Spreading and Migration of Human Glioma and Rat C6 Cells on Central Nervous System Myelin in Vitro Is Correlated with Tumor Malignancy and Involves a Metalloproteolytic Activity

Verena R. Amberger, Tamara Hensel, Nobuyoshi Ogata, and Martin E. Schwab

Brain Research Institute, University of Zurich, 8029 Zurich, Switzerland (V. R. A., T. H., M. E. S.), and Department of Neurosurgery, University Hospital of Zurich, 8091 Zurich, Switzerland [N. O.]

ABSTRACT

Malignant gliomas infiltrate the brain preferentially along myelinated fiber tracts. Central nervous system (CNS) myelin, however, contains inhibitory proteins that block axon regeneration, neurite outgrowth, and cell spreading of astrocytes and fibroblasts. We tested 5 human brain tumor cell lines, 1 rat brain tumor cell line, and 29 short-term cultured specimens from human brain tumors for their ability to spread and migrate on a CNS myelin substrate. Low-grade and pilocytic astrocytoma, ependymoma, medulloblastoma, and meningioma cell lines as well as primary cultures were strongly sensitive to the inhibitory proteins present in the CNS myelin. In contrast, glioblastomas, anaplastic astrocytomas, and oligodendrogliomas were able to spread and migrate on CNS myelin-coated culture dishes, demonstrating that within the gliomas, the ability to overcome the inhibitory effects of the CNS myelin is correlated with the grade of malignancy of the original tumor. Cell spreading of glioblastomas and anaplastic astrocytomas specifically on a CNS myelin substrate was strongly inhibited by the metalloprotease blocker O-phenanthroline and the peptide derivative carbobenzoxy-Phe-Ala-Phe-Tyr-amide, whereas blockers for serine, aspartyl, and cysteine proteases had no effect. Enzymatic peptide degradation assays revealed the presence of a phosphoramidon-sensitive and thiorphan-insensitive metalloproteolytic activity in the plasma membranes of high-grade glioma cells. These results suggest a crucial involvement of a membrane-bound metalloendoprotease in the process of invasive migration of malignant gliomas along CNS white matter fiber tracts.

INTRODUCTION

Malignant primary brain tumors, especially gliomas, are characterized by extensive infiltration of the surrounding normal brain tissue (1). It is therefore of primary importance to achieve a better understanding of the mechanisms involved in brain tumor cell migration. Single tumor cells migrate along blood vessels and seem to be arrested by the glial limitans. Basement membrane proteins, which are present around large blood vessels, have been shown to inhibit proliferation, and some of them stimulate migration of glioma cell lines (2–7) and primary glioma cells (8). The main route for glioma dissemination, however, is along white matter fiber tracts and can lead to distant spread, e.g., through the corpus callosum into the other hemisphere (1, 9, 10).

One important component of the brain white matter is myelin. In addition to structural proteins, myelin contains specific membrane proteins, the neurite growth inhibitors N133/250, which inhibit axon regeneration, neurite outgrowth, and cell spreading of astrocytes and fibroblasts (11–13). These inhibitory effects can be blocked with monoclonal antibodies in vitro and in vivo (14–16). The fact that the main route of malignant brain tumor infiltration is along white matter fiber tracts suggests that gliomas use a specific mechanism to overcome the effect of these neurite growth-inhibitory proteins. Rat C6 glioma, a chemically induced glial brain tumor cell line (17), shows rapid and diffuse infiltration of brain tissue in vivo (18, 19), very similar to human glioblastomas. In vitro, C6 glioma cells use a metalloproteolytic activity to spread and migrate on CNS tissue (20) and could be blocked specifically by the metalloprotease blocker O-phenanthroline or the newly designed tetrapeptide Cbz-Phe-Ala-Phe-Tyr-amide, whereas blockers of serine, aspartyl, or cysteine proteases had no effect (13, 20). We characterized the C6 enzyme as a membrane-bound metalloendoprotease with a Mr between 60,000 and 90,000 that is highly sensitive to phosphoramidon but insensitive to thiorphan and to blockers of matrix metalloproteases (13). A similar metalloproteolytic activity combined with the capability to spread and migrate on CNS myelin and white matter was found recently in CNS glial precursor cells (21). These cells are known to be highly migratory cells in the developing and adult CNS (22–25).

