

## Laboratory Investigation

# The Effect of Hyaluronic Acid on the Invasiveness of Malignant Glioma Cells : Comparison of Invasion Potential at Hyaluronic Acid Hydrogel and Matrigel

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**Objective :** Hyaluronidase (HAse), a degrading enzyme of hyaluronic acid (HA), is highly expressed in patients with malignant glioma. The purpose of this study was to verify whether HAse is related to the invasion of glioma cells. We also investigated if glioma cells with higher mobility in 2-dimensional (2-D) method have also higher mobility at 3-dimensional (3-D) environment.

**Methods :** Malignant glioma cell lines (U87MG, U251MG, U343MG-A, and U373MG) were used, and their HAse expressions were evaluated by HA zymography. The migration ability was evaluated by simple scratch technique. The invasiveness of each cell lines was evaluated by Matrigel invasion assay and HA hydrogel invasion assay. In HA hydrogel invasion assay, colonies larger than 150 µm were regarded as positive ones and counted. Statistical analysis of migration ability and invasion properties of each cell lines was performed using t-test.

**Results :** In scratch test to examine migration ability of each cell lines, U87MG cells were most motile than others, and U343MG-A least motile. The HAse was expressed in U251MG and U343MG-A cell lines. However, U87MG and U373MG cell lines did not express HAse activity. In Matrigel invasion assay, the cell lines expressing HAse (U251MG and U343MG-A) were more invasive in the presence of HA than HAse deficient cell lines (U87MG and U373MG). In HA hydrogel invasion assay, the HAse-expressing cell lines formed colonies more invasively than HAse-deficient ones.

**Conclusion :** Malignant Glioma cells expressing HAse were more invasive than HAse-deficient ones in 3-dimensional environment. Therefore, it might be suggested that invasion of malignant gliomas is suppressed by inhibition of HAse expression or HA secretion. Additionally, the ability of 2-D migration and 3-D invasion might not be always coincident to each other in malignant glioma cells.

**KEY WORDS :** Hyaluronic acid · Hyaluronidase · Invasion · Migration · Malignant glioma · Hyaluronic acid hydrogel.

## INTRODUCTION

One of the most lethal properties of high grade gliomas is their ability to invade surrounding normal brain tissue. Local invasion of the brain tumors is the major factor for the tumor growth and widespread rather than metastasis<sup>24</sup>. It is rare for glioma cells to enter the subarachnoid space or to intravasate into the cerebral microvasculature<sup>2</sup>. In the

treatment of brain tumor, the first barrier to overcome is to understand invasive characteristics of brain tumor cells on the extracellular matrix (ECM) of brain. Understanding of brain tumor cell invasion to the distant location is required invasion properties of brain tumor cells on the ECM in the brain. It was suggested that specific protein in the ECM may influence on the cell morphology or plasma membrane through chemical and mechanical reaction<sup>20</sup>. Although the properties and contents of brain ECM was not completely clarified, amorphous ECM in the brain contains HA and glycosaminoglycan (GAG) instead of collagen or fibrous protein such as fibronectin, and elastin<sup>3</sup>.

Hyaluronic acid (HA), a linear polysaccharide composed of alternating D-glucuronic acid and N-acetyl-D-glucosa-

• Received : May 11, 2009 • Revised : July 16, 2009

• Accepted : October 25, 2009

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mine units, is one of the main glycosaminoglycan components of the ECM, the synovial fluid of joints, and the scaffolding comprising cartilage<sup>11,15</sup>. In particular, high concentration of HA in the cell and ECM may create microenvironment to facilitate migration, proliferation, and invasiveness<sup>9,22</sup>. Hyaluronidase (HAse), a degrading enzyme of HA, is classified to HYAL1, HYAL2, HYAL3, HYAL4, PH20, and HYALP1 in human genome. Although the precise mechanism of HAse expression or secretion on invasive properties of glioma cells are not yet fully investigated, it was reported that the expression of HAse was positively correlated with invasion of tumor cells<sup>5</sup>. Liu et al.<sup>16</sup>, reported that HAse was expressed only at four invasive malignant glioma cell lines while it was not expressed at normal tissues of brain.

