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Modified Explant Culture is the Effective Technique for Isolating Mesenchymal Stem Cells from Glioblastoma

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ABSTRACT

Background and Aim: Until now, mesenchymal stem cells (MSCs) have been obtained from a variety of sources, including several tumor types. In order to acquire cells with the best quality and function while also taking economic factors into account, selecting an appropriate isolation procedure is a crucial step. The goal of this study was to use a modified explant culture approach to isolation of pure mesenchymal stem cells from human glioblastoma.

Methods: Six samples of glioblastoma tumors were cut into cube - like fragments of 1-3 mm in diameter (explants). The explants were put into the wells of a 24-well plate, cultured in Dulbecco's Modified Eagle's medium (DMEM), and kept at 37°C with 5% CO_2 in an incubator. Inverted microscopy was used to analyze the morphological phenotypes of the cells. Wells with uniform fibroblast-like cell morphology were considered positive and chosen for further expansion and identification.

Results: Totally, 38.46% of the wells were chosen for development after testing positive for all grown explants. According to flow cytometry analyses, the isolated cells were positive for CD73, CD105, and CD90 but negative for CD45 and CD34. The cells also successfully underwent multipotential differentiation into adipocytes and osteoblasts.

Conclusion: Overall, our research demonstrated that GA - MSC could successfully be isolated from human Glioblastoma tumor tissues utilizing a modified explant culture method. With this approach, we were able to rapidly and efficiently develop a more pure MSC population by choosing tumor pieces with the potential to produce a population of homogenous cells with fibroblast - like morphology and removing pieces whose heterogeneous cells were moved. The approach is also workable and affordable. The results might serve as a framework for more investigation into GA - MSCs, glioblastoma TME, and their mechanism of development.

INTRODUCTION

Probably the hardest malignancy in adults is glioblastoma multiforme (GBM) which is a grade IV brain tumor according to the WHO. Glioblastoma is more common among the oldest people and men are more likely to experience it than women (1). There are evidences that oligodendrocyte precursor cells, neural stem cells (NSCs), and astrocytes produced from NSCs are the sources of GBM (2). After surgical intervention, Glioblastoma is often treated with temozolomide - based chemoradiotherapy. Nearly all GBM tumors locally return following treatment, despite vigorous adjuvant therapy and maximum surgical resection (3). The partial removal represents a major genetic diversity, GBM's unique immunomodulatory microenvironment and blood-brain barrier present continuous treatment challenges (4). The tumor microenvironment (TME), which is formed of

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normal cells including mesenchymal stem cells (MSCs), cancer cells, and elements of the surrounding environment, is now widely recognized to have an important role in the evolution of tumors (5). Malignant cells interact with and stimulate nearby mesenchymal stromal cells, which causes the stromal cells to produce pro - inflammatory agents and factors that help the tumor to grow. The TME is created by stromal cell activation and partially resembles chronic inflammation (6, 7).

MSCs may be obtained from adult and fetal tissues and have significant proliferative potential, a particular CD - antigen profile, and multipotency (8). They can be isolated from blood, amniotic fluid, tooth pulp, bone marrow, and adipose tissue (7, 9, 10). Because of their availability, multilineage differentiation capability, and fairly simple and scalable preparation and native or altered MSCs are being carefully explored for medicinal purposes globally, such as adjuvant intervention for tumors and blood neoplasia (7, 11). Up to now, MSCs have been obtained from various tumor tissues. including stomach, colon, breast cancers, and glioblastoma (12). Researchers have used a variety of alternative methods for isolating MSC from human tissues. For example, enzymatic digestion is an expensive and time - consuming procedure that is a traditional approach for isolating tumor MSCs. Additionally, isolation - related mechanical stress reduces cell viability. This technique has a risk of endotoxin and xenoantigen contamination (12). Additionally, in this approach contamination of isolated MSCs by cancer cells is common. According to this, we used a unique explant culture approach in the current study as a quick and efficient method for separating the pure MSC population from human glioblastoma. Our results were shown that explant cultures is an appropriate way to gain MSCs from tumor tissues efficiently and purely. Explant procedures were also shown to be more economical than enzymatic cell separation approaches. Furthermore, there is no requirement to use pricey growth factors in the methods for cell growth and culture. This might have benefits for future studies on the glioblastoma microenvironment and glioblastoma - associated MSCs (GA-MSCs).

