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***In vitro* and *in vivo* interactions between glioma and Marrow-Isolated Adult Multilineage Inducible (MIAMI) cells**

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Abstract

The prognosis of patients with malignant glioma remains extremely poor despite surgery and improvements in radio- and chemo-therapies. We recently showed that marrow-isolated adult multilineage inducible (MIAMI) cells, a subpopulation of human mesenchymal stromal cells (MSCs), can serve as cellular carriers of drug-loaded nanoparticles to brain tumors. However, the safety of MIAMI cells as cellular treatment vectors in glioma therapy must be evaluated, in particular their effect on glioma growth and their fate in a tumor environment. In this study, we showed that MIAMI cells were able to specifically migrate toward the orthotopic U87MG tumor model and did not influence its growth. In this model, MIAMI cells did not give rise to cells resembling endothelial cells, pericytes, cancer-associated fibroblasts (CAFs), or astrocytes. Despite these encouraging results, the effects of MIAMI cells may be glioma-dependent. MIAMI cells did not migrate toward the orthotopic Lab1 tumor and they can induce the proliferation of other glioma cell lines *in vitro*. Furthermore, a fraction of MIAMI cells was found to be in a state of proliferation in the U87MG tumor environment. These findings indicate that the use of MIAMI cells as cellular treatment vectors for malignant tumors must be controlled. These cells may be used as “suicide vectors”: vectors for killing not only tumor cells but themselves.

Key words: mesenchymal stromal cells, glioma, cancer-associated fibroblasts, tumor microenvironment

1. Introduction

Glioblastoma (GB) is a highly infiltrative tumor with a recurrence rate of 90% in the first few centimeters of the resection cavity, even in cases of complete tumor resection and adjuvant chemo/radiotherapy (Giese et al., 2004; Liang et al., 1991; Wallner et al., 1989). After optimal treatment, median survival is approximately 16 months and less than 10% of these patients survive more than five years (Stupp et al., 2009).

The failure of existing treatment methods is partly due to the inability to target tumor cells infiltrating the healthy brain tissues surrounding the tumor. The discovery of the special tropism of adult multipotent mesenchymal stromal cells (MSCs) for brain tumors has identified a new potential treatment strategy. MSCs distribute throughout the primary tumor bed and migrate toward the outgrowing tumor microsatellites after they are implanted either in the tumor or at sites distant from the tumor (Kosztowski et al., 2009; Motaln et al., 2010). This behavior has been exploited as a glioma-targeting therapeutic strategy. Strong antitumor effects have been reported following intracranial administration of MSCs genetically modified by viruses conferring the expression of immunostimulator or toxic molecules in experimental glioma models (Bexell et al., 2010; Grisendi et al., 2011; Hu et al., 2010; Roger et al., 2011).

Recently, we showed that a subpopulation of adult human MSCs called marrow-isolated adult multilineage inducible (MIAMI) cells can deliver drug-loaded lipid nanocapsules (LNCs) to brain tumors (Roger et al., 2012; Roger et al., 2010). LNCs offer several advantages over viral vectors. They have a low risk of immunogenicity, are easy to produce without high manufacturing costs, and can carry multiple types of therapeutic agents, such as drugs (Allard et al., 2008; Huynh et al., 2011; Lacoeyille et al., 2007), radionuclides (Vanpouille-Box et al., 2011) or DNA (Morille et al., 2011).

In contrast to MSCs, MIAMI cells are a homogenous population of cells that express certain embryonic stem cell markers and are capable of differentiating into cell lineages derived from all three germ layers, including immature neurons that exhibit neuronal ionic channel activity *in vitro* (Delcroix et al., 2010; D'Ippolito et al., 2004; Tatard et al., 2007). MIAMI cells have already shown promise as an autologous human cell population for tissue regeneration in skeletal and nervous system disorders (Delcroix et al., 2011; Garbayo et al., 2011).

Transforming MIAMI cell-based anti-cancer strategies into clinical therapies requires defining their interactions with glioma cells to assure the safety of this cellular vector. Indeed, potential hazards have been linked to the use of MSCs as tumor-targeting delivery vehicles (Bexell et al., 2010; Grisendi et al., 2011). In this study, we examined the influence of MIAMI cells on glioma cell line growth *in vitro* and *in vivo*, and analyzed their fate in terms of proliferation, differentiation and migration in a glioma environment.