In the present study, we tested five human cell lines and one rat glioma cell line in three different in vitro assays: (a) a spreading assay on CNS myelin extract; (b) a migration assay on CNS myelin extract; and (c) a peptide degradation assay to evaluate metalloproteolytic activity. Additionally, short-term cultures from human brain tumor biopsies were tested for their ability to spread on purified CNS myelin. We collected 24 specimens from glial tumors and 5 specimens from meningiomas as a nonglial control tissue. We show evidence for a direct correlation between the in vivo grade of malignancy of a given glioma and the ability to spread and migrate on a CNS myelin substrate. Cell spreading and migration of malignant glioma cells were inhibited on CNS myelin by the general metalloprotease blocker O-phenanthroline and by the tetrapeptide Cbz-Phe-Ala-Phe-Tyr-amide, suggesting the involvement of a metalloprotease similar to that of C6 glioma cells and glial precursor cells.

MATERIALS AND METHODS

Reagents and Materials. Cell culture media were obtained from Life Technologies, Inc. (Basle, Switzerland); pepstatin A, leupeptin, and aprotinin were obtained from Fluka (Buchs, Switzerland). The tetrapeptide Cbz-Phe-Ala-Phe-Tyr-amide was synthesized by Bachem AG (Basle, Switzerland). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). The CSM (Apex Manufacturing, Inc.; Phoenix, AZ) used for the cell migration assay was a kind gift of Dr. M. Berens (Neuro-Oncology Laboratory, Barrow Neurological Institute of St. Joseph’s Hospital, Phoenix, AZ).

Cell Lines. C6 glioma cells (passage 335) were a kind gift from Dr. D. Monard (Friedrich Miescher Institute, Basle, Switzerland). U251 glioblastoma cells (passage 580) were provided by Dr. R. Del Maestro (Brain Research Laboratories, London, Canada), and four permanent cell lines derived from biopsy cultures [IPSB-18 (passage 35; Ref. 26), IPNT-H (passage 10), IPPS-11 (passage 15), and IPNN-8 (passage 15; Refs. 27–30)] were provided by Dr. G.
Pilkington (Institute of Psychiatry, London, United Kingdom). All cell lines were grown as monolayer cultures in DMEM supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (0.5 mg/ml) in the following called culture medium under 5% CO₂ at 37°C. Cells were trypsinized every fifth day using 0.1% trypsin in Ca²⁺-free, Mg²⁺-free PBS and 0.025% EDTA.

**Biopsy Tissue.** Fresh specimens from primary brain tumors were taken at time of operation, and, in parallel, tissue samples were sent for neuropathological diagnosis. The tissue was washed in ice-cold Ringer solution, and blood vessels and necrotic tissue were removed. After mincing the tissue in culture medium, the cells were dissociated by passing them through a 70-μm mesh size filter. After centrifugation, the cells were resuspended in culture medium, seeded on culture dishes (100 × 20 mm; Nunclon; Nunc, Roskilde, Denmark), and kept under 9% CO₂ at 37°C for 7–10 days. The culture medium was changed at day 2 and day 5 in culture. For the spreading assay, the cells were trypsinized with 0.1% trypsin in Ca²⁺-free, Mg²⁺-free PBS and 0.025% EDTA before plating.

**Cell Spreading Assay and Quantification.** CNS myelin was prepared from rat spinal cord by sucrose density gradient centrifugation (12), extracted with buffer containing 20 mM Tris and 60 mM 3-[3-cholamidopropyl]dimethylamino]-1-propanesulfonate, and routinely tested for inhibitory activity for cell spreading with 3T3 fibroblasts (21). The myelin protein concentration resulting in 80% inhibited fibroblasts was used for the spreading and migration assays. CNS myelin extract or extraction buffer alone (as control) was coated on 4-well culture dishes (Greiner, Nürtingen, Germany) and incubated over night at 4°C. After washing the plates three times with HBSS, 10,000 cells/well (1 cm²) were plated on coated culture dishes, and as soon as the attached cells on the control substrate were well spread, the assay was stopped by the addition of 4% formaldehyde in 0.1 M phosphate buffer supplemented with 4% sucrose. Protease blockers (3 μM aprotinin, 10 μM pepstatin A, and 100 μM leupeptin) were added directly to the cell suspensions from 10× solutions 10 min before plating. Cell spreading was quantified by counting flat, spread cells versus round cells in 12 randomly chosen areas of each well (= about 12% of the total well surface). Each experimental condition was performed in triplicate (cell lines) or duplicate (primary cells). The statistical significance was evaluated using the unpaired t test.