METHODS

Tumor specimens

Six Glioblastoma tumor samples were collected from patients (2 men and 4 women) who underwent surgery in Shohada'e Tajrish hospital. Patients had not received another treatment including chemo or radiotherapy before surgery. The age of five patients was between 55-65 years old and one patient was 30. The protocol of our study has been approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (SBMU) with IR.SBMU.MSP.REC.1398.875 ethical code, and every method was performed according to ethical committee approval. All patients who took part in the current investigation signed informed consent forms.

Explant culture

Tumor samples were delivered to the laboratory less than 30 minutes after surgery in cold sterile phosphate buffer saline (PBS). To separation of MSCs from tumor tissues, first, we washed the tumor sample with 2 ml of sterile PBS containing penicillin / streptomycin 1% 2 times to remove the blood. After that, we removed the necrotic and non - tumor tissues from the tumors. Then we cut the alive parts of each tumor into 1-3 mm cube - like pieces called explant tissue in a sterile Petri dish by scalpel and then placed each explant in a well of 24 well plates. Then we added 0.5 ml DMEM / F12 containing FBS 15% and penicillin/streptomycin 1% to each well how the specimens do not be floated. The plate was then incubated for 2 to 3 weeks at 37˚c with 5% CO_2. At first, after 3 days, and then after every 4-5 days, the medium was changed. The morphological phenotype of cells in wells was observed by an inverted microscope. Positive wells were those with a homogeneous cell population with a fibroblast - like appearance. Following removal of the explant tissue, the expanded cells were cultivated in a fresh medium for a further 4-6 days, resulting in the formation of a confluent monolayer (P0). Afterward, cells were harvested by trypsin - EDTA, and then monolayers were seeded onto T25 flasks (P1). Conversely, Negative wells were those that either contained no developing cells or a community of cells that were phenotypically mixed.

Flow cytometry surface marker analysis

Utilizing trypsin - EDTA, passage 3 cells were collected, centrifuged at 1500 rpm for five minutes, and then washed twice with PBS and prepared for flow cytometry examination. Anti - human antibodies were used to identify the cell phenotypes of the cell surface markers including CD73, CD105, CD90, CD45, and CD34. After a 40-45-min incubation at room temperature, we washed cells using PBS, three times. The BD FACS Calibur equipment was used to count about 10,000 events, which were then further analyzed using FlowJo Software.

Adipogenic differentiation

We seeded 5,000 fibroblast - like cells derived from explants in each well of 4-well plates then adipogenic differentiation was induced using a complete medium supplemented with 200 mM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10 mg / ml insulin, and 1 mM dexamethasone (all from Sigma - Aldrich, USA). Adipogenic differentiation was evaluated after 14 days by the lipid droplets staining by oil red O.

Osteogenic differentiation

After being harvested, the 5,000 cells were cultured in each well of 4-well plates in complete media supplemented by

dexamethasone 0.1 mM, b- glycerophosphate 10 mM, and ascorbic acid 0.2 mM (all from Sigma - Aldrich) at 37°C in the humid environment with 5% CO_2. Alizarin red S staining after 21 days allowed for the detection of mineralization (Sigma - Aldrich, USA)

RESULTS

Explant culture was used to extract MSC from six human glioblastoma cancer samples. Initially, tumors were fragmented into pieces between 1-3 mm in diameter (so - called explants). After that, the explants were incubated at 37°C with 5% CO_2 in the 24-well plates. After about 3 days, cells started to migrate from the borders of tumor explants. On days 3-5, spindle - shaped cells were initially detected in several wells. Colonies started to develop and cell densities started to rise after 5 days in various wells. However, cell densities and homogeneity in diverse explants were different when examined by the inverted microscope. Then explant tissues were collected from wells with uniform fibroblast - like morphology cells around two weeks later, and the cells were kept in cultivation for a total of 4-5 days so that they could continue to proliferate and reach confluence. The majority of the positive wells showed 80-100% cell confluency after about 3 weeks (P0). Eventually, the cells were passaged and transferred into T25 flasks following trypsinization (P1). We eventually enumerated 65 wells (explants) from all six tumor samples. 38.46% (25 wells) of the wells were confirmed to be positive and were selected for expansion, whereas 43.07% (28 wells) exhibited mixed cells, 15.38% (10 wells) had only tumor - like cells, and the remaining 3.07% (2 wells) wells contained non - growing cells (chart1). Explants' surrounding migrating cells initially exhibited short, fibroblast - like morphologies at P0 (Fig 1), but during passages 1 and 2, they formed longer bodies (long and spindle - shaped). The cells had typical mesenchymal morphologies and were arranged in a parallel configuration at high cell densities.