2. Results

2.1. Effect of MIAMI cells on glioma cell line growth *in vitro* and *in vivo*

We studied the effect of diploid MIAMI cells on the proliferative activity of several aneuploid glioma cell lines *in vitro* (Fig. 1). The A172 and U118MG cell lines co-cultured for 72 hr with MIAMI cells displayed increased proliferation, whereas proliferation of the Lab2 and U138MG cell lines co-cultured with MIAMI cells was inhibited. No significant changes were observed for HS683 and Lab1 cell lines co-cultured with MIAMI cells. The diploid U87MG cell line was not tested in these conditions. However, previous results using transwell system showed that MIAMI cells inhibited slightly the proliferation of U87MG cells (Roger et al., 2012).

We examined the influence of MIAMI cells on tumor growth *in vivo* using the two tumorigenic human GB cell lines, U87MG and Lab1. The other glioma cell lines tested *in vitro* were not analyzed *in vivo* because they were not tumorigenic or they induced an immune rejection in nude mice. U87MG tumor-bearing mice had a median survival of 30 ± 2.1 days (Fig. 2A) and Lab1-bearing mice had a median survival of 20 ± 2.3 days (Fig. 2B). The median survival times of tumor-bearing animals were not statistically different from those of tumor-bearing mice receiving an intratumoral injection of MIAMI cells (29.5 ± 2.5 days for U87MG tumor-bearing mice, and 20 ± 1.5 days for Lab1 tumor-bearing mice).

2.2. Analysis of the migration of MIAMI cells toward GB

MIAMI cells derived from a male donor were injected into six-day-old striatal U87MG and Lab1 tumors (from female donors) in female nude mice. The migration of MIAMI cells toward the tumor cells was detected by Y-chromosome fluorescence *in situ* hybridization (FISH) on day seven (Fig. 3A). For U87MG tumors, MIAMI cells were largely distributed at the border zone between the tumor and normal parenchyma and were found near tumor infiltrates (Fig. 3B). No Y-chromosome-positive MIAMI cells were observed in the opposite hemisphere (data not shown). In contrast, after injection into the Lab1 tumor model, MIAMI cells were not distributed in the tumor mass (Fig. 3C) and remained at the injection site (data not shown). These results were confirmed *in vitro* where MIAMI cells were able to migrate specifically toward the U87MG tumor conditioned medium (TCM) whereas the Lab1 TCM did not affect migration at all in comparison with the control medium (Supplementary data). MIAMI cells also migrated toward U87MG tumors if they were inoculated into the contralateral hemisphere. At three and seven days after injection, MIAMI cells were found to have migrated away from the initial injection site and toward the contralateral

tumor and were located in the peri-tumoral zone (Fig. 4). Some MIAMI cells remained at the injection site (Fig. 4).

2.3. Analysis of the fate of MIAMI cells in a GB environment *in vitro* and *in vivo*

We studied the proliferation and differentiation of MIAMI cells cultured for seven days in U87MG TCM. MIAMI cells cultured in this medium contained higher levels of cancer-associated fibroblasts (CAF)-associated markers (alpha-smooth muscle actin, [α -SMA] and fibroblast specific protein, [FSP]) and the Ki-67 proliferation marker than those of control MIAMI cells (Fig. 5). Markers associated with endothelial cells (CD31), astrocytes (glial fibrillary acidic protein, [GFAP]) and pericytes (chondroitin sulfate proteoglycan, [NG2]) were not detected in MIAMI cells cultured in the U87MG TCM medium or in control medium.

We assessed the fate of MIAMI cells *in vivo* seven days after being injected into U87MG tumors and compared it to the fate of MIAMI cells injected into the striatum of healthy mice (Fig. 6). Most of the MIAMI cells in the healthy mice and in the U87MG tumor environment expressed CAF-associated markers (α -SMA and vimentin). In contrast to the *in vitro* data, with the immunofluorescence technique we used, it was difficult to determine whether the expression levels of these CAF markers were different in these two *in vivo* environments. As observed *in vitro*, MIAMI cells did not express desmin (a pericyte marker), CD31 or GFAP. In the U87MG tumor environment, MIAMI cells were principally localized in the glial scar and $36 \pm 2\%$ of MIAMI cells were close to endothelial cells. We noted that a fraction of injected MIAMI cells ($19.5 \pm 3.6\%$) were found in a proliferative state in the U87MG tumor environment. No double-labeled Y⁺/Ki-67⁺ MIAMI cells were detected in healthy mouse striatum.

