Hyaluronidase-2 Overexpression Accelerates Intracerebral but not Subcutaneous Tumor Formation of Murine Astrocytoma Cells

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ABSTRACT

Gliomas are highly invasive, invariably fatal intracerebral tumors. It seems that receptors for hyaluronan are required for the invasive process. Hyaluronan is a major component of the extracellular matrix in the brain, and all of the gliomas express CD44, the principal receptor for hyaluronan. To investigate the role of lysosomal hyaluronidases on tumor invasion we overexpressed hyaluronidase-2 (HYAL2) in murine astrocytoma cells. We found that high expression of HYAL2 accelerated intracerebral tumor growth dramatically, whereas the same cells formed s.c. tumors within the same time as the parental cells. The brain tumors were highly vascularized and more invasive than the control tumors. It seems that the interactions of the HYAL2-expressing tumor cells with the hyaluronan-containing extracellular matrix in the brain mediate these effects, whereas the same cells in a s.c. environment, which lacks the high hyaluronan level, behave like the parental cells.

INTRODUCTION

High-grade gliomas are highly invasive brain tumors that are invariably fatal. The factors controlling the invasive behavior of these tumors are poorly understood. There is, however, good evidence that interactions between hyaluronan, a highly polymerized glycosaminoglycan, which is abundantly present in the brain, and receptors of hyaluronan play an important role in the invasiveness of the tumor (1–5). There are several hyaluronan-binding proteins in the extracellular matrix of the brain, including link protein and other members of a family of related proteins such as BEHAB/brevican, neurocan, versican, the glial hyaluronate-binding protein (GHAP), and hyaluronectin, both of the latter proteins derived by proteolytic degradation of versican (6–11). In addition there are several cell-associated hyaluronan-binding proteins including: (a) CD44, the predominant hyaluronan receptor; (b) BEHAB/brevican; (c) a COOH-terminal fragment of BEHAB/brevican; and (d) RHAMM/IHABP (12–16). These cell-associated hyaluronan receptors have been reported to mediate cell migration in response to hyaluronan during fetal development and during tumor formation (3–5, 17).

Hyaluronan can be taken up by cells via CD44 interactions and transported into lysosomes, in which lysosomal hyaluronidases cause its degradation (18, 19). HYAL2 degrades hyaluronan from $M_r \sim 8,000,000$ to $M_r \sim 20,000$, or about 100 sugar moieties (19). The hyaluronidase, found in the serum HYAL1, similar to the sperm-derived hyaluronidase, PH-20/SPAM1, cleaves hyaluronan into small oligosaccharides of two to three tandem repeats of $\beta$-glucuronic acid and N-acetyl-glucosamine (20). There is some evidence that degradation products of hyaluronan with intermediate size have biological activity (21–23). In addition, they are good substrates for other hyaluronidases that degrade these fragments further (19). On the other hand, small oligosaccharides have been described as having angiogenic activity (24, 25).

The hyaluronidases HYAL1, HYAL2, and HYAL3 have been cloned recently (19, 20, 26). They are related to the sperm-derived hyaluronidase, PH-20 (or SPAM1), which is an enzyme bound to the cell surface by a glycosylphosphatidylinositol (GPI)-anchor (27). The $M_r 64,000$ form of PH-20 has a neutral pH optimum for catalytic activity, whereas the $M_r 53,000$ form has an acidic pH optimum (28). Although HYAL1 and HYAL2 are expressed ubiquitously, PH-20 is expressed only in the testes and in some tumors (19, 29, 30). The newly described HYAL3 is expressed predominantly in the liver and the brain, but presently there is no information about the functional activity of HYAL3 (31).

Our experiments were aimed at the elucidation of the role of HYAL2 in glioma growth and invasion in cells that express high levels of the hyaluronan receptor, CD44. The results indicate that HYAL2 very potently activates tumor growth and the invasion of intracerebral tumors but has no such activity in s.c. tumors formed by the same cells.