Regardless of its pathologic grade, each glioma cell line may exhibit a particular invasiveness. The simple scratch technique was found to be a rapid and quantitative method and was performed in a confluent cell monolayer. However, its relevance to invasion has occasionally been called into question. The invasive potential of cancer cell lines has commonly been tested *in vitro* by using matrigel as a barrier to invasion and HA hydrogel invasion assay<sup>13</sup>.

The purpose of this study was to prove that HAse is related to the invasion of glioma cells. We also investigated if glioma cells with higher mobility at 2-dimensional has also higher mobility at 3-dimensional environment.

## MATERIALS AND METHODS

### Astrocytoma cell lines and culture conditions

Human malignant astrocytoma cell lines [U87 MG (obtained from The Korean cell line bank, Seoul, Korea), U251 MG, U343MG-A, U373MG (obtained from Brain Tumor Research Center, University of California, San Francisco, Calif., USA)] were grown in monolayer culture. Cell lines were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Gaithersburg, Md., USA) supplemented with 10% fetal bovine serum (FBS).

### Migration assay

In order to compare the motility in each cell lines, the media of culture cells were replaced with medium containing 5 mM hydroxyurea (HU). A 24 h treatment with 5 mM hydroxyurea resulted in complete inhibition of cell proliferation. After 24 h of hydroxyurea treatment, the cultures are scraped with a single-edged razor blade. Cells are washed twice with PBS and placed in medium containing hydroxyurea. After 48 h of incubation, cells are washed twice with PBS, fixed with absolute alcohol, and stained

with 0.1% toluidine blue. Three microscopic fields were evaluated for each wound injury. The number of cells migrating across the wound edge and the maximum distance migrated were determined in each field and averaged for each injury, these experiments were repeated three times.

### HA zymography

HA (1 mg/mL, SIGMA) was incorporated into a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Serum-free conditioned media (SFM) was concentrated 30 or 50 times by use of Centrifugal Filter Device (VIVASCIENCE) and sample 50 µg was loaded in the gel. HAse (SIGMA) was added to the gel, and heated HAse was used as negative control. After electrophoresis, the gel was incubated in 3% Triton-X 100 for 1 h with kindly agitation in order to remove SDS. The gel were then transferred into the HAse assay buffer (10.5 g 50 mM citric acid, 2.92 g 0.05 M NaCl, pH 4.0) and then incubation on 37°C for 16 h. The gels were rinsed twice with distilled water, and stained in the Alcian blue solution (0.5% Alcian blue in 3% acetic acid solution) for 2 h. The gels were destained in destain solution (20% methanol, 10% acetic acid, 70% H<sub>2</sub>O) for 4 h. HAse activity is seen as white bands on a clear blue background. In order to investigate the effect of HU to HAse, we performed the HA zymography after addition of 0.1-5 mM concentration of HU into HAse solution.

We also investigated the HAse activity in different grade astrocytomas (grade I-IV), metastatic tumors, and normal brain tissues.

### Matrigel invasion assay

Invasion assay was performed using a transwell chamber and 8 µm pore size polycarbonate membranes (Costar, Cambridge, MA) coated with serum-free DMEM diluted ECM (Matrigel; Becton Dickson, Bedford, MA)(DMEM to ECM 3 : 1). Cells were seeded at a density of  $1 \times 10^4$  cell in 350 µl of serum-free DMEM in the upper compartment of the transwell, and allowed to invade polycarbonate membrane for 24 hours. The lower chamber was filled with DMEM that contained 10% FBS. After incubation, non-invaded cells on the upper surface of the membrane were removed, and the invaded cells on the lower surface of the membrane were stained with HEMACOLOR® (GERMANY). The number of invaded cells in four randomly selected microscopic fields (100<sup>TM</sup>) per membrane was counted.

To examine the effect of HA on the invasion of glioma cells, 100 µg or 800 µg of HA was added to serum-free DMEM diluted ECM solution and tested it similar way as described above.

