Release of collagen type IV degrading activity from C6 astrocytoma cells and cell density

MASASHI TAMAKI, M.D., WARREN MCDONALD, B.SC., R.T., AND ROLANDO F. DEL MAESTRO, M.D., PH.D., F.R.C.S.(C)

Brain Research Laboratories, Experimental Research Unit, Division of Neurosurgery, Victoria Hospital Research Institute, University of Western Ontario, London, Ontario, Canada

Type IV collagen is a major protein component of the vascular basement membrane and its degradation is crucial to the initiation of tumor-associated angiogenesis. The authors have investigated the influence of cell density on the release of collagen type IV degrading activity by C6 astrocytoma cells in monolayer culture. The release of collagen type IV degrading activity was assessed biochemically, immunocytochemically, and by Western blot analysis. The results demonstrate that increasing plating density and increasing cell density are associated with decreased collagen type IV degrading activity released per tumor cell. These findings indicate the existence of regulatory mechanisms dependent on cell–cell communication, which modulate release of collagen type IV degrading activity. The extrapolation of these results to the in vivo tumor microenvironment would suggest that individual and/or small groups of invading tumor cells, distant from the main tumor mass, would release substantial collagen type IV degrading activity, which may be crucial to their continued invasion and to angiogenesis.

KEY WORDS • collagen type IV degrading activity • C6 rat astrocytoma cell • cell density • angiogenesis • gelatinase A • extracellular proteasome
between cell density and the release of collagen type IV degrading activity by C6 astrocytoma cells in monolayer culture.

Materials and Methods

Cultures of C6 Astrocytoma Cells

The C6 astrocytoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin at 37°C in a humidified atmosphere of 95% air/5% CO₂.

Seeding of C6 Astrocytoma Cells at Different Cell Densities and Preparation of C6 Cell Conditioned Medium

Subconfluent C6 astrocytoma cells were removed from the dish by treatment with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA) in DMEM. After pipetting with whole medium (10% FCS), C6 astrocytoma cells were seeded onto 60-mm dishes at three different cell densities (5 × 10³, 10⁴, and 10⁵ cells per dish). Cell growth was monitored daily and the condition media were collected for each of the 5 days in monolayer culture.

Collagen Type IV Degrading Activity Assay

Collagen type IV degrading activity was assayed by measuring the degradation of 3H-collagen IV to products soluble in 100% trichloroacetic acid. Human collagen type IV was radiolabeled with [3H]formaldehyde as described previously. The activity per 1 ml of conditioned medium of extracellular proteasome (total activity) was calculated according to the following formula and represented the amount of activity released per 10⁶ cells: cell activity (U/cell) = total activity / number of cells × 10⁶.

Extracellular Proteasome Activity: General Protease Assay

This assay was used to assess extracellular proteasome activity released from C6 cells. Because the general proteolytic activity of extracellular proteasome is metallo-dependent and inhibitable by EDTA, the activity per 1 ml of conditioned medium of extracellular proteasome (total activity) was calculated according to the following formula: total activity = general proteolytic activity – remaining activity after preincubation of EDTA.

General proteolytic activity was assayed by measuring the degradation of [3H]collagen IV to products soluble in trichloroacetic acid. This assay was performed in the manner described previously. In inhibitory experiments using EDTA, each sample was preincubated with EDTA (10 mM/sample) for 15 minutes at room temperature. The activity per cell was calculated in the same way as that described for the collagen type IV degradation assay.

Immunocytochemical Analysis of Gelatinase A

The C6 cells were grown on glass coverslips at the three different cell densities studied (5 × 10³, 10⁴, and 10⁵ cells). Samples were assessed immunocytochemically on Days 1, 3, and 5 at each cell density. Negative controls for each experiment were performed by omitting the first antibody. The coverslips were fixed with 95% ethanol in the refrigerator for 20 minutes and then washed in phosphate-buffered saline (PBS). The samples were incubated for 1 hour at 37°C with the first antibody after inactivation of intrinsic peroxidase with 3% hydrogen peroxide for 5 minutes and blocking of nonspecific binding with 5% casein for 30 minutes. Subsequently the samples were incubated with biotin-labeled anti-rabbit immunoglobulin (IgG), followed by incubation with biotin-labeled ExtrAvidin–Peroxidase (1:20) for 30 minutes and a chromogen kit. The reaction was stopped by rinsing the slides with distilled water, and the samples were counterstained with hematoxylin for 5 minutes. Slides were assessed by counting the number of cells that stained positively with the antibody. From 700 to 1000 cells were randomly assessed from each coverslip and assigned a score from 0 to 2+. The scoring system consisted of the following: 0 meant no staining of the cytoplasm, 1+ slight positive cytoplasmic reaction, and 2+ no reaction to antibody.