MATERIALS AND METHODS

Cells. SMA560 cells, derived from a spontaneous murine astrocytoma, kindly provided by Dr. D. Ashley (Royal Children’s Hospital, Melbourne, Australia), were cultured in DMEM, supplemented with 10% fetal bovine serum. To generate cell lines that express human HYAL2, the cells were lipofected with a linearized HYAL2 expression vector, pCATCH (a gift from Dr. C. Hovens, University of Melbourne, Melbourne, Australia) using the Lipofectamim Reagent (Life Technologies, Rockville, MD) together with pPGK-Puro (a gift from Dr. S. Cory, Walter and Eliza Hall Institute, Melbourne, Australia). After selection with puromycin for 2 weeks, individual colonies were isolated and expanded.

Tumorigenicity of Cells after Intracerebral or s.c. Injection. For in vivo studies, the cells were injected into adult (8-12-week-old) syngeneic VM/Dk mice. Intracerebral inoculations of $10^3$ cells in 10 pl of PBS were given through the coronal suture approximately 2 mm from the midline with a 27-gauge needle fitted with a plastic sleeve, which allowed penetration into the frontal cortex to a depth of 3 mm. The mice were monitored daily, and, when they showed neurological symptoms, they were monitored every 2 h. All of the mice in one cohort were killed when they became moribund, which occurred within 12 h of the first appearance of symptoms. The brains were excised, cut in half through the inoculation site, and embedded in Jung tissue-embedding medium (Leica Instruments, Nussloch, Germany). s.c. inoculations of $10^5$ cells in 100 pl of PBS were given in the flank of the mice. The s.c. tumors were measured in three dimensions daily. The animals were killed as soon as the tumors had a volume exceeding 1 cm$^3$, in accordance with our local animal ethics regulations. The s.c. tumors were excised and frozen in Jung tissue-embedding medium. Frozen sections were cut at a thickness of 7 mm and collected on 3-aminopropyltriethoxysilane-coated slides. The sections were then either stained with H&E or used for immunohistochemical analyses.

Northern Blot Analysis of mRNA. RNA was prepared from cells or tissues following standard procedures (32). Total RNA was separated on 1% agarose gels, blotted onto Hybond-N membranes (Amersham, Amersham, United Kingdom) and hybridized with $^{32}$P-labeled DNA probe, generated by

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3 The abbreviations used are: BEHAB, brain-enriched hyaluronan-binding; HYAL1, hyaluronidase-1; HYAL2, hyaluronidase-2; HYAL3, hyaluronidase-3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PECAM, platelet endothelial cell adhesion molecule.
random primer extension as described previously (32). After exposure to X-ray film, the membranes were boiled in 1% SDS and were reprobed.

**Immunohistochemistry.** Cells grown on glass chamber slides (Nunc, Roskilde, Denmark) and frozen tissue sections of 7 μm, were air-dried for 1 h, fixed in cold acetone for 10 min, and incubated with antibodies to murine CD44 (a gift from Dr. R. Bischof, University of Melbourne, Melbourne, Australia) and murine PECAM (PharMingen, San Diego, CA) following standard procedures. After incubation with horseradish peroxidase-conjugated secondary antibodies (DAKO, Botany, Australia), the cells and sections were incubated with 3,3′-diaminobenzidine tetrahydrochloride, dihydrate (DAB). After several washes in water, the sections were lightly counterstained with hematoxylin. The antibody binding was detected by microscopy.

**RESULTS**

**In vitro Characteristics of HYAL2-expressing Cells.** Two cell lines derived from the murine astrocytoma SMA560 cells that express high levels (2s-2 and 2s-7) and one cell line that expresses low levels (2s-6) of HYAL2 mRNA were selected for all of the subsequent studies (Fig. 1). The cells had the same morphology as the parental SMA560 cells. However, unlike the parental cells, the HYAL2-expressing cells tended to form foci on the culture dish and easily lifted off the dish within 3 days of incubation, never forming confluent monolayers of cells (Fig. 2). All of the cell lines had the same doubling time of about 13 h when grown in the absence of hyaluronan, which increased slightly in the presence of 80 μg/ml hyaluronan (data not shown).