### Synthesis of HA hydrogels

The HA hydrogels consisted of a long-chain of HA cross-linked with adipic dihydrazide (ADH)(SIGMA) as cross-linking agent and 1-Ethyl- 3-[3-(dimethylamino)-propyl] carbodiimide (EDCI)(JAPAN) as reagent. All hydrogels were prepared from high molecular weight ( $> 1 \times 10^6$  Da) HA (SIGMA), similar to the procedure described by Prestwich et al.<sup>17</sup>. Briefly, the ratios of ADH to HA and HA to EDCI were adjusted to obtain hydrogels optimized for cell adhesion and culture. The best results were obtained with ratios of ADH to HA equal to 10 : 1 and ratios of HA to EDCI equal to 1 : 1. HA and hydrazide cross-linker (ADH) were dissolved in milliQ-water and the pH adjusted to 4 by adding 0.1 N HCl. The carbodiimide reagent (EDCI) was dissolved in milliQ-water and added to the mixture, and allowed to gel for 2 h with gentle agitation. HA hydrogels were equilibrated in 0.1 N NaCl for 2 days, then in a mixture wateryethanol (3/1, v/v) for 2 days, and in milliQ-water for 2 days to remove ADH. HA hydrogels were lyophilized, then stored. Before use for cell culture, squares of the HA hydrogels were cut (to approximately  $5 \times 5 \times 1.5$  mm) then sterilized (60°C for 2 day) and rehydrated with culture medium and swelled roughly 100-fold in mass. After rehydration, the properties of the HA hydrogels were similar to the properties before lyophilization.

### HA hydrogel invasion assay

Tumor cells were trypsinized, centrifuged at 1500 rev/min for 10 min and the pellet was resuspended in culture medium to disperse cells for counting.  $4 \times 10^5$  cells in 2 mL DMEM 10% FBS were seeded in a well containing a HA hydrogel. Cells were allowed to migrate and colonize the HA hydrogel for 24 h in humidified atmosphere under 5% CO<sub>2</sub> in air at 37°C. Then the HA hydrogel was transposed in a new well with fresh culture medium and incubated in humidified atmosphere under 5% CO<sub>2</sub> in air at 37°C for 5 days. Experiments were made in duplicates. The HA hydrogels were examined using an inverted microscope. The measurements were performed by tracing the diameter of colonies on digitalized images.

The invasiveness of each cancer cell lines was estimated by the ability of cells to invade through reconstituted HA matrix. Colonies with more than 150 μm in diameter were regarded as positive ones and counted<sup>8</sup>.

### Doubling-time of the cell lines

$3 \times 10^4$  cells of each cell lines were seeded on in 30-mm culture dishes. The cells were harvested and counted every 24 h after 48 h of serum starvation. They were trypsinized and the number of viable cells was counted with a hemocy-

tometer. The doubling-time was calculated from the cell growth curve over five days. Equation for doubling time =  $(t-t^{\circ})\log 2 / \log N - \log N^{\circ}$  (t: final time, unit: hour, t<sup>°</sup>: initial times, N: final cell number, N<sup>°</sup>: initial cell number).

### Data analysis

Statistical analysis of invasion and HA hydrogel invasion assay properties of each cells was performed by paired samples t-test using the SPSS software program (version 12.0 for Windows; SPSS INC., Chicago, IL).

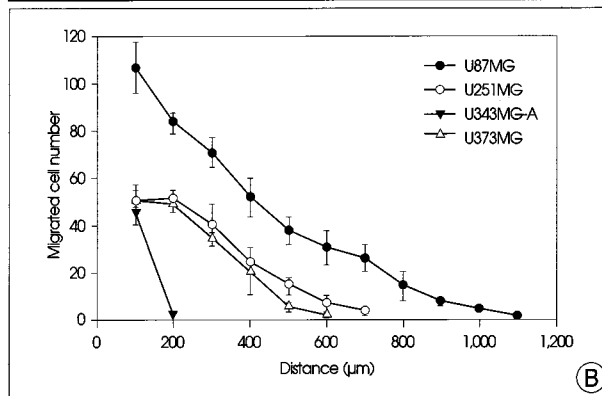
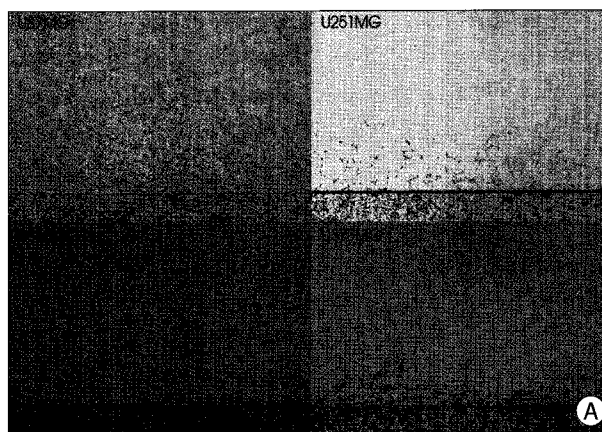
## RESULTS