Western Blot Analysis

Serum-free conditioned media from C6 cells were collected from the three different cell plating densities assessed on Days 1, 3, and 5. After being concentrated with a microconcentrator, each sample was diluted to the appropriate volume, an adjustment to equalize for cell number. Following electrophoresis on 12% (w/v) sodium dodecylsulfate (SDS) gel, samples were electrophoresed onto transfer membrane. After blocking nonspecific binding with a mixture of 5% casein and a small molecular weight blocker, the polyclonal antibody to gelatinase A was used at a concentration of 1:1500 and incubated with the blot for 1 hour to detect gelatinase A. All processes were performed using an immunoblot assay procedure in accordance with the manufacturer’s instructions.

Examination of Released Tissue Inhibitor Metalloprotease–2 From C6 Astrocytoma Cells

Conditioned media from C6 astrocytoma cells were assessed using Western blot analysis and reverse-phase high-performance liquid chromatography (HPLC) analysis to determine whether these cells release tissue inhibitor of metalloprotease (TIMP-2) into the media. For Western blot analysis, conditioned media obtained on Days 1, 3, and 5 at a seeding density of 10⁵ cells were examined using 15% (w/v) SDS gel. For reverse-phase HPLC analysis, conditioned media obtained on Days 1, 2, and 4 at a seeding density of 10⁵ cells were tested using a linear acetonitrile gradient. Recombinant TIMP-2 was used as a standard in the reverse-phase HPLC experiment and an antibody to this protein was used for Western blot analysis as described above.

Sources of Supplies and Equipment

The C6 rat astrocytoma cells were obtained from American Type Culture Collection, Rockville, MD. Human collagen type IV, ExtrAvidin–Peroxidase, and the AEC Chromogen Kit were provided by Sigma Chemical Co., St. Louis, MO. The polyclonal antibody to gelatinase A was kindly provided by Dr. W. G. Stetler-Stevenson as was the small molecular weight blocker used in the Western blot analysis. Recombinant TIMP-2 and antibody to TIMP-2 were gifts from Dr. K. E. Langley, Amgen, Thousand Oaks, CA. The Centricron 3 microconcentrator was obtained from Amicon, Beverly, MA; the Immobilon-P transfer membrane from Millipore, Bedford, MA; and the Amplified Alkaline Phosphatase Immunoblot Assay Procedure from Bio-Rad, Hercules, CA.
**Statistical Analysis**

For the linear portion of the growth curves, a simple linear regression of the log (cell number) as a function of time was calculated. All values reported are expressed as mean ± standard deviation. Statistical significance was assessed using Student’s t-test for the doubling times and one-way analysis of variance (ANOVA) and Dunnett’s multiple t-test for the results of the enzyme assay; probability values less than 0.05 were considered significant.

**Results**

**Growth of C6 Astrocytoma Cells and Collagen Type IV Degrading Activity**

The growth curves of the three seeding densities of C6 cells ($5 \times 10^3$, $10^4$, and $10^5$ cells) in monolayer culture are shown in Fig. 1. The doubling times of each cell density were $21.4 \pm 1.6$, $21.6 \pm 1.6$, and $22.2 \pm 2.6$, respectively, and no statistically significant differences were found among these values. Total activities and cell activities are shown in Fig. 2. The magnitudes of changes in activity after each day were variable for each cell density series and statistical significances were not observed except for activities on the last 3 days in the $10^5$ seeding density (Fig. 2A). However, activities per cell significantly decreased after each day in monolayer culture for all three series. Moreover, the activity for each day in the $5 \times 10^3$ series was greater than that in the $10^4$ series, and, in turn, this was greater than that in the $10^5$ series (Fig. 2B).

**Extracellular Proteasome Activity of C6 Astrocytoma Cell**

Extracellular proteasome activities were measured on Days 1, 3, and 5 for each seeding series. The highest activity was found on Day 1 at all the seeding densities and decreased in a similar manner to that for collagen type IV degrading activity (Fig. 3A and B).

**Immunocytochemical Analysis of Gelatinase A**

The majority of cells stained positive for gelatinase A in the Day 1 sample of the $5 \times 10^3$ series with many cells showing 2+ staining (Fig. 4A). This reactivity decreased on Days 3 and 5 (Fig. 4B and C). These results were consistent with what was found in the $10^4$ and $10^5$ series (Fig. 4D and E). On Day 5 in the $10^5$ series, positive cells were sparse and were surrounded by a large number of negative cells (Fig. 4F).