**Overexpression of HYAL2 Causes Accelerated Intracerebral Tumor Growth but Has No Effect on s.c. Tumor Growth.** Inoculation of syngeneic VM/Dk mice intracerebrally with 10^5 SMA560 cells resulted in tumor formation within 18 days and gave a range of tumor sizes, with most mice harboring medium to large size tumors (Table 1). 2s-6 cells, which express low levels of HYAL2 mRNA, formed tumors faster, requiring only 13 days for 8 of 10 mice to develop large tumors. However, intracerebral inoculation of mice with 2s-2 or 2s-7 cells resulted in rapid tumor formation, giving 10 of 10 large tumors within 10 days using 2s-2 cells and 7 of 9 large tumors in 9 days using 2s-7 cells (Fig. 3; Table 1). The differences in survival time of the animals were highly significant. All of the tumors resulting from inoculation with HYAL2-expressing cells expressed CD44 similar to the parental SMA560 cells (Fig. 3 and data not shown). In addition, the tumors derived from 2s-2 and 2s-7 cells were more invasive than the tumors resulting from the parental SMA560 cells (Fig. 4).

s.c. inoculation of mice with the HYAL2-expressing and the parental cell lines resulted in tumor formation as well, with the first tumors being measurable within 7 days. However, the s.c. tumor growth rate was comparable using the parental SMA560 cells or the 2s-2 and 2s-7 cells, which both express HYAL2 at high levels (Fig. 5).

**Increased Vascularization in HYAL2-overexpressing Intracranial Tumors.** The 2s-2- and 2s-7-cell-derived intracerebral tumors were red in color and contained small necrotic areas, whereas SMA560- and 2s-6 cell-derived tumors were faint pink in color and did not contain necrotic areas (Fig. 6a). Staining of intracerebral tumor-derived sections with the endothelial cell-specific anti-PECAM antibodies showed that 2s-2 and 2s-7 cells formed highly vascularized...
tumors (Fig. 6b), whereas 2s-6 cells formed tumors that had the same density of blood vessels as the tumors formed by the parental SMA560 cells.

Expression of HYAL2 in Human Brain Tumor Specimens. HYAL2 is not expressed in normal adult brain, but is expressed in fetal brain (19, 33). However, because our experiments indicated that HYAL2 overexpression in murine glioma cells enhances tumorigenicity, we analyzed several primary human brain tumor specimens by Northern blot hybridization. One ganglioglioma (667), one glioblastoma multiforme (674), and two meningioma specimens (611 and 673) were found to express HYAL2. However, three anaplastic astrocytomas (228, 433, and 446) and one glioblastoma multiforme (675) were found not to express HYAL2 (Fig. 7). This result indicates that HYAL2 expression may occur in human brain tumors, although it does not seem to correlate well with invasiveness or stage.

DISCUSSION

Our results indicate that overexpression of HYAL2 increases the tumorigenicity of the murine astrocytoma cells, SMA560, after inoculation of the brain but not after s.c. inoculation. Furthermore, the HYAL2 overexpressing brain tumors are very well vascularized.

Hyaluronidase genes have been proposed to be a family of tumor suppressor genes or genes that may facilitate tumor growth and invasiveness, but evidence in most publications has been circumstantial. A deletion of part of the human chromosome 3, which contains the gene loci for HYAL1, HYAL2, and HYAL3, is often associated with small cell lung cancer (30, 31, 33). Therefore, this chromosomal location was thought to encode a tumor suppressor. However, in most instances, these deletions are very large, and many other genes could be on the same deleted region of the chromosome. Similarly, in a murine model, two congenic strains of mice that differ only in the HYAL1 locus on chromosome 9 were used as recipients for tumor cells. The mice expressing higher levels of hyaluronidase activity were found to be more resistant to tumor development (34). However, at least one of the cell lines used for the inoculations, B16F10, expressed very high levels of hyaluronidase activity, and the results may, thus, be difficult to explain. On the other hand, it has been suggested that hyaluronidases may be involved in breast tumor metastasis formation because metastases express about four times higher levels of hyaluronidase as the primary tumor (35). In addition, it has been proposed that elevated urinary hyaluronidase levels can be used as diagnostic markers for high-grade bladder cancer because of a strict correlation of hyaluronidase levels with tumor grade (36). The aberrant expression of the gene, located on chromosome 7, encoding the testicular hyaluronidase, PH-20/SPAM1, has been reported to occur in high-grade gliomas, breast cancers, and colon cancers but not in low-grade tumors of the same organs (21). A third chromosomal