### Doubling time and migration assay

The doubling time of U343MG-A, U373MG, U87MG, and U251MG were 46.6 h, 46.6 h, 41.4 h, and 30.1 h,

**Table 1.** Doubling-time of four cell lines

Cell line	Doubling time (h)
U87MG	41.4
U251MG	30.1
U343MG-A	46.6
U373MG	46.6



**Fig. 1.** Migration of cell lines at 2-D environment evaluated by simple scratch test. Migrated cells by light microscopy (A) and count of cells migrated from start line according to the distance (B). Error bar expressed standard deviation (S.D.).

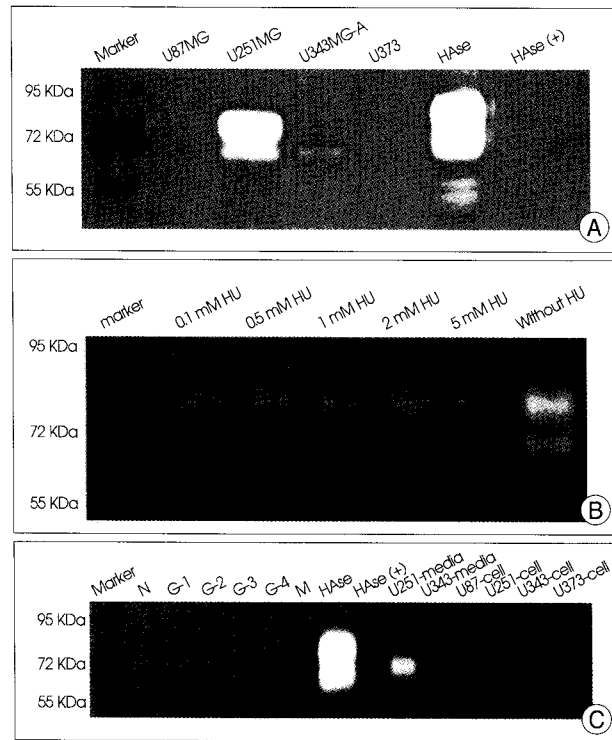
respectively (Table 1).

On simple scratch test, which is one of 2-D methods, U373MG cells and U251MG were more motile than U343MG-A, and U87 MG cells demonstrated the fastest motility (Fig. 1A). The four groups were different for the cell number and the cell distance (Fig. 1B). In the descending order of motility, U87MG, U251MG, U373MG and U343MG-A were shown.

**HA zymography**

Hase activity was observed as white bands on a clear background. Among the four cell lines, only U251MG and U343MG-A showed the expression of Hase activity. The U251MG cell line showed higher degree of expression than U343MG-A (Fig. 2A). Neither U87MG nor U373MG cell line expressed Hase activity.