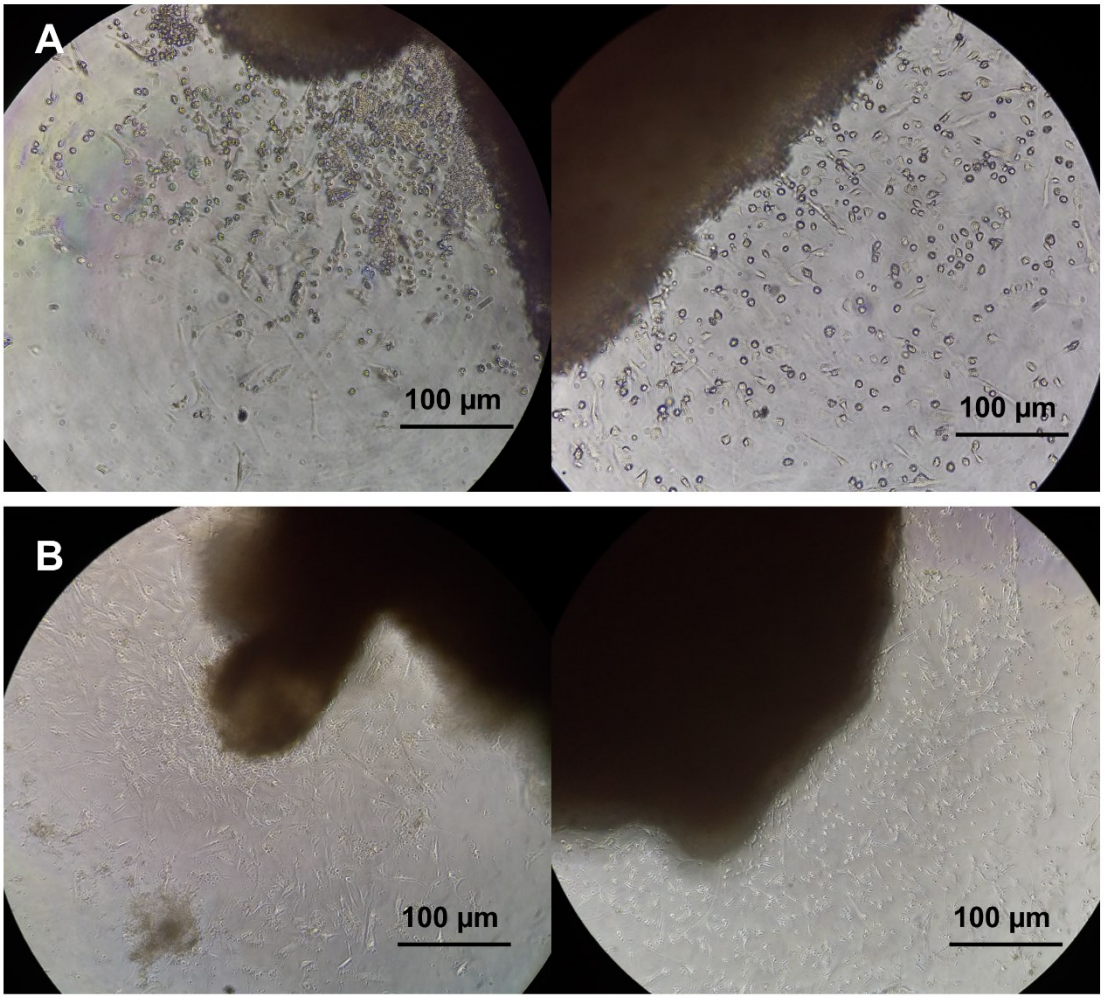

FIGURE 1. Migration of cells from tissues of glioblastoma explants.
A) Migration of fibroblast - like cells and cancer cells from two distinct explants (negative wells).
B) Fibroblast - like cells migration and their accumulation around the explants (positive wells). Original magnification ×40.

Antigen expression analysis of MSCs

Flow cytometry analysis had shown that antigen expression of cells derived from tumor explants is similar to typical MSCs (Fig 2). These cells were positive for CD73 (99.9%), CD90 (99.2%), and CD105 (99.7%) and negative for CD34 (98.8%), and CD45 (95.3%).