<table>
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<th>Intracerebral tumor formation</th>
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<tr>
<td>SMA560</td>
<td>6 of 10 large, 2 medium, and 2 small tumors after 18 days</td>
</tr>
<tr>
<td>2s-2</td>
<td>10 of 10 large tumors after 10 days, ( P &lt; 0.0001 )</td>
</tr>
<tr>
<td>2s-6</td>
<td>8 of 10 large, 1 medium, and 1 no tumor after 13 days, ( P = 0.001 )</td>
</tr>
<tr>
<td>2s-7</td>
<td>7 of 9 large tumors, 1 small, and 1 no tumor after 9 days, ( P = 0.001 )</td>
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Table 1. Intracerebral tumor formation

The data represent one experiment. Each experiment was terminated, and the cohort was culled when the first mice showed neurological symptoms. Each experiment was repeated several times with varying numbers of animals and was found to be reproducible.

The survival time of the animals in each group was compared with the control group of mice inoculated with SMA560 cells and was calculated using the \( \chi^2 \) test.

Fig. 3. Photomicrograph of 2s-2-derived brain tumor. The brain of a 2s-2 tumor-bearing animal was sectioned, and consecutive sections were stained with anti-CD44 antibodies (a) or with H&E (b).

Fig. 4. Increased invasiveness in HYAL2-expressing tumors. Representative sections of a SMA560-derived tumor (a) and a 2s-2-derived tumor (b) were stained with H&E.

Fig. 5. HYAL2-expressing cells and parental SMA560 cells form s.c. tumors within the same time period. Cells (10⁶) of each cell line—SMA560, 2s-2, and 2s-7—were injected s.c. into syngeneic mice. The size of the tumors was measured daily. The results shown represent ten mice. Bar, SE.
location mapping to 10q24.1-q24.3 and encoding a hyaluronidase expressed in meningiomas has been described recently (37).

The extracellular matrix of the brain is rich in hyaluronan, and high-grade human gliomas contain elevated levels of hyaluronan and the hyaluronan receptor, CD44, which is required for the uptake of hyaluronan (2, 38). In addition, the levels of hyaluronectin, a hyaluronan-binding protein that is proposed to counteract the effects of hyaluronan fragments, are drastically reduced in high-grade gliomas (11). Hyaluronan fragments are described to be angiogenic, but the molecular mechanisms mediating this effect are not understood as yet (24, 25).

Although the serum hyaluronidase HYAL1 and other lysosomal hyaluronidases that require an acidic pH for enzymatic activity are found in a secreted form, they are inactive in the extracellular environment because the pH is close to neutral. This would imply that only hyaluronan, which is taken up by the cell and translocated into the lysosomal compartment, can be degraded by these enzymes. Mutations in HYAL1 that led to the inactivation of the protein were found to be the cause of a lysosomal storage disorder, mucopolysaccharidosis IX (31). As SMA560 cells express high levels of CD44, uptake of hyaluronan from the extracellular matrix seems to be possible in these cells. The parental SMA560 cells seem not to contain hyaluronidase activity as assayed by zymography gels (data not shown). It is interesting to speculate, whether the rather large hyaluronan fragments that are generated by HYAL2 have biological activity or whether further degradation by low levels of other hyaluronidases took place in the HYAL2-expressing SMA560 cell clones. The increased vascularization of the tumors may have been caused by smaller hyaluronan fragments or may be due to increased micronecrosis within the tumor. Necrosis is known to stimulate the synthesis of vascular endothelial growth factor, VEGF, which in turn activates neovascularization (39).

The tumor growth rate of the HYAL2-expressing cells was enhanced only in the brain and not if grown s.c. This may reflect the different extracellular environment, with higher levels of hyaluronan,
found in the brain. Although the skin itself is very rich in hyaluronan, the s.c. space may well have no, or very low, levels of hyaluronan. Similarly, the vascularization level, which may depend on small hyaluronan fragments being generated, is only increased in the intracerebral tumors.

It is interesting that although normal adult brain is deficient in HYAL2 expression as judged by Northern blot analysis and by reverse transcription-PCR (19, 33), we have observed HYAL2 transcripts in Northern blots of several glial cell tumors and in meningiomas. There seemed, however, no clear correlation with the tumor stage. A study using more tumor specimens may clarify this matter.

It seems that HYAL2 not only increases the growth rate of intracerebral glioma but enhances invasiveness and vascularization. These effects seem to depend on the environment encountered in the brain because the same cells grown s.c. do not behave differently from the parental cells, which do not express HYAL2.

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