**Cell Migration Assay and Quantification.** Teflon-printed microscope slides (Cell-Line Associates, Inc., Newfield, NJ), which are subdivided into 10 wells, were precoated with poly-L-lysine (5 μg/well) overnight. After blocking the unspecific binding with 1% BSA in PBS for 30 min, the slides were washed with Ca²⁺-free, Mg²⁺-free PBS, dried, and stored at room temperature. Some slides were additionally coated with rat CNS myelin extract (15 μg/well) as described above. The migration assay was performed according to Berens et al. (3) with some modifications: coated slides were covered with 70 μl of culture medium each; the CSMs were placed on the slides; and 1 μl of cell suspension (2,000 cells) was placed in the sedimentation cylinder. Protease blockers (3 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 100 μM O-phenanthroline, and 40 μM Cbz-Phe-Ala-Phe-Tyr-amide) were added directly to the cell suspensions from 10× solutions 10 min before seeding. After incubating the cells for 2 h at 37°C under 5% CO₂, the CSMs were removed, fresh culture medium containing protease blockers was added, and the area covered with cells was recorded immediately and 6, 12, and 24 h after seeding with a black and white video camera (CCD: Sony) attached to an inverted microscope (×2.5 objective; Olympus, IMT-2). Cell migration was measured and quantified with an image analysis system (Image Access; image-1), and the values obtained at 2 h after seeding were used as a baseline and subtracted from the values measured at later time points. The numbers represent the mean measurement of three wells, and each experiment was performed three times. The significance was evaluated using the unpaired t test.

**Plasma Membrane Preparation and Cbz-Phe-Ala-Phe-125I-Tyr-amide Degradation Assay.** Plasma membrane preparation, radioiodination of Cbz-Phe-Ala-Phe-Tyr-amide, and the degradation assay were performed as described previously (13). Briefly, Cbz-Phe-Ala-Phe-Tyr-amine was iodinated with Na²¹ (500 Ci/μl; 2000 Ci/mmol) using chloramin T and purified by sequentially running the sample on a C₁₈-Sep-Pak column (Waters, Millipore, Volketswil, Switzerland) and on a C₁₈ reversed-phase high-performance liquid chromatography column (Brownlee, Applied Biosystem). The specific activity of the purified probe was 2000 cpm/μmol peptide.

Cell membranes from the different tumor cell lines were purified on a discontinuous sucrose gradient, resuspended in 20 mM 2-(N-morpholino)ethanesulfonic acid/HC1 buffer (pH 6.0), and stored at —70°C. Plasma membranes were preincubated in the presence of a blocker mixture containing 10 μM leupeptin, 10 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride for 15 min at 37°C. O-Phenanthroline and phosphoramidon were added to the mixture when indicated. The reaction was started with 100 fmol of Cbz-Phe-Ala-Phe-1²¹I-Tyr-amine, and after 1 h of incubation at 37°C, the components in the incubation medium were separated by TLC, and iodinated peptides were visualized by autoradiography. For quantification, the gel regions corresponding to the bands on the autoradiogram were excised and counted in a gamma counter (LKB Wallace 1282 compu-gamma counter). Each experimental condition was performed in duplicate and was tested four times. The significance was evaluated using the unpaired t test.