3. Discussion

Recently, we showed that a subpopulation of MSCs, MIAMI cells, can deliver drug-loaded LNCs to brain tumors (Roger et al., 2012; Roger et al., 2010). However, several reports have indicated that MSCs can modulate the tumor microenvironment and may impact tumor behavior. For example, Yu *et al.* (Yu et al., 2008) showed that subcutaneous or intracranial co-injection of MSCs derived from human adipose tissues (hADSCs) together with U87MG cells in BALB/c nude mice promoted outgrowth of the U87MG tumors. Similarly, Nakamiso *et al.* (Nakamizo et al., 2005) observed that nude mice bearing U87MG xenografts and receiving β -gal-labeled hMSC survived for shorter times than did tumor-bearing mice that received saline treatments. On the other hand, some studies have demonstrated either opposite effects (Nakamura et al., 2004) or no effect at all (Bexell et al., 2009; Kucerova et al., 2010).

In the present study, we analyzed the effect of MIAMI cells on the proliferation of a panel of aneuploid glioma cell lines *in vitro*. The use of aneuploid cells allowed tumor cell proliferation in co-cultures to be distinguished from that of the diploid MIAMI cells. We found the effects of MIAMI cells on glioma cell growth *in vitro* differed depending on the tumor cell line used. MIAMI cells increased A172 and U118MG cell growth, decreased Lab2 and U138MG cell growth, and had no effect on Lab1 and HS683 cell growth. Although the growth rate effects of MIAMI cells were statistically significant, the magnitudes of the effects on U118MG, A172, Lab2 and U138MG cells were quite modest and may not be physiologically relevant. Consistent with these results, we found that MIAMI cells grafted into the two tumorigenic GB cell lines (U87MG and Lab1) did not affect the survival of tumor-bearing mice. However, we can not exclude the possibility that in the case of U118MG and A172 cells, where there is a MIAMI

cell-mediated proliferation support, *in vivo* injection of MIAMI cells with these glioma cells could decrease the extent of immune rejection and/or result in abrogation of tumor dormancy leading to tumor growth. This property was described for CAFs which can induce the transformation of non tumorigenic prostatic epithelial cell lines (Hayward et al., 2001). Future studies are needed to address this possibility.

Whereas the effects of MSCs in tumor environments are unclear, concerns are also accumulating regarding their fates in these environments. In a glioma environment, MSCs gave rise to pericyte-like cells contributing to the stabilization of tumor-vessel walls (Bexell et al., 2009; Birnbaum et al., 2011). In this study, we found 36% of the injected MIAMI cells near mouse endothelial cells. However, we did not find MIAMI cells expressing the pericyte marker, desmin, or the endothelial cell marker CD31. These results were confirmed *in vitro*, as U87MG TCM exposure did not induce expression of CD31 or NG2 (another pericyte marker) in MIAMI cells. The differences between our results and those of other studies could be due to the use of different tumor models, such as xenografts rather than syngenic models. MSCs have also been shown to differentiate into astrocytes after being injected into neonatal mouse brains (Kopen et al., 1999). In this study, we did not find the astrocyte marker GFAP to be expressed by MIAMI cells after they were injected into either the healthy environment or the U87MG tumor environment. Several reports describe the capacity of MSCs to differentiate into CAFs (Cho et al., 2011; Cho et al., 2012; Mishra et al., 2008; Spaeth et al., 2009). CAFs may have an important role in the growth of tumors (Bhowmick et al., 2004; Franco et al., 2010). Unfortunately, no CAF-specific markers have been identified and the markers typically used to identify these cells, such as α -SMA and FSP, are also expressed by MSCs, vascular smooth muscle cells and pericytes in different conditions *in vitro* and *in vivo*. We found that MIAMI cells

expressed these CAF markers and that exposure to U87MG TCM for seven days increased their expression levels. Although MIAMI cells expressed these CAF markers, they did not appear to share their functional properties because intratumoral injection of these cells did not impact the survival of U87MG- or Lab1-tumor-bearing mice.