In order to investigate the effect of HU to activity change



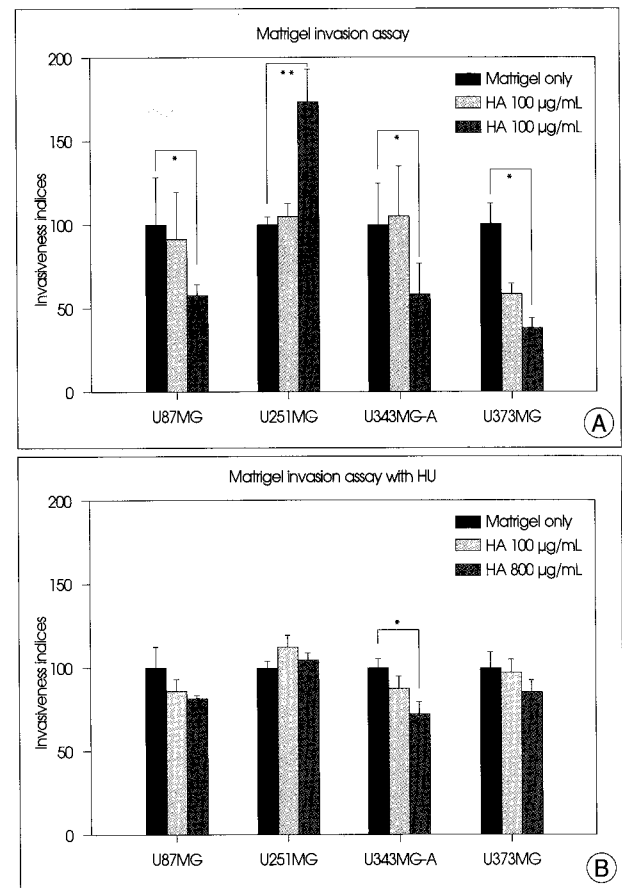
**Fig. 2** A : Hyaluronic acid (HA) zymography in serum-free media of various malignant glioma cell lines. Hyaluronidase (Hase) activity is observed only in the media of U251MG and U343MG-A cell lines. B : The activity of Hase is inhibited with the addition of hydroxyurea (HU) to the Hase solution. To test the effect of HU on the Hase activity, 0.1-5 mM of HU was added to the Hase solution [1,000 unit Hase/1 mL PBS (0.1 M, pH 7.4)]. C : HA zymography in tissue or cell extraction of astrocytic tumors and malignant glioma cell lines. Intracellular activity of Hase is not observed in astrocytic tumor and malignant glioma cell lines. But, it is observed in grade IV and metastatic brain tumor. Each one sample was used for HA zymography of N and G-I, respectively. More than three samples were used for G-II, G-III, G-IV, and M, respectively. Hase : positive control, Hase (+) : negative control, heated Hase, G-I : pilocytic astrocytoma, G-II : fibrillary astrocytoma, G-III : anaplastic astrocytoma, G-IV : glioblastoma, M : brain metastatic tumor, N : normal brain.

of Hase, 0.1-5 mM concentration of HU were added to the Hase solution, and same HA zymography was performed. Using Hase solution without HU as a control, we found that Hase activity was inhibited with the addition of HU to the Hase solution (Fig. 2B).

Hase activity was observed in tissue of the glioblastoma (grade IV) and metastatic brain tumor, but it was not observed in other astrocytic tumors (grade I-III), malignant glioma cell lines and normal brain tissue (Fig. 2C).

**Matrigel invasion assay in vitro**

In order to produce the similar environment to ECM (extracellular matrix), HA was added to matrigel. To eliminate cell proliferated influence, the cell was divided into



**Fig. 3** A : The effect of hyaluronic acid (HA) (HA 100 g/mL and 800 g/mL) on the invasion of glioma cell lines at matrigel invasion assay without hydroxyurea (HU). \*U87MG ( $p = 0.021$ ), U343MG-A ( $p = 0.01$ ) and U373MG ( $p = 0.001$ ) cells show significant decreased invasiveness at HA concentration of 800 g/mL, \*\*But U251MG cells which secreted more Hyaluronidase show increased invasiveness at same condition ( $p = 0.002$ ). B : The effect of HA addition (HA 100 g/mL and 800 g/mL) on the invasion of glioma cell lines at matrigel invasion assay with HU. Error bar expressed standard deviation (SD). \*U343MG-A cell line shows significant decrease in invasiveness when the HA concentration is 800 g/mL ( $p < 0.001$ ). U87MG and U373GM also show decreased invasiveness but it is not statistically significant. U251MG also shows inverse relationship of increased invasiveness.

two groups; with HU and without HU. The result of the invasion assay has been studied with the number of invaded cells in matrigel without HA set as 100%. As shown in Fig. 3, the extent of invaded cells in matrigel with HA was compared to matrigel only.