**RESULTS**

Spreading of Human Glioma Cell Lines on Purified CNS Myelin. Five characterized human glioma cell lines generated from an ependymoma (IPPS-11), a medulloblastoma (IPNN-8), a pilocytic astrocytoma (IPNT-H; Refs. 27–30), an anaplastic astrocytoma (IPSB-18; Ref. 26), and a glioblastoma (U251; Refs. 31 and 32) were tested for their ability to attach and spread on culture dishes coated with a CNS myelin protein extract containing the neurite growth-inhibitory proteins. The tumor cells were plated either on control substrate coated with extraction buffer or on culture dishes coated with CNS myelin proteins. After 1 h of incubation, most of the IPNT-H cells (pilocytic astrocytoma; Figs. 1a and 2A), the IPSB-18 cells (anaplastic astrocytoma; Figs. 1c and 2A), and the U251 glioblastoma cells (Fig. 2A) were firmly attached to the control substrate, and the spreading process was advanced, as reflected by the flattening of the cells. On the CNS myelin-coated dishes, the pilocytic astrocytoma cells (IPNT-H) attached poorly, and spreading was strongly inhibited (Figs. 1b and 2A), whereas the anaplastic astrocytoma cells (IPSB-18; Figs. 1d and 2A) as well as the U251 glioblastoma cells...
Fig. 2. Pilocytic astrocytoma (IPNT-H), ependymoma (IPPS-11), and medulloblastoma (IPNN-8) cell spreading on CNS myelin-coated culture dishes is inhibited, whereas glioblastoma (U251) and anaplastic astrocytoma (IPSB-18) spread equally well on CNS myelin and on control substrate and can be inhibited by metalloprotease blockers specifically on CNS myelin. A, cells were plated on control substrate (open bars) and on CNS myelin-coated culture dishes (hatched bars). After 1 (IPNT-H, IPSB-18, and U251), 3 (IPPS-11), and 3 h (IPNN-8), the cells were fixed, and the percentage of spread cells was determined. B, before plating, U251 and IPSB-18 cells were preincubated for 15 min with a blocker mixture (10 μM pepstatin A, 10 μM leupeptin, and 3 μM aprotinin) and, where indicated, with 200 μM O-phenanthroline or 20 μM Cbz-Phe-Ala-Phe-Tyr-amide. After 1 h, the cells were fixed and quantified. The values are the means of counted cells in 12 randomly chosen areas of 3 experiments. Bars, SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

(Fig. 2A) were attached and spread, demonstrating that the high-grade glioma cells were not affected by the inhibitory proteins present in the CNS myelin. Medulloblastoma cells (IPNN-8) and ependymoma cells (IPPS-11) slowly attached to and spread on the control substrate (3 and 5 h, respectively) and, at that time point, showed significantly reduced cell spreading on CNS myelin (Fig. 2A; IPNN-8, P < 0.001; IPPS-11, P < 0.05). These results show that glioblastoma and anaplastic astrocytoma cells have a mechanism that enables them to spread on CNS myelin under conditions that are strongly inhibitory for tumor cells derived from pilocytic astrocytomas, ependymomas, and medulloblastomas.

Spreading of Glioblastoma and Anaplastic Astrocytoma Cells on CNS Myelin Is Inhibited by Metalloprotease Blockers but not by Blockers for Serine, Aspartyl, and Cysteine Proteases. IPSB-18 astrocytoma and U251 glioblastoma cells were plated on CNS myelin-coated culture dishes in the presence of a mixture containing blockers...
for serine (3 μM aprotinin), aspartyl (10 μM pepstatin A), and cysteine proteases (10 μM leupeptin). After 1 h, the cells were attached and spread equally well on both control and CNS myelin substrates (Figs. 2B and 3a), showing that this mixture of protease blockers did not affect IPSB-18 and U251 cell spreading on neither CNS myelin nor on control substrate. In contrast, 200 μM O-phenanthroline (Figs. 2B and 3b), a general metalloprotease blocker, or 20 μM Cbz-Phe-Ala-Phe-Tyr-amide (Figs. 2B and 3c), a protease blocker inhibiting specifically the metalloproteolytic activity involved in C6 glioma cell spreading on CNS myelin (13), both strongly blocked cell spreading on CNS myelin but had no effect on control substrate (Figs. 2B and 3d). The strong blocking effects by O-phenanthroline and Cbz-Phe-Ala-Phe-Tyr-amide but not by the blocker mixture inhibiting serine, cysteine, and aspartyl proteases suggest the involvement of a metalloproteolytic activity that enables malignant astrocytoma cells to spread on CNS myelin.

Spreading of Primary Human Brain Tumor Cells on CNS Myelin. Short-term cultures from 29 specimens collected at the time of operation, which were neuropathologically identified as 11 glioblastoma multiforme, 2 anaplastic astrocytomas, 3 low-grade astrocytomas, 3 pilocytic astrocytomas, 5 oligodendrogliomas, and 5 meningiomas, were tested for their ability to spread on CNS myelin-coated culture dishes. Cell spreading of pilocytic and low-grade astrocytomas was significantly inhibited by the CNS myelin (P < 0.001 and P < 0.01, respectively) compared to anaplastic astrocytomas and glioblastomas, which easily attached to and spread on the CNS myelin substrate (Fig. 4A). These results reveal a correlation between the ability to spread on CNS myelin and the in vivo malignancy of the original astroglial tumors. Oligodendroglioma cells were not affected; they attached and spread equally well on CNS myelin substrate and on control substrate (Fig. 4A). Meningiomas originating from the arachnoid cells of the meninges were strongly impaired in cell spreading on CNS myelin, and this inhibition was highly significant compared to glioblastoma cell spreading (P < 0.001; Fig. 4A).