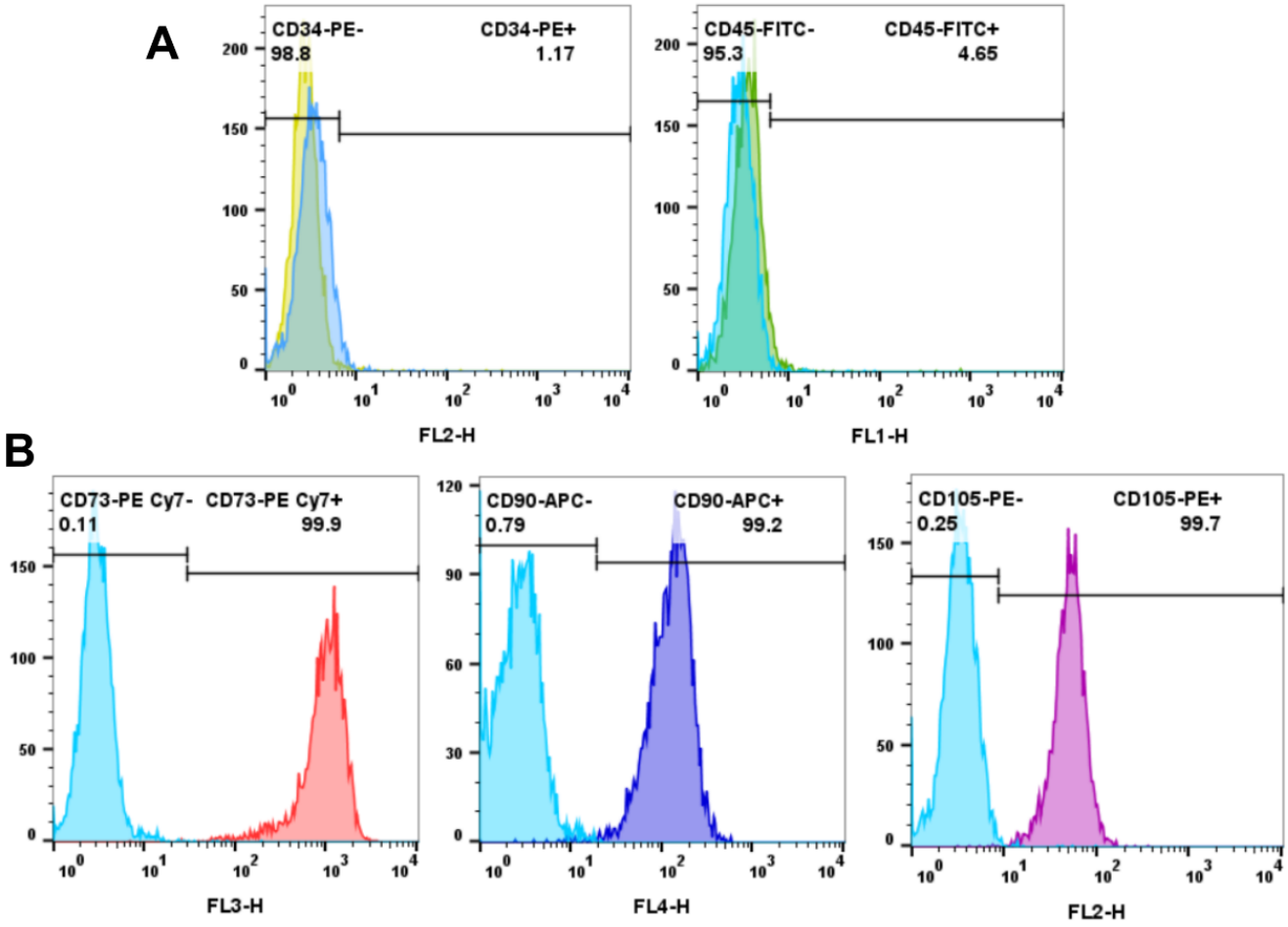

FIGURE 2. Detection of cell surface markers expressed by explants derived GA - MSC at P (3) by flow cytometric analysis. A) GA - MSCs were negative for, CD45 and CD34 and B) positive for, CD73, CD105 and CD90 and GA - MSCs: Glioblastoma associted Mesenchymal Stem Cells.

Adipogenic differentiation of MSCs

In the adipogenesis analyses, the morphological alterations began after induction and progressed. After a few days, lipid vacuoles started to accumulate in the cells (Fig 3). Their numbers continued to increase. On day 14, Oil Red O staining of lipid vacuoles revealed that the cells were adipocytes.