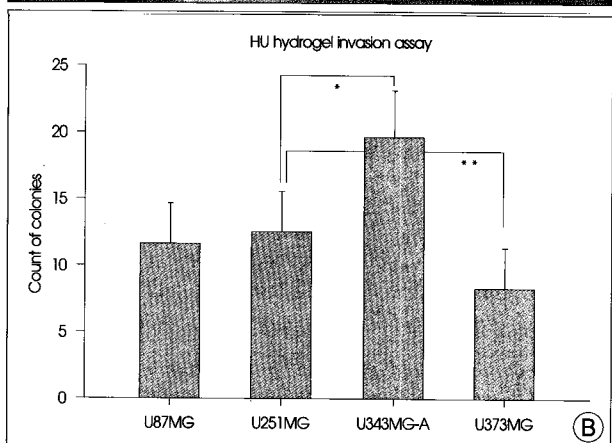
As shown in Fig. 3A, there was no significant difference in the invasiveness between matrigel with HA 100 µg/mL and matrigel only except for U373MG.) U251MG cell lines in matrigel with HA concentration of 800 µg/mL showed significant increase in the invasiveness compared with matrigel only ( $173.53 \pm 19.57\%$ ,  $p = 0.002$ ). In other cell lines, decrease in invasiveness has been observed U87MG ( $57.66 \pm 6.73\%$ ,  $p = 0.021$ ), U343MG-A ( $57.65 \pm 19.1\%$ ,  $p = 0.01$ ) and U373MG ( $38 \pm 5.66\%$ ,  $p = 0.001$ ) at HA concentration of 800 µg/mL (Fig. 3A).

Among HU-treated cell lines (Fig. 3B), U343MG-A cell line showed significant decrease in invasiveness when the

HA concentration was 800 µg/mL ( $p < 0.001$ ). U87MG ( $p = 0.21$ ) and U373MG also showed decreased invasiveness but it was not statistically significant ( $p = 0.41$ ) as shown in Fig. 3B. U251MG also showed inverse relationship of increased invasiveness ( $p = 0.225$ ) (Fig. 3B).

### HA hydrogel invasion assay

In HA hydrogel invasion assay, both cell lines which secrete HAse and cell lines which do not secrete HAse, were cultured in the presence of HA hydrogel in order to observe the effect of HAse secretion of the cell to the invasiveness. We observed that cell lines invaded HA hydrogel and formed clusters (with diameter less than 150 µm) and colonies (diameter larger than 150 µm) (Fig. 4A). The number of colonies after 5-day culture in HA hydrogel was a good indicator of quantitatively expressing invasiveness. The ability to form colonies, which indicates the invasiveness, was in the order of U343MG-A, U251MG, U87MG and U373MG (Fig. 4B) (Among the four cell lines, U343MG-A cell formed colonies significantly higher than other three cell lines ( $p < 0.001$ ). Furthermore, HAse secreted U251MG showed higher colony formation than U373MG while both of U251MG and U373MG cells showed similar motility ( $p = 0.006$ ).



**Fig. 4.** A : Hyaluronic acid (HA) hydrogel invasion assay using various cell lines. U343MG-A Colonies formed in the HA hydrogel observed by light microscope and counting colonies according to the various cell line (Magnification,  $\times 100$  Bar = 150 m). B : The ability to form colonies is in the order of U343MG-A, U251MG, U87MG and U373MG. Error bar expressed standard deviation (SD). \*Hyaluronidase (HAse) secreted U343MG-A shows highest colony formation ability than other cell lines ( $p < 0.001$ ). \*\*Furthermore, HAse secreted U251MG shows higher colony formation than U373MG while both showed similar motility ( $p = 0.006$ ).

### DISCUSSION

The microenvironment is essential for tumor cells to grow and divide in which the tumor cell interacts with many substances from supporting cell, degradative enzyme, and ECM. These ECM mediated receptor cell interactions are responsible for the regulation of cell proliferation, migration, and adhesion<sup>12</sup>. HA is an essential substance in brain cell whose concentration increases in brain tumor cell's stroma and advancing edge. It is synthesized by HA-synthases in the plasma membrane and its molecular weight exceeds 107 KDa. Synthesis of HA is promoted by various growth factors such as epidermal growth factor, basic fibroblast growth factor, and transforming growth factor<sup>14</sup>. HA-protein interactions play crucial roles in cell adhesion, growth, and migration, and HA acts as a signaling molecule in cell motility, inflammation, wound healing and cancer metastasis<sup>4,22</sup>.