Spreading of Glioblastoma Multiforme on CNS Myelin Is Impaired by Metalloprotease Blockers. Seven short-term cultured human glioblastoma multiforme specimens were tested for cell spreading on CNS myelin and on control substrate in the presence of various protease blockers. The presence of a mixture blocking serine, aspartyl, and cysteine proteases (3 μM aprotinin, 10 μM pepstatin A, and 10 μM leupeptin) did not affect cell spreading on either CNS myelin or control substrate (data not shown). In contrast, the metalloprotease blockers O-phenanthroline (150 μM) or Cbz-Phe-Ala-Phe-Tyr-amide (20 μM) inhibited cell spreading on CNS myelin (Fig. 4B), and these effects were highly significant (P < 0.001), whereas there was no significant effect on cell spreading on control substrate. From these results, we can conclude that primary glioblastoma cells use a metalloproteolytic activity to spread on a CNS myelin substrate, reflecting similar results obtained with glioma cell lines. Cell spreading of three oligodendroglioma specimens in the presence of both metalloprotease blockers was not significantly affected (data not shown), suggesting a different mechanism for oligodendroglioma cell spreading on CNS myelin.

Cell Migration of Five Human Cell Lines and One Rat Cell Line on CNS Myelin. Five human (U251, IPSB-18, IPNT-H, IPPS-11, and IPNN-8) cell lines and one rat (C6) brain tumor cell line were tested in a migration assay using slides that were coated with either CNS myelin extract or poly-L-lysine as a control substrate. Areas covered with migrating cells were measured after 2, 6, 12, and 24 h (Fig. 5), and the value measured at 2 h was defined as the baseline level, which was subtracted from the values measured at later time points. At 24 h after plating on the control substrate U251 glioblastoma, the anaplastic astrocytoma (IPSB-18) and the ependymoma cells (IPPS-11) showed the highest migration activity (2.6 mm²), followed by the C6 glioma (2.1 mm²) and the medulloblastoma cells (IPNN-8; 1.7 mm²; Fig. 6). The pilocytic astrocytoma cells (IPNT-H) showed very little migration (0.8 mm²; Fig. 6). After 24 h on the CNS myelin substrate, pilocytic astrocytoma, medulloblastoma, and ependymoma cells showed hardly any migration activity (0.3, 0.4, and 0.5 mm², respectively; Fig. 6), and migration of the anaplastic astrocytoma cells was reduced by 50% compared to that of the control substrate (1.2 mm²). In contrast, C6 and U251 glioblastoma cells were not affected by CNS myelin. The cells migrated equally well on both CNS myelin and control substrate (Fig. 6). These results demonstrate that CNS myelin is a nonpermissive substrate for cell migration of pilocytic astrocytomas, ependymomas, and medulloblastomas. The sensitivity to the inhibitory effect of CNS myelin is correlated with the malignancy of the original tumor, being lowest in the most malignant glial tumors.