While these findings suggest MIAMI cells might be used safely in malignant conditions, caution must be exercised in their use as treatment vectors. Firstly, the tumor models we used in this study are aggressive, so the effects of MIAMI cells may not have had time to occur. Recently, a secretome analysis indicated that MIAMI cells secrete high levels of vascular endothelial growth factor (VEGF), fractalkine, interleukin (IL)-6, IL-8 and a growth-related oncogene (Roche et al., 2012). All these soluble mediators play key roles in angiogenesis, arteriogenesis, immunomodulation, neuroprotection, cell proliferation and cell survival. These features of the MIAMI cell secretome are of particular interest for their use in tissue repair and regeneration. In cancer therapy, however, these soluble factors could unfavorably affect tumor growth. Secondly, although we detected no specific MIAMI cell differentiation in the U87MG tumor environment, we observed that 20% of MIAMI cells expressed Ki-67 proliferation marker in this environment. In the healthy environment, we found no MIAMI cells in a proliferative state suggesting that factors produced by the U87MG cells induced MIAMI cell proliferation. Thirdly, we observed that MIAMI cells migrated to and dispersed throughout the tumor mass in the peri-tumoral zone whether they were injected intratumorally or in the contralateral brain hemisphere of mice bearing the U87MG tumor. However, we noted that this behavior seemed to be glioma dependent, as MIAMI cells injected into Lab1 tumor-bearing mice remained at the injection point and did not distribute throughout the tumor mass. This different

migration pattern of MIAMI cells underlines the specific molecular expression pattern and cell signaling crosstalk of GB cell lines. The molecular characterization of the MIAMI cell migration should be evaluated before beginning the clinical study of MIAMI cells.

4. Methods and Materials

4.1. Cell culture

MIAMI cells were isolated by differential adhesion of iliac crest aspirates from human male post-mortem organ donors (median age: 66 years) after obtaining family consent (protocol approved by the Ethics Committee of the French Agency of Biomedicine). The cells were cultured *in vitro* under conditions described previously (D'Ippolito et al., 2006). Briefly, iliac crest-derived cells were plated at a density of 10^5 cells/cm² in DMEM low-glucose media (DMEM-LG, Gibco, Cergy Pontoise, France) containing 3% fetal bovine serum (FBS, Hyclone, PerbioScience, Bredières, France) and 1% antibiotics (Sigma-Aldrich, St. Quentin Fallavier, France) on a fibronectin substrate (10 ng/mL, Sigma-Aldrich) under low-oxygen conditions (3% O₂, 5% CO₂, and 92% N₂). Fourteen days after the initial plating, non-adherent cells were removed. Pooled colonies of adherent cells were plated at 100 cells/cm² and expanded until reaching 30% confluence. These cells are negative for hematopoietic lineage markers (CD45, CD34) but are positive for CD13; CD29; CD44; CD49b, c and e; CD63; CD73; CD81; CD90; CD105; CD140b; CD164; CD166; and SSEA4. Furthermore, MIAMI cells are able to differentiate into osteoblasts and adipocytes under appropriate culture conditions (data not shown).

Five human glioma cell lines were obtained from the ATCC (A172, HS683, U118MG, U138MG, U87MG; LGC Promochem, Molsheim, France) and two (Lab1 and Lab2)

were generated from patients undergoing surgery for GB in the Department of Neurosurgery from CHU Angers (Table 1). Briefly, after surgery, tumor samples were mechanically dissociated into a suspension which was passed through tissue culture and seeded into flasks, as previously described (Clavreul et al., 2009). Lab1 and Lab2 were used at passage 20. All the cell lines were maintained in DMEM high-glucose (HG) (Lonza, Verviers France) containing 10% FBS (Lonza), and 1% antibiotics (Sigma-Aldrich) in a humidified incubator containing 5% CO₂ (37°C) until reaching 80% confluence.

4.2. *In vitro* proliferation assay

We assessed the ability of MIAMI cells to affect the proliferation of glioma cells by using aneuploid glioma cell lines (A172, U118MG, U138MG, HS683, Lab1, and Lab2) to allow tumor cells to be distinguished from diploid MIAMI cells. Glioma cell lines were seeded into 6-well plates at 10⁵ cells/well and cultured in DMEM-HG supplemented with 10% FCS and 1% antibiotics. After 24 hr, 10⁵ MIAMI cells were added to each well. The cells were co-cultured for three days and then harvested. Vindelov's protocol was used to stain chromosomal DNA (Vindelov et al., 1983) and the cells were analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences, Le Pont-de-Claix, France). DNA index (DI) and the percentages of the cell populations in each culture identified on the basis of their DNA content were calculated with Modfit version 5.2 software (Verity Software House, Topsham, Maine). The diploid DNA peak was located on DNA histograms, according to an external standardization procedure using normal human lymphocytes and an internal standardization procedure using trout red blood cells. DI was calculated as the ratio of the mean DNA content of tumor cells to the mean DNA content of diploid cells. Cultures with a DI value of one were classified as DNA-diploid and cultures with DI

