell bodies which are surrounded by the positive granular background. (b) An intracellular location of HN was seen in a unique case (glioma 7). Cell bodies were not stained for HA. In that glioma, the extracellular matrix labeling for HN was slighter than in the general case. (c, d) Two examples of perivascular staining for HA (c) and HN (d). HA and HN were superimposable in the perivascular locations up to the vascular wall. HN staining of the tumour section allowed frequently the disclosure of capillaries which were not seen with the classical histological staining. Some deposits were seen in close contact to endothelial cells. It is unclear, however, if HN is taken up or secreted by capillary cells. Bar = 30 μm.

the presence of extracellular matrix proteins is not related to the mesenchymal differentiation of tumours. The protein composition of tumour extracellular matrix is close to that of normal nervous tissue [18]. This is not the case of hyaluronan. Although it is also released by fibroblasts, hyaluronan is clearly increased in gliomas regardless of the grade, when compared to the HA content of normal adult tissue. This observation is not restricted to gliomas and elevation of HA has been observed in all types of malignant tumours studied: breast carcinomas, gastric adenocarcinoma, colonic carcinomas and prostate carcinomas [19]. The results obtained show that HA is increased in cerebral gliomas as it is generally in cancer. Consequently the HA concentration in glioma tissue is close to that of embryonic brain tissue which is also much richer in hyaluronan than adult tissue.

Tumour HA originates from several cells. Fibroblasts are HA-producing cells and we were able to verify that, in the case of non-nervous tumours, tumour-derived fibroblasts produce high molecular weight HA (unshown). On the other hand, cancer cells most often produce high molecular mass HA and this is particularly evident in gliomas. In the series of 11 human glioma-derived cell lines studied, HA production by cultivated glioma cells was comparable to that of fibroblasts. Along with fibroblastic (perivascular) production, this accounts for the high HA content of tumours.

Several roles have been proposed for hyaluronan. The secretion of HA by glioma cells may protect glioma cells against attack by immune cells which have been shown to be unable to cross the pericellular HA barrier [6]. Our results support that this can occur *in vivo*, since HA is found in the extracellular matrix surrounding cancer cells, thereby separating the cancer cells from immune cells. Another hypothesis suggests a role for hyaluronan in cell migration, which would be facilitated by HA-rich extracellular matrix [20]. This could account for the cellular infiltration in normal tissue surrounding glioblastomas, which is the main obstacle to the surgical eradication of glioblastomas. At last, it was shown that medium sized HA-derived oligosaccharides might have an angiogenetic activity [21]. Our results, however, do not support that HA-derived oligosaccharides are significantly present in gliomas.

The concentration of HA-associated glycoprotein HN was not modified in the same way. The more malignant grades (glioblastomas, grade III and IV) correlate with a low level of the protein which is significantly lower than the HN concentration in astrocytomas and in normal tissue. Interestingly, although in some cases the HA content reached extreme values (e.g. 51–810 for glioblastomas 3.3–300 for astrocytomas grade I, II) the HN/HA ratios were similar within both groups which suggests that HN and HA synthesis may be mutually dependent. Our results, which show a clear-cut quantitative difference between astrocytomas (grade I and II) and glioblastomas (grade III and IV) are, therefore, in agreement with the morphological classification.

As for HA, several HN-producing cells have been identified:cancer astrocytes [22], normal or leukaemic human blood monocytes [23] and fibroblasts which could be stimulated by contact with cancer cells [24]. This would explain the perivascular staining often seen, since in brain tumours fibroblasts are essentially located around capillaries.

We can speculate that HN activity is related to that of HA in several physiopathological events. Perris and Johansson [25] showed *in vitro* that the migration of neural crest cell of the axolotl was inhibited by the addition of a HA-binding protein to the medium. The migration of cancer cells in normal brain tissue, at distance of the tumour mass, is responsible of recurrences in the more severe forms of brain tumours. In that view, we can hypothesise that as far as HA is involved in the migration of glioma cells, HN could have an opposite effect and, therefore, could minimise the severity of tumour evolution. This hypothesis fits with our quantitative results since HN was relatively more abundant in the lower grades of gliomas, and with our histological observations showing that HA and HN were associated with cell migration areas of the developing brain [7].

The close contact between HA and HN, and tumour capillaries suggests that HA and HN could be produced also by tumour-associated vascular cells. The production of hyaluronan by transformed endothelial cells [26] and in developing vessels of the developing yolk sack [27] was shown recently. Our observations fit with these results. Neoangiogenesis, though an important feature of glioblastomas, is not restricted to glioblas-