Cell Migration of Glioblastoma and Anaplastic Astrocytoma Cells Is Inhibited in the Presence of Metalloprotease Blockers. C6, U251, and IPSB-18 cells were placed on CNS myelin-coated slides and on control slides. The presence of a mixture blocking serine (1 μM aprotinin), cysteine (10 μM leupeptin), and aspartyl proteases (10 μM pepstatin A) did not affect cell migration on both substrates (Fig. 6). In contrast, the presence of 100 μM O-phenanthroline strongly inhibited cell migration of the anaplastic astrocytoma and the two glioblastoma cell lines on CNS myelin-coated wells (Figs. 5 and 6). Most of the cells remained round, and actively migrating cells were rare. The blocking effects of the tetrapeptide Cbz-Phe-Ala-Phe-Tyr-amide were much less pronounced. Cell migration of all three cell lines was impaired within the first 12 h but was hardly affected after 24 h (Fig. 6). A similar short-term inhibition by Cbz-Phe-Ala-Phe-Tyr-amide was seen on cell spreading of C6 cells (13) and cell spreading and migration of glial precursor cells (21) on CNS myelin. Cbz-Phe-Ala-Phe-Tyr-amide is a substrate for the C6 metalloprotease and is degraded with a slow time course (13). The partial and transi-
Malignant Gliomas Degrade Cbz-Phe-Ala-Phe-Tyr-amide Using a Phosphoramidon-sensitive and Thiorphan-insensitive Metalloprotease. To investigate the type of metalloproteolytic activity involved in cell migration and spreading of high-grade gliomas, plasma membranes were prepared from U251, IPSB-18, IPNT-H, IPPS-11, and IPNN-8 cells. The plasma membrane proteins were incubated with 100 fmol of Cbz-Phe-Ala-Phe-Tyr-amide for 1 h. To restrict peptide degradation to metallodependent enzymes, a mixture containing blockers for serine, aspartyl, and cysteine proteases (1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A) was added 15 min before the incubation. U251 plasma membranes degraded 75 fmol peptide/h/10 μg protein (Fig. 7A). Membrane proteins from the anaplastic astrocytoma (IPSB-18) and the pilocytic astrocytoma (IPNT-H; 60 and 55 fmol/h) as well as membranes from the ependymoma (IPPS-11) and the medulloblastoma (IPNN-8; 48 and 56 fmol/h) showed a significantly lower degradation rate compared to U251 membranes (Fig. 7A). The peptide derivative Cbz-Phe-Ala-Phe-Tyr-amide inhibits glioblastoma cell spreading in short-term ass-
HUMAN AND RAT GLIOMAS ON CNS MYELIN

Fig. 5. U251 cells migrate on a CNS myelin substrate, a process that is inhibited by the metalloprotease blocker O-phenanthroline. U251 cells were preincubated in the absence or presence of 80 μM O-phenanthroline for 15 min and placed into the CSMs. At 2 (immediately after removing the CSMs), 6, 12, and 24 h, cells were fixed and stained with H&E, and pictures were taken at a magnification of ×35 (details, ×350). Bar, 300 μm.

DISCUSSION

In the present study, we tested five human cell lines, one rat brain tumor cell line, and short-term cultured human brain tumor specimens in their ability to spread and migrate on rat CNS myelin protein extract that is strongly inhibitory for cell spreading of astrocytes and fibroblasts (11–13). In our experiments, CNS myelin turned out to be a nonpermissive substrate for cells from glial tumors, such as the pilocytic and low-grade astrocytomas, ependymomas, and medulloblastomas, and for cells from a mesenchymal tumor, such as the meningioma, whereas anaplastic astrocytoma, glioblastoma, and rat C6 glioma cells rapidly spread and migrated on this substrate. This ability seems to correlate with the grade of malignancy of the glial tumors and was strongly reduced by the metalloprotease blocker O-phenanthroline and the peptide Cbz-Phe-Ala-Phe-Tyr-amide but not by blockers for serine, cysteine, and aspartyl proteases. Oligodendroglioma cells spread rapidly on CNS myelin-coated culture dishes but were not affected by metalloprotease blockers or any other tested protease blocker. In the recent past, research on glioma invasion had been focused mainly on the interaction between tumor cells and the extracellular matrix and the production of matrix metalloproteases by the tumor itself or neighboring cells (for reviews, see Refs. 35 and 36). Extraacellular matrix proteins such as collagens, fibronectin, and laminin are predominantly found in the basement membrane around blood vessels, at the leptomeninges, and in the glial limitans externa (37). In the gray and white matter of the CNS, glycosaminoglycans, also called lecticans (38), hyaluronic acid, and tenascin are expressed (35, 39, 40).
Fig. 6. Time course of glioma and medulloblastoma cell migration on a control and on a CNS myelin substrate. C6 and U251 glioblastoma and the anaplastic astrocytoma (IPSB-18) cells are able to migrate on a CNS myelin substrate, whereas pilocytic astrocytoma (IPNT-H), ependymoma (IPPS-11), and medulloblastoma (IPNN-8) cell migration was strongly inhibited by CNS myelin. Glioblastoma (C6 and U251) and anaplastic astrocytoma (IPSB-18) cell migration is strongly inhibited by O-phenanthroline and is partially impaired by Cbz-Phe-Ala-Phe-Tyr-amide. Cells were placed on control substrate and on CNS myelin-coated culture dishes, and the migrated distance was measured at 6, 12, and 24 h. When indicated, the cells were preincubated with a blocker mixture (10 μM pepstatin A, 10 μM leupeptin, and 1 μM aprotinin) and either 100 μM O-phenanthroline or 40 μM Cbz-Phe-Ala-Phe-Tyr-amide. The values represent the mean measurement of three slides and three independent experiments. Bars, SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
that interact with a multitude of membrane proteins of neuronal and glial cells. Two membrane proteins of oligodendrocytes and myelin, the neurite growth inhibitors N135/250, are strongly inhibitory molecules for neurons, fibroblasts, and astrocytes (11—13). These negative signals in the CNS myelin have been found in many higher vertebrates including rats, cattle, and humans (11, 12, 41).