values of <1 or >1 were classified as aneuploid. Cell-cycle analysis was carried out on the aneuploid glioma cells and the proliferation index (PI) of these cells was evaluated as the percentage of cells in S+G2M. *In vitro* proliferation assays were performed in triplicate for each cell line.

4.3. Exposure of MIAMI cells to U87MG TCM

U87MG cells were grown in DMEM-HG containing 10% FBS and 1% antibiotics and conditioned medium from these cells (TCM) was harvested after 72 hr. TCM was centrifuged, filtered through a 0.45 µm pore filter (Millipore, Molsheim, France) and stored at 4°C before use. MIAMI cells were exposed to TCM or control medium (DMEM-HG containing 10% FBS and 1% antibiotics) for seven days. Cells were then analyzed by flow cytometry for the presence of astrocyte, pericyte, CAF, endothelial cell and proliferative cell markers.

4.4. Flow cytometry analysis

MIAMI cells grown in TCM or control medium were suspended in PBS containing 5% FBS and 0.02% NaN₃ and incubated on ice for 1 hr with primary antibodies against markers associated for endothelial cells (human CD31, BD Biosciences), astrocytes (GFAP, Life Technologies SAS, Saint Aubin, France), pericytes (NG2, Millipore), CAFs (α -SMA, R&D Systems, Lille, France; FSP, Sigma-Aldrich), and proliferative cells (Ki-67, Dako, Trappes, France) as well as with isotype control antibodies. Following incubation, cells were washed and stained with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Dako, Trappes, France) for 30 min on ice. After washing, the cells were fixed in 2% formaldehyde. The stained cells were analyzed using a FACScan flow cytometer with CellQuest Software (BD Biosciences).

4.5. *In vivo* experiments

Female athymic Swiss nude mice, aged 8-9 weeks were obtained from Charles River (L'Arbresle, France). Experiments were conducted in accordance with the regulations of the French Ministry of Agriculture and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Animals were anesthetized by an intraperitoneal injection of xylazine (50 mg/kg body weight) and ketamine (10 mg/kg body weight) and were positioned in a Kopf stereotaxic instrument.

Two GB models were used in this study: U87MG and Lab1. These GB cells were obtained from female human donors.

Analysis of MIAMI cells effects on tumor growth

On day zero, 5×10^4 U87MG cells ($n = 20$) or 2×10^4 Lab1 cells ($n = 14$), in 5 μ l HBSS with Ca^{2+} and Mg^{2+} , were implanted into the striatum [coordinates: posterior to Bregma (AP), -0.5 mm; lateral (L), +2 mm; deep from the outer border of the cranium (D), -3 mm]. At day six, either 5 μ l HBSS with Ca^{2+} and Mg^{2+} containing 3×10^5 MIAMI cells or 5 μ l of HBSS with Ca^{2+} and Mg^{2+} alone (negative control) were injected into the tumors of the mice at the same coordinates. Animals were sacrificed when they had lost more than 10% of their body weight which was directly attributed to the tumor development.

Analysis of MIAMI cell migration toward U87MG and Lab1 tumors

Mice were implanted in the striatum at day zero with 5×10^4 U87MG cells ($n = 12$) or 2×10^4 Lab1 cells ($n = 3$) as described above. At day six, 3×10^5 MIAMI cells were injected into the U87MG or Lab1 tumor at either the same coordinates as the tumor or in the brain hemisphere contralateral to the U87MG tumor mass (coordinates: AP, -0.5 mm; L, -2 mm; D, -3 mm). Mice were killed three or seven days after injection of the MIAMI cells.

Analysis of the fate of MIAMI cells in vivo

MIAMI cells (3×10^5 cells) were injected into the striatum of healthy mice ($n = 3$) or in mice bearing an intracranial U87MG tumor (on day 6 after U87MG cell injection) ($n = 6$) (coordinates: AP, -0.5 mm; L, +2 mm; D, -3 mm). Mice were killed seven days after MIAMI cell injection.