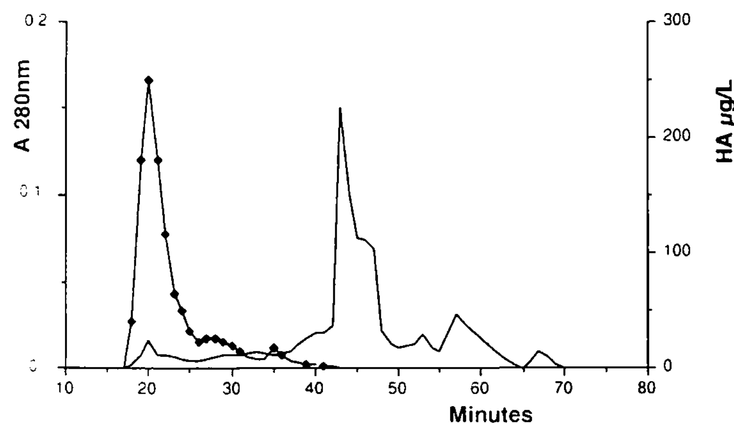

Fig. 2. HPLC of glioblastoma culture medium for HA molecular mass determination. Culture medium of human glioma derived cell line CB 109 was concentrated 40 times under pressure and 0.2 ml was injected. HA (dark points, right scale) was found in the void volume, indicating a molecular mass above 4 × 10^6 d. Proteins were recorded at 280 nm (unbroken line).

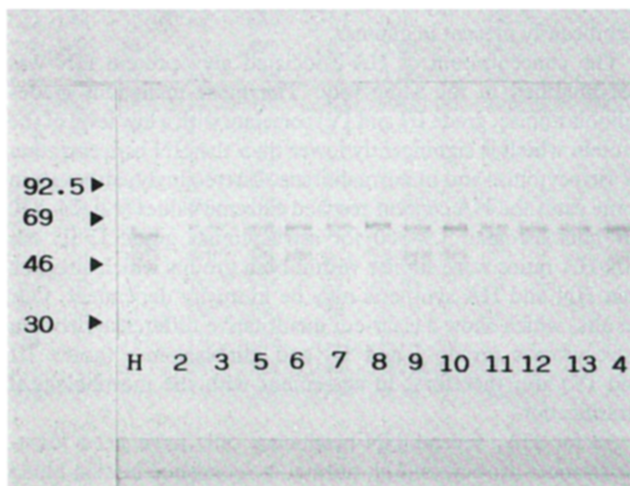

Fig. 3. Western blotting of tumour HN. Extracts were adjusted at 0.5 µg HN per ml. Samples were 15 µl. H : human brain HN. Other numbers refer to the tumour number (see Table 1). Vertical scale points the molecular mass in kD.

Table 2. HA production by glioma cells lines was measured after 2 days of culture in medium without serum

Cells	Origin	Passage	Duration (h)	Cells/ml	HA (pg/cell/h)
CB74	Glioblastoma	16	48	85200	0.05
CB109	Glioblastoma	95	67	42240	0.11
CB191	Glioblastoma	57	48	39560	0.06
CB192	Glioblastoma	2	48	100000	0.0005
CB193	Glioblastoma	30	24	60280	0.54
SA4	Gliosarcoma	34	48	35690	0.24
SA39	Astrocytoma	25	48	38040	0.02
SA45	Glioblastoma	43	48	40000	0.18
SA146	Glioblastoma	17	48	48720	0.02
CB201	Oligodendroglioma	11	48	35000	0.05
CB216	Oligodendroglioma	2	48	34000	0.01
Fibroblasts	Fetal skin	11	48	30352	0.14

tomas. Neither is HN association with glioma vessels, since a faint but positive vessel labelling was also seen in meningiomas. It is likely that HA and HN content of meningiomas reflects only the presence of connective tissue in the fragment extracted. Whether HN is also produced by endothelial cells or not was not shown. In view of functional similarity which was suggested for tumour invasion and angiogenesis [28], the presence of HA and HN could be considered as promoting condition for invasion.

One of our observations indicated the presence of HN within a cancer cell body. HN was detected within cultured cells of one glioma cell line, the cell line CB 109 [22] suggesting that HN could originate from malignant astrocytes. Interestingly, the CB 109 line also retained its capacity to synthesise the glial fibrillary acidic protein GFAP, which usually disappears rapidly from cultured glioma cells [29]. This suggests that HN synthesis may often be lost by cultivated cells. If HN is produced together with HA by the astrocytes, then we could assume that HN synthesis is in part disregulated in malignant astrocytes of grade III and IV gliomas compared to its synthesis in lower grade astrocytomas and in normal tissue. Another explanation for the lower content of HN in grade III/IV gliomas could be the degradation by tumour proteases.

Both studies, quantitative and cytochemical, showed that in every case HA was not saturated by HN. We showed previously [7, 11] that the weight of HN required to saturate HA molecules was 10 times that of HA. Thus glioma HA is far from being saturated by HN. This point seems of interest in so far as tumour HA could be used as a focus for methods aimed at tumour localisation or drug targetting.

Table 3. HA and HN concentration in meningiomas

	Sex	Age	HA (µg/g)	HN (µg/g)
1	F	45	0.3	0
2	F	51	0	3.6
3	F	53	0	0.7
4	F	42	2.6	3
5	F	53	27	2.7
6	F	42	4.6	0.6
7	M	29	1.5	1.4
8	M	71	0.9	0

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