In previous studies, we have shown that C6 glioma cells, a highly invasive rat brain tumor cell line, rapidly spread and migrate on CNS tissue and on a CNS myelin substrate, and that they express a membrane-bound metalloprotease that neutralizes the inhibitory components present in the CNS myelin (13, 20), especially a highly purified inhibitory protein from bovine bNI-220.5 Recently, Giese et al. (42) could show that four different human glioma cell lines, including U251 cells, and primary cell suspensions from two tumors were able to attach to and migrate on CNS myelin. This cellular behavior could not be inhibited by antibodies against the neuronal cell adhesion molecule NCAM and was only slightly inhibited with anti-β1 integrin antibodies, demonstrating that these membrane constituents are not major players in these processes.

In our present experiments, human glioblastomas, anaplastic astrocytomas, and rat C6 glioma rapidly spread and migrated on a CNS myelin substrate under conditions that were inhibitory for cell spreading and migration of pilocytic and low-grade astrocytomas, ependymomas, and medulloblastomas. Ependymoma cell spreading and migration were strongly inhibited on a CNS myelin substrate, and the pilocytic astrocytoma cells showed hardly any migration activity on either control or CNS myelin substrate; both effects might be explained by the noninvasive, benign behavior of these tumors in vivo (1). In contrast, the low-grade astrocytomas and the medulloblastomas are known as malignant, invasive tumors (1); however, in our experiments, their cell spreading and migration were significantly inhibited on a CNS myelin substrate. Low-grade astrocytomas show a number of differences in their invasive character compared to glioblastomas,
e.g., the invasion of the brain parenchyma is a much slower process that lacks any evidence of tissue destruction (1). Furthermore, the expression levels of the proteases cathepsin B and D, which are high in glioblastomas, are low in grade II astrocytomas (43, 44), whereas the tissue inhibitors for metalloproteinases 1 and 2 are highly expressed in low-grade astrocytomas and are almost absent in glioblastoma tissue (45). These findings, together with our results, suggest that low-grade astrocytomas use a different mechanism to invade surrounding brain tissue. Medulloblastomas are highly malignant brain tumors in young children that mainly migrate along blood vessels and invade the subarachnoid space; however, infiltration of the brain parenchyma has been found as well (1). The reason for the pressed in low-grade astrocytomas and are almost absent in glioblastoma.

Myelination is a late developmental process; during the first year of age, only about 20% of the nerve structures in the CNS are myelinated, during the second year, it reaches about 60%, and most parts of the CNS are not myelinated before the age of 7 years (46).

These results demonstrate a correlation between the ability to spread and migrate on CNS myelin and the grade of malignancy of a glial tumor. Cell spreading and migration of glioblastomas and anaplastic astrocytomas on CNS myelin were highly sensitive to the metalloprotease blocker O-phenanthroline, whereas blockers for serine, aspartyl, and cysteine proteases had no effect. This blocker profile suggests the involvement of a metalloproteolytic activity that enables glioblastomas and anaplastic astrocytomas to spread and migrate on a CNS myelin substrate. Rossi et al. (47) have investigated the influence of sequential in vitro passaging on human brain tumor cell lines, especially on IPSB-18, IPNN-8, and IPPS-11, which were used for the present study. They described changes in the enzyme secretion of four matrix metalloproteinases, but without a defined pattern. In our study, we found that the results obtained with the short-term cultured brain tumor specimens clearly corresponded to those of the cell lines, showing the validity of these cell lines as a model. The only minor difference was in the required inhibitory concentration of the metalloprotease blocker O-phenanthroline (200 μM for cell lines U251 and IPSB-18 versus 150 μM for primary human glioblastoma cells). A similar difference had been found earlier between a glial precursor cell line (CG-4) and primary glial precursor cells (21).