Osteogenic differentiation of MSCs

After the treatment, osteogenic activity was initially noticed on day 3, and mineral deposits were visible after 7 days. After 21 days of treatment, by Alizarin Red staining, large mineral deposits of Extracellular calcium (Ca2+) were visible (Fig 3). As negative controls for adipo and osteogenic differentiation assays, Primary fibroblast - like cells in wells containing DMEM / F12 did not exhibit any morphological changes, and no lipid vacuoles or Ca deposit was seen after staining.

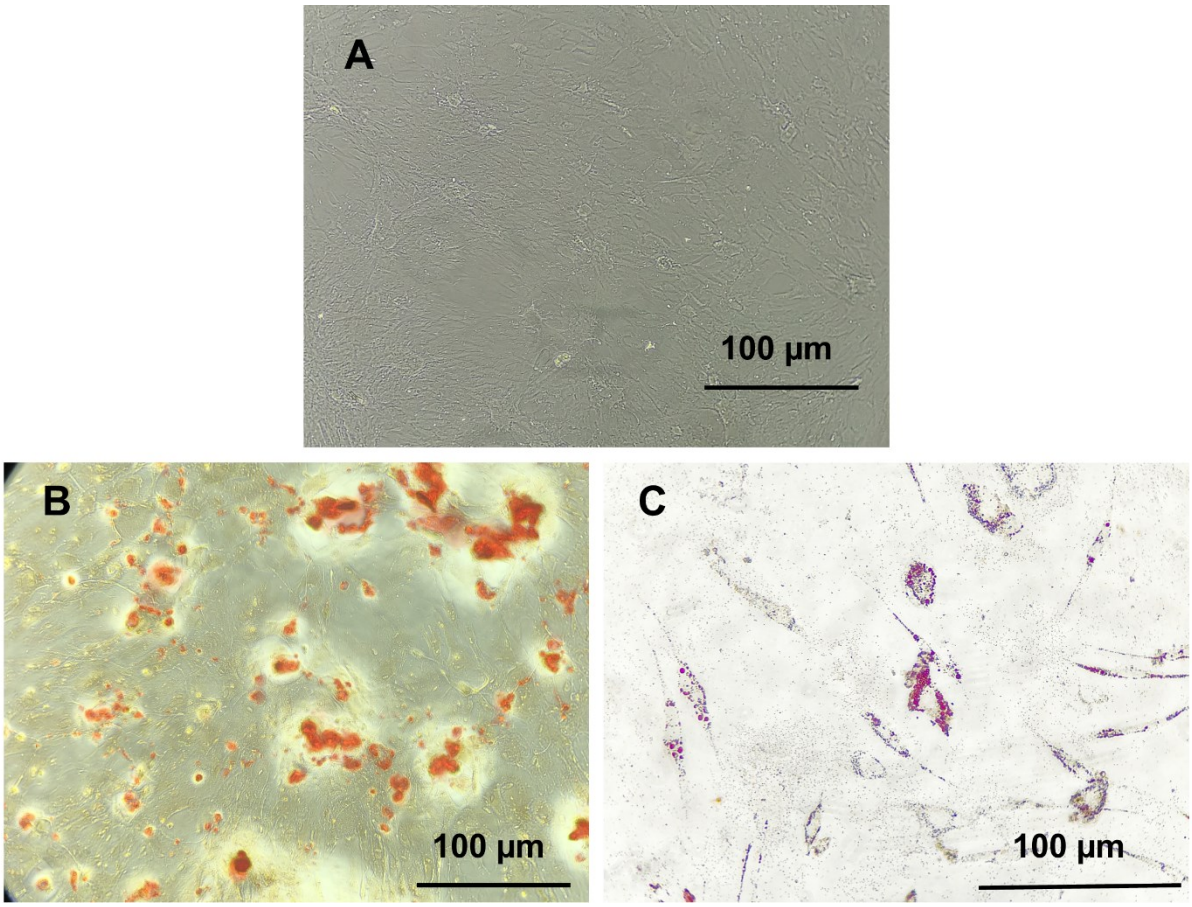

FIGURE 3. The ability of extracted GA - MSCs to differentiate into adipo and osteocytes.
A) GA - MSCs that migrated from explant tissues and had a spindle form. B) Alizarin red S staining was used to observe the differentiation into osteocytes following induction culture. B) Following induction, adipogenic differentiation was evaluated by Oil Red O staining. Original magnification ×40 GA - MSCs; Glioblastoma - associated mesenchymal stem cells.

