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The