In a previous study, we characterized a metalloendoprotease that is expressed on plasma membranes of rat C6 glioma cells and that enables these cells to spread and migrate on a CNS myelin substrate (13). This metalloproteolytic activity degrades the peptide Cbz-Phe-Ala-Phe-125I-Tyr-amide with a slow time course and is highly sensitive to phosphoramidon but is insensitive to thiorphan (both are very potent blockers for endopeptidase 24.11, enkephalinase; Refs. 33 and 34) and to matrix metalloproteinase blockers. This blocker profile differentiates the C6 enzyme from most known metalloproteinases (13). The peptide Cbz-Phe-Ala-Phe-Tyr-amide, which blocks the spread of C6 cells and partially blocks their cell migration on a CNS myelin substrate, acts as a competitive substrate for a metalloprotease and is itself slowly degraded (13), which could explain the transient blocking effect on cell migration found in this study: 20 μM Cbz-Phe-Ala-Phe-Tyr-amide strongly blocked cell spreading of malignant gliomas within 1 h of incubation, but even a higher concentration of the tetrapeptide (40 μM) only slightly affected cell migration within the first 12 h and had almost no effect at 24 h, suggesting that the peptide was degraded by C6, U251, and anaplastic astrocytoma cells (IPSB-18). The results obtained in the peptide degradation assay confirmed this assumption: plasma membranes of rat C6 and human U251 cells degraded the iodinated peptide Cbz-Phe-Ala-Phe-125I-Tyr-amide, whereas membranes from the anaplastic astrocytoma (IPSB-18) and the pilocytic astrocytoma (IPNT-H) degraded significantly less peptide per hour, showing a correlation between the peptide-degrading activity and the grade of malignancy of the original tumor.

The peptide-degrading activities of all tested cell lines were inhibited by O-phenanthroline, identifying them as metalloendoproteinase activities. Further characterization of the peptide-degrading enzymes revealed a high sensitivity to phosphoramidon in U251 glioblastoma cells, very similar to results found with C6 cells membranes (13), whereas in pilocytic astrocytoma (IPNT-H), ependymoma (IPPS-11), and medulloblastoma (IPNN-8), phosphoramidon had no major effect on peptide degradation. Thiorphan did not affect peptide degradation in all tested cell lines, differentiating the involved enzymes from endopeptidase 24.11 (enkephalinase).

In conclusion, spreading and migration of human primary glioma cells and human and rat glioma cell lines on a CNS myelin substrate are strongly dependent on the action of a membrane-bound metalloendoprotease. This enzyme degrades the peptide Cbz-Phe-Ala-Phe-Tyr-amide and is phosphoramidon sensitive and thiorphan insensitive, showing similarities to the metalloendependent enzymes found in C6 glioma (13) and in glial precursor cells (21). The activity of the metalloprotease seems to correlate with the grade of malignancy of the original tumor and is highest in the most malignant glioma. Future high-affinity blockers for this type of protease may allow us to inhibit or significantly slow down the infiltration of white matter by malignant glioma.

ACKNOWLEDGMENTS

We thank G. J. Pilkington (Department of Neuropathology, Institute of Psychiatry, London, United Kingdom) for providing four human tumor cell lines and Prof. Y. Yonekawa, Dr. Könti, Dr. R. Bernais, and Dr. K. von Ammon (Department of Neurosurgery, University Hospital, Zurich, Switzerland) for providing tumor specimens. Special thanks are due to Dr. Dr. W. Born (Calcium Laboratory, Orthopaedic Hospital Balgrist, University of Zurich, Switzerland) for iodinating the tetrapeptide and to R. Schoeb and R. Foster for preparing the figures.

REFERENCES

12. Caroni, P., and Schwab, M. E. Two membrane protein fractions from rat central nervous...
Spreading and Migration of Human Glioma and Rat C6 Cells on Central Nervous System Myelin in Vitro Is Correlated with Tumor Malignancy and Involves a Metalloproteolytic Activity

Cancer Res 1998;58:149-158.

Updated version
Access the most recent version of this article at:
hhttp://cancerres.aacrjournals.org/content/58/1/149

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.