Table 1. Positive wells derived from each cultivated tumor sample. The number of early pieces for each tumor samples is shown in second row. In every well placed just one tumor piece. In third row, the number of final wells just containing fibroblast like cells (positive wells) are separately shown for each tumor sample.

Tumor sample	1	2	3	4	5	6
All cultured wells	9	13	9	10	13	11
Positive wells	4	5	5	4	4	3

DISCUSSION

According to recent investigations, MSCs have been found in several types of human malignancies (13). It has been established that MSCs, as significant stromal cells in tumors, are crucial to the TME and regulate the growth and evolution of tumors (14). Primary culture of tumor tissue is one of the potential techniques for cell function studies engaged in cancer development (15). Ex vivo analysis of primary MSCs derived from patients represented the closest in vivo conditions, however, it is necessary to use the right technique to separate MSCs from other cells in tumor tissues (16). Enzymatic and mechanical digestion are the main components of traditional techniques to separate MSCs from human tumor tissue (17, 18). In enzymatic digesting processes, cells are subjected to three main stresses: detachment from the body, rapid separation from adjacent tissues, and enzymatic breakdown (12). Additionally, the lytic activity of the enzymes may reduce cell viability (19).

The explant approach, allows cells to be moved into culture condition from a piece of the original source tissue without being quickly severed from their associated tissue (20). Therefore, under such circumstances, the cells can dependably survive isolation stress while maintaining their features (21). Obtaining cells from tissue explants is

a traditional method that has recently been applied to achieve human MSCs from many body tissues such as adipose, dental pulp, umbilical cord, and recently tumor (22-24).

According to some studies, tissue fragments may be able to secrete growth factors and cytokines into the culture medium, stimulating the growth of cells and maintaining MSCs in their stemness state. This may explain why in the presence of the source tissue fragments MSC expansion and stemness are maintained during long-term in vitro culture (25).

To separate MSCs from primary glioblastoma tumor tissues without the requirement for enzymatic treatment, we have created a modified explant culture approach. We select small explant sizes (1-3 mm) to aid in the separation and emigration of MSCs from the explants into the culture medium. In the next stage, we placed each explant in one well on a 24-well plate and observed its activity independently due to the diverse ways that explants behaved when the various cell types were separated from anyone. After 15-17 days, the homogeneous population - containing wells were picked for future development, whereas the mixed - cell, cancer - cell, and cell - free wells were considered to be negative.

By using this enhanced technique, we were able to separate tumor pieces that had a more uniform fibroblast - like cell population that had moved from them. The tumor tissues, however, were collected from the wells containing a uniform fibroblast - like cell population after around 20 days (positive wells). In this manner, we prevented the eslishment of a heterogeneous cell population by preventing other cell types' migration from the explants into the wells. Because of challenges that are associated with traditional ways to separate MSC from the tumor, a tumor sample may be wasted in some cases without gaining MSC, but by our method, despite just around 38% of the explants being positive, we succeeded to isolate pure GA - MSCs from all of six tumor samples. According to our findings, mesenchymal stem cells were uniformly positive for the mesenchymal stem cell markers including CD73, CD90, and CD105, and were also negative for hematopoietic markers (CD45, CD34). However, it is necessary to evaluate classifying MSCs merely based on the expression of surface antigens, these cells also need to be capable of in vitro osteoblast and adipocyte differentiation. We employed the second passage from GA - MSCs for culture in the circumstances that promoted the osteogenic and adipogenic differentiation of MSCs to examine the differentiation capacity of GA - MSCs. The outcomes demonstrated that induced GA-MSCs were Alizarin Red and Oil Red O positive. We had also some limitations for doing this study. Some of the most important problems that we had first were the deficiency of the glioblastoma sample. We propose researchers to use more samples in similar studies to gain more reliable results. Another important challenge in our study was passaging of p0 cells. Because of a low number of early cells, this step needs to caution in using trypsin to detach cells.

CONCLUSION

Overall, our research demonstrated that GA - MSC could successfully be isolated from human Glioblastoma tumor tissues utilizing a modified explant culture method. With this approach, we were able to rapidly and efficiently develop a more pure MSC population by choosing tumor pieces with the potential to produce a population of homogenous cells with fibroblast-like morphology and removing pieces whose heterogeneous cells were moved. The approach is also workable and affordable. The results might serve as a framework for more investigation into GA - MSCs, glioblastoma TME, and their mechanism of development.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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