For all of the *in vivo* experiments, mouse brains were snap-frozen in isopentane cooled by liquid nitrogen and stored at -80°C . Brain coronal sections were cut at 10 μm and collected onto silanized slides.

4.6. Fluorescence *in situ* hybridization

MIAMI cells were detected by FISH using a Y-chromosome probe. The DNA probe was complementary to the highly repetitive human satellite III sequences near the centromeric region of the human Y-chromosome DYZ1 locus (CEPY) and was labeled with the fluorochrome SpectrumOrange (Vysis, Abbott Molecular, Rungis, France). Frozen sections were air-dried, rehydrated in Dulbecco's PBS (DPBS) and fixed for 10 min in 4% paraformaldehyde (PFA) pH 7.4 at 4°C . Nuclei were denatured for 5 min using 70% formamide (Sigma-Aldrich)/2X saline-sodium citrate buffer (SSC, Vysis) at 73°C and then dehydrated in alcohol gradient baths (EtOH 70%, 80%, 90% and 100%) for 2 min each. The Y-chromosome probe was diluted with hybridization buffer according to the manufacturer's instructions, added onto the tissue sections, covered with coverslip and sealed with rubber cement. The hybridization reaction was performed in three steps: 10 min at 73°C to denature the DNA and the probe, 2 min at 4°C to fix the denaturation, and overnight incubation in a humid chamber at 45°C to hybridize DNA with the probe. After three washes for 10 min in baths of 50% formamide/2X SSC and 5 min in 2X SSC/0.1% NP-40, nuclei were counterstained in DAPI II and antifade (Vysis).

4.7. Immunofluorescence

Briefly, cryosections were air-dried, rehydrated in PBS and fixed for 10 min in 4% PFA pH 7.4 at 4°C. To block nonspecific binding, sections were incubated in PBS containing 4% BSA and 10% normal goat serum. Incubations with isotype controls and primary antibodies against endothelial cells (human and mouse CD31, BD Biosciences), astrocytes (GFAP, BD Biosciences), pericytes (desmin, Dako), CAFs (α -SMA, R&D Systems; vimentin, Dako), and proliferative cells (Ki-67, Dako) were performed overnight at 4°C. Primary antibodies were detected using biotinylated secondary antibody and amplified using streptavidin-FITC (Dako). After washing, MIAMI cells were detected by FISH using a human Y-chromosome probe as described above.

4.8. Image analysis

Brain cryosections stained by FISH and by immunofluorescence from three mice injected intracerebrally with MIAMI cells and three U87MG tumor-bearing mice injected intratumorally with MIAMI cells were analyzed under a fluorescence microscope (Axioscope® 2 optical, Zeiss, Le Pecq, Germany) and under a confocal microscope (LSM 700, Zeiss). Labeled MIAMI cells (Y^+) and double-labeled MIAMI cells ($Y^+/Ki-67^+$, Y^+/α -SMA $^+$, $Y^+/vimentin^+$, $Y^+/desmin^+$, $Y^+/GFAP^+$ and $Y^+/CD31^+$) were quantified using a computerized image-analysis system (MetaView, Roper Scientific, Evry, France). Results were expressed as the mean percentage of double-labeled MIAMI cells in a representative slice from three animals \pm standard error of the mean (SEM).

4.9. Statistical analysis

Results are provided as mean \pm SEM and median \pm SEM for *in vivo* experiments. Student's *t* tests were used to determine statistically significant differences. Survival curves were obtained using the Kaplan-Meier method. Comparison of these curves was conducted using the log-rank test. Differences were considered statistically significant for *P* values < 0.05 .

5. Conclusions

We have shown that MIAMI cells are able to migrate toward the orthotopic U87MG tumor model and that their intratumoral injection did not affect mice survival. Furthermore, in this model, MIAMI cells did not give rise to endothelial-like, pericyte-like, CAF-like, or astrocyte-like cells. However, our analysis of interactions between MIAMI cells and other glioma cell lines indicated that some MIAMI cell properties may be glioma-dependent. Furthermore, the proliferation of MIAMI cells in the U87MG environment should not be ignored. All these data indicate that MIAMI cells used as cellular treatment vectors in malignant conditions must be controlled. For example, it may be preferable that the cells carry molecules that are toxic not only to tumor cells but also to themselves. Such a suicidal strategy could prevent their unwanted proliferation and secretion of growth and angiogenic factors. Moreover, supplementary studies are needed to identify factors produced by glioma cells that foster MIAMI cell migration.