Many cell-based studies with 2-D space on a glass or plastic surface have been used, but they may not produce characteristic results of *in vivo*<sup>7</sup>. Recent studies have shown that cell-based assays in 3-D substrates may provide physiologically relevant results<sup>6,12</sup>. HA hydrogel is hydrophilic and its physical properties resemble those of biological tissues. Many researchers reported that vascular smooth

muscle and endothelial cell grew successfully on HA hydrogel<sup>19,21,23</sup>. HA hydrogel contains HA in sufficient amount, but it lacks substances such as vitronectin, osteopontin, tenascin-C, SPARC, neurocan and BEHAB which are present in brain tumor ECM.

In this study, simple scratch test, which is a 2-D space, showed that U87MG cell line had the most mobility, but, in 3-dimensional HA hydrogel invasion assay, HAse-expressing U343MG-A cell line produced more colonies, which may indicate that U343MG-A has higher invasiveness *in vivo*. Even though U251MG and U373MG cell lines showed similar mobility, HAse-expressing U251MG formed more colonies in HA hydrogel than U373MG cells. The difference in doubling time of each cell lines did not coincide with the number of colony formation. Therefore, we conclude that the difference of colony formation is not due to the doubling time in each cell lines.

Delpech et al.<sup>10</sup> reported that HAse activation occurred only in pH range of 3.5-4, and Laurent et al.<sup>8</sup> reported that HAse activation can also occur in physiological status of pH 7.5. HAse reacts with HA hydrogel to make it easier for the invasion, and the small molecule of HA promotes migration of cells<sup>1</sup>. In the report of Delpech et al.<sup>9</sup>, HAse is elevated more in human brain metastases than in primary brain tumors. The fact that HAse activity has been observed in both high grade astrocytic tumors (grade IV) and metastatic brain tumors suggest that HAse might be related to tumor invasiveness.

Matrigel is used for *in vitro* invasion assay. It is the basement membrane matrix extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma and contains many proteins such as laminin, collagen IV, heparan sulfate proteoglycan, entactin, nidogen and growth factors such as TGF-beta, fibroblast growth factor, tissue plasminogen activator while it contains little glycosaminoglycan (GAG) such as HA. Although it cannot reflect ECM completely, it has been used as a good model to quantitative analysis. In this study, U251MG cell lines showed significant increase in the invasiveness in matrigel with HA concentration of 800 µg/mL than matrigel only ( $p = 0.002$ ) (Fig. 3A). Radotra and McCormick<sup>18</sup> reported that invasion of glioma cell line was increased 70 % in comparison of matrigel only with dose-dependent manner up to 200 µg/mL HA concentration. This effect was diminished higher than 200 µg/mL and invasion was slightly decreased. As the HAse expression was repressed, invasiveness was lowered, which was stated in the report of David et al.<sup>8</sup>. Among HU-treated cell lines (Fig. 3B), invasiveness of U251MG cells was not significantly changed at matrigel with HA 800 µg/mL, indicating that HAse expression might be suppressed by addition of HU

and then invasiveness was not changed. U343MG-A cells showed significant decrease in invasiveness at HA concentration of 800 µg/mL ( $p < 0.001$ ). These results indicate that suppression of HAse expression induce decrease of invasiveness of HAse-expression cells.

At HA invasion assay using HA hydrogel, U343MG-A formed most number of colonies among all of the cells despite U251MG showed higher invasiveness at Matrigel assay test. At this moment, even though this result is not evident, HA hydrogel is in the absence of matrigel component and then invasiveness of tumor cells in HA hydrogel may be different with HA + matrigel assay. Furthermore, the differences of HA-related receptors such as CD44 and RHAMM may affect the invasiveness of glioma cells<sup>11,14,18</sup>.

## CONCLUSION

In malignant glioma cell lines, mobility in 2-D space and invasiveness in 3-D did not correlate. It was found that HAse secreting glioma cells had higher invasiveness in 3-D invasion assay systems. Any means of repressing HAse secretion and activation is expected to reduce the invasiveness of malignant glioma cell.

### Acknowledgements

This work was supported by the Brain Korea 21 Project, Center for Biomedical Human Resources at Chonnam National University.

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