The decrease in reactivity from Day 1 to Day 5 with the different cell seeding series is shown in Fig. 5. The numbers of positive cells on Day 1 in the $5 \times 10^3$, $10^4$, and $10^5$ series were $77\%$, $72\%$, and $76\%$, respectively; those on Day 5 were $63\%$, $61\%$, and $9\%$. The number of positive staining cells decreased during monolayer culture and was especially dramatic in the $10^5$ series. These results indicate that intracellular gelatinase A was inversely related to cell number.
Western Blot Analysis

Western blot analysis of conditioned media from C6 astrocytoma cells reveals immunoreactivity at 68 kD, which corresponds to the activated form of gelatinase A. The change in the amount of the gelatinase A per cell between Days 1 and 5 in each seeding series is shown in Fig. 6A. The result of comparative studies between different seeding series on Day 1 is shown in Fig. 6B. Immunoreactivity decreased with increasing cell density in both these experiments. These results are consistent with the biochemical results for collagen type IV degrading activity discussed previously (Fig. 2B).

Tissue Inhibitor of Metalloprotease–2 Release From C6 Astrocytoma Cells

No evidence of TIMP-2 release was found using reverse-phase HPLC or Western blot analysis (results not shown).

Discussion

Our results have demonstrated that the release of collagen type IV degrading activity per C6 astrocytoma cell is dependent both on the initial plating density and on the cell density in monolayer culture. Increasing plating density and increasing cell number were associated with statistically significant decreases in collagen type IV degrading activity released per C6 astrocytoma cell. A number of hypotheses are available to explain these results, including 1) decreased production and/or release of collagen type IV degrading activity per C6 astrocytoma cell; 2) decreased activation of gelatinase A and/or extracellular proteasome; 3) increased gelatinase A and extracellular proteasome inhibitor production and/or release; 4) increased degradation of activated gelatinase A and/or extracellular proteasome; and 5) some combination of these.

Release of Collagen Type IV Degrading Activity From C6 Cells

We have reported that C6 astrocytoma cells release two predominant types of collagen type IV degrading activity into the medium during monolayer culture: 68-kD activity that represents the activated form of the MMP gelatinase A and a 1000-kD serine-sensitive activity associated with a novel extracellular proteasome.13,28 For the first 2 days after initial plating of C6 cells, the extracellular proteasome appears to be the predominant activity found, whereas with continued growth in monolayer culture gelatinase A activity predominates.13,28 Our biochemical results support the concept that the extracellular activity of collagen type IV decreases during monolayer culture. This could be the result of decreased activity of gelatinase A, extracellular proteasome, or both.

The cellular content of gelatinase A was assessed immunocytochemically using an antibody that recognizes both the proenzyme (72 kD) and the activated (68 kD) forms of gelatinase A.5 Although this method does not allow for the differentiation of the latent and activated forms of the enzyme, our results suggest that the total intracellular content of latent and/or activated gelatinase A decreases during monolayer growth. The Western blot analysis system used to assess gelatinase A content in the media of C6 cells can differentiate the latent and activated...
forms of gelatinase A because of their size difference. As has been reported previously, the majority of detectable gelatinase A activity found in the media of C6 cells is in the activated form.\(^{14}\) The results of the Western blot experiments demonstrate two points: 1) at the three plating densities tested, an increase in plating density was associated with a decrease in the level of immunoreactive activated gelatinase A activity; 2) when adjusted for cell number, C6 growth in monolayer culture was associated with decreased activated gelatinase A immunoreactivity. The results of these experiments support the hypothesis that C6 astrocytoma cells release less activated gelatinase A per cell into the media during monolayer growth.

Extracellular proteasome release was assessed biochemically by taking advantage of the finding that the major metallodependent general protease activity released by C6 cells appears to be associated with the extracellular proteasome. These results also support the hypothesis that extracellular proteasome release also decreases during monolayer growth. Together these results are consistent with the concepts that: 1) both the cellular content and the extracellular release of activated gelatinase A decrease per cell during C6 monolayer growth; 2) extracellular proteasome release by C6 cells decreases during monolayer growth; and 3) at the plating densities assessed, increasing plating density is associated with decreased activated gelatinase A and extracellular proteasome activity.

**Activation of Collagen Type IV Degrading Activity**

Gelatinase A is a member of the MMP family and was previously called MMP-2.\(^{4,9}\) The chief characteristics of the MMPs are: 1) their catalytic mechanism is dependent on Zn\(^{2+}\) at the active site; 2) these enzymes are produced as latent proenzymes that must be activated to have maximum activity; and 3) their activities are inhibited by TIMP-1 and TIMP-2.\(^{5,24,25}\) The regulation of these enzymes may occur at the level of gene expression, the activation of these metalloproteinases, and the control of their proteolytic activity.\(^{24}\) Although a number of mechanisms are available to activate the MMP of C6 cells, the majority of the gelatinase A found in the media is in the activated form, and this appears to be dependent on the cosecretion of a factor(s) called collagenase-activating factor(s) (CAFs).\(^{14}\) Two results suggest that decreased activation of gelatinase A is not the reason for the decreased collagen type IV degrading activity found. The Western blot analysis of the media from C6 cells did not demonstrate increasing amounts of the 72-kD proenzyme of gelatinase A, and treatment of the media with CAF did not result in significant activation of latent gelatinase A (RF Del Maestro, unpublished results). No information is presently available on activation mechanisms for the extracellular proteasome and, therefore, the present data can not exclude the possibility that a substantial amount of the extracellular proteasome is present in an inactivated form. However, because the extracellular proteasome is predominantly responsible for the collagen type IV degrading activity present during the first 2 to 3 days of C6 monolayer culture\(^{6,28}\) and the activity per cell decreases during this time period, we could speculate that, like gelatinase A, total extracellular proteasome release is decreasing. The decreased activation of extracellular proteasome would not appear to be the major cause of decreased collagen type IV degrading activity that was observed.

**Increased Inhibition of Collagen Type IV Degrading Activity**

Increasing secretion of an inhibitor of gelatinase A, specifically TIMP-2, could be responsible for the decreased activity seen. This TIMP-2 was undetectable in the conditioned media from C6 cells using both reverse-phase HPLC analysis and Western blot analysis. These results do not eliminate the possibility that other inhibitors of gelatinase A, such as TIMP-1, could be present.

**Increased Inactivation of Proteolytic Activity**

A number of enzymatic mechanisms could degrade gelatinase A and/or the extracellular proteasome resulting in decreased activity. The immunocytochemical and

---

**Fig. 5.** Graph showing the percent change in the rate of reaction to anti-gelatinase A in immunocytochemical analysis. From 700 to 1000 cells were assessed for each time point. The decrease in the rate of 2+ reactive cells and the increase in the rate of 1+ reactive cells were seen from Day 1 to Day 3 in all three different seeding series. Overall, the rate of the positive reactive cells decreased during culture. This change was very dramatic in the 10\(^5\) cell seeding series. Black bars = 2+ reaction; hatched bars = 1+ reaction; white bars = no reaction.

**Fig. 6.** Results of Western blot analysis. The analysis of conditioned media from C6 astrocytoma cells on Days 1, 3, and 5 in three different seeding densities was performed using anti-gelatinase A. Each sample set was diluted to an appropriate volume to equalize for cell number. Group A shows the change of immunoreactivity in gelatinase A per cell on Day 1 and Day 5 for each seeding series, and Group B shows the changes of immunoreactivity on Day 1 in all seeding series. In both experiments, the immunoreactivity of gelatinase A decreased with increasing cell density.
Western blot analyses do not support the presence of modified gelatinase A or enzymatic degradation secondary to the presence of increased enzymatic proteolytic activity.

**Matrix Metalloproteinase and Malignant Glial Tumors**

Apodaca, *et al.*,2 have shown that malignant glioma cell lines in culture release gelatinase A (MMP-2), gelatinase B (MMP-9), and interstitial collagenase (MMP-1). Abe, *et al.*,1 investigated the correlation between invasive activity and MMP-2 using nine human glioma cell lines. Generally it was found that MMP-2 is related to invasive activity and may be important to the invasion of basement membrane. Nakagawa and coworkers30 demonstrated that human brain tumors in vivo produced MMP-1, MMP-2, MMP-3, MMP-9, and TIMP-1. These researchers concluded that MMPs play an important role in human brain tumor invasion, probably due to an imbalance between the production of MMPs and TIMP-1.

We have also reported that the rat C6 astrocytoma cell and the human glioma cell line U251 secrete gelatinase A (MMP-2) in tissue cultures.10,11,30 One can speculate that there should be a correlation between the secretion of gelatinase A, other MMPs, and extracellular proteasome and glial tumor-associated angiogenesis. The initiation stage of angiogenesis is crucially dependent on the localized degradation of the basement membrane.2,13,18 However, at the present time the exact relationship between tumor cell release of collagen type IV degrading activity and angiogenesis in cerebral tumors remains unclear.

**Regulatory Mechanism of Production of Collagen Type IV Degrading Activity**

In this study we have shown that the release of collagen type IV degrading activity per cell by C6 astrocytoma cells is inversely correlated to cell number and to initial plating density. Simultaneously, in the experimental conditions assessed, the total amount of collagen type IV degrading activity in the media is maintained at a relatively constant level until confluence is attained (Fig. 2A). Our results support the concept that C6 astrocytoma cells in monolayer culture regulate their production and thus their release of collagen type IV degrading activity to maintain a defined level of activity in the medium at least until substantial cell crowding exists. This could only be accomplished by the presence of a feedback system by which C6 cells regulate their transcription and/or release of collagen type IV degrading activity extracellularly.

Xie and colleagues25 have shown that gelatinase B, a 92-kD type IV collagen degrading activity, was induced by external signals, such as cytokines and growth factors, only in sparsely populated cultures of A431 human epidermoid carcinoma cells. These authors concluded that it was important for tumor cells to be located in the noncontact-inhibited microenvironments, namely areas of lower cell density, to maximize the production of enzyme. The results of our immunocytochemical analysis and other studies would support the concept that cells in a lower cell density population possess and release substantially more collagen type IV degrading activity. Direct cell–cell communication may downregulate the transcription of gelatinase A, resulting in the cellular immunocytochemical results that we observed and in decreased release of enzyme. In conditions of confluence, downregulation is so pronounced that the total media content of degrading activity is also substantially decreased.

Naus, *et al.*,21 examined the role of intercellular communication via gap junctions in the control of tumor growth by using the transfection technique of connexin43 complementary DNA, which is a gap-junction protein. They showed a correlation of tumorigenesis with the lack of gap junctions in C6 astrocytoma cells. We have examined the collagen type IV degrading activity of C6 cells transfected with connexin43, but no significant differences in collagen type IV degrading activity between nontransfected and transfected C6 cells was observed (RF Del Maestro, unpublished results). Therefore, gap-junction cell–cell communication that is dependent on connexin43 does not appear to be involved in the regulation of collagen type IV degrading activity. We have tested other hypotheses related to the feedback control but have not been able to ascertain the molecular mechanism by which C6 cells regulate their transcription and/or release of collagen type IV degrading activity in monolayer culture.

**Synthesis With Other Studies**

If these in vitro results can be extrapolated to the in vivo context, individual and/or small groups of tumor cells may release equivalent or substantially more collagen type IV degrading activity than the primary tumor mass. This would predict two in vivo results: 1) individual cells distant from the main tumor mass may express more enzymatic activity and this should be immunohistochemically evident; 2) as tumors grow in size they should contain less overall collagen type IV degrading activity.

We have investigated the distribution of gelatinase A in human malignant astrocytic tumors using the same antibody to gelatinase A that we used in the studies reported in this communication. In both malignant astrocytomas and glioblastomas multiforme increased gelatinase A expression was localized to cells at the growing edge of the tumor and to what appeared to be invading malignant astrocytic cells some distance from the tumor edge.30 These results are consistent with those of other researchers using nonglial tumor systems.15,22

In another group of experiments from our laboratory collagen type IV degrading activity was assessed serially during the growth of C6 astrocytoma spheroids in vitro.50 The spheroid content of the collagen type IV degrading activity decreased as the spheroids grew from 350 to 750 μm in vitro.30

We have previously assessed tumor angiogenesis in the rat C6 astrocytoma spheroid implantation model using a number of techniques.8,17 In this model two specific angiogenic events appear to occur. The C6 astrocytoma cells grow along previously established normal microvessels and alter their morphology (tumor-induced vascular modification)12,17 and new tumor microvessels also develop, which vascularize the spheroid and allow for continued growth (tumor-associated angiogenesis).12,16,17 When we assessed tumor content of collagen type IV degrading activity during the growth of C6 astrocytoma spheroids in vivo, we found that this enzymatic activity decreased...
Collagen type IV degrading activity

with continued tumor growth. Tumor-induced vascular modification and tumor-associated angiogenesis have both been documented during growth of human malignant astrocytomas and glioblastomas multiforme, but a detailed biochemical study of the distribution of collagen type IV degrading activity in these tumors has not been conducted. Our results would tend to support the concept that an individual biochemical measurement of total collagen type IV degrading activity in tumor tissue or cell culture may not be predictive of subsequent tumor growth or angiogenic behavior. For these values to be useful, care must be taken to define the boundary zones between the tumor mass and invading tumor cells, and the enzymatic activity in these areas must be specifically defined. Angiostatic therapy may need to be targeted at these boundary zones where migrating individual tumor cells or small groups of tumor cells are secreting large amounts of collagen type IV degrading activity into their immediate microenvironment.

References