Author disclosure statement

No potential conflicts of interest.

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Appendix A. Supplementary data

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Figure legends

Fig. 1: Direct co-culture assay of MIAMI cells and aneuploid glioma cell lines. The DNA contents of glioma cells and MIAMI cells were analyzed by flow cytometry and the PI of aneuploid cells alone or co-cultured with MIAMI cells was calculated. (A) Example of flow cytometry histograms showing the DNA content of a co-culture of MIAMI cells (DI = 1) with aneuploid glioma cells (HS683, DI = 1.79). (B) Bar graph showing the PI of aneuploid cells alone or co-cultured with MIAMI cells. The proliferation of A172 and U118MG cell lines co-cultured for 72 hr with MIAMI cells was significantly higher, and the proliferation of Lab2 and U138MG cell lines was significantly lower than control cultures. No significant differences were observed for the HS683 and Lab1 cell lines (mean \pm SEM from triplicate wells). *, P<0.05 versus glioma cells alone.

Fig. 2: Effect of MIAMI cells on the proliferation of GB cell lines *in vivo*. Kaplan-Meier graphs show that the survival times of U87MG (A) or Lab1 (B) tumor-bearing mice that received an intratumoral injection of 3×10^5 MIAMI cells six days after the intracerebral inoculation with tumor cells were not different from those of control tumor-bearing animals.

Fig. 3: Fluorescent microscopy images of tissue sections after intratumoral injection of MIAMI cells in U87MG or Lab1 tumor-bearing mice. A) Schematic representation of the experimental model. B) MIAMI cells were distributed at the border of the tumor seven days after they were injected into the U87MG tumor model. C) MIAMI cells were not found at the border of the tumor seven days after they were injected into the Lab1 tumor model. MIAMI cells were detected by labeling the Y-chromosome with a red fluorescent probe. Nuclei were stained with DAPI. Scale bars = 100 μm and 50 μm (inset) for magnifications.

Fig. 4: Fluorescent microscopy images of tissue sections after contralateral injection of MIAMI cells in U87MG tumor-bearing mice. A) Schematic representation of the experimental model. Three days (day 9) and seven days (day 13) after injecting MIAMI cells into the left brain hemisphere contralateral to the tumor, MIAMI cells had migrated and were localized within the tumor mass. MIAMI cells were detected by red fluorescent labeling of the Y-chromosome. Nuclei were stained with DAPI. Scale bars = 200 μm for low and 50 μm for high magnifications.

Fig. 5: Analysis by flow cytometry of the proliferation and differentiation of MIAMI cells grown in U87MG TCM for seven days. Grey and white histograms show the level of the expression of isotype controls, CD31, NG2, GFAP, FSP, α -SMA and Ki-67 in MIAMI cells grown in TCM and in control medium respectively. No expression of CD31, NG2 and GFAP by MIAMI cells was observed in TCM and control medium. MIAMI cells expressed FSP, α -SMA and Ki-67 in control medium and this expression increased when they were grown in U87MG TCM. A representative experiment is shown.

Fig. 6: Analysis of the proliferation and differentiation of MIAMI cells in healthy (A-F) and U87MG tumor environments (G-N). MIAMI cells were detected by red fluorescent

labeling of the Y-chromosome, and markers (Ki-67, α -SMA, vimentin, GFAP, desmin, murine CD31 and human CD31) were detected by green fluorescent labeling with antibodies. Nuclei were stained with DAPI. No expression of Ki-67 by MIAMI cells was detected in the healthy environment (A). However, 20% of MIAMI cells expressed Ki-67 in the U87MG tumor environment. An example of a double-stained cell is shown in the magnified photo. (G). In the healthy environment and in the U87MG tumor environment, MIAMI cells expressed α -SMA (B, H) and vimentin (C, I), but did not express GFAP (D, J), desmin (E, K) or human CD31 (F, L). Note the localization of MIAMI cells in the glial scar (M) and close to mouse endothelial cells (N). The percentages of double-stained MIAMI cells are indicated on the respective panels. Scale bars = 100 μ m for low and 10 μ m for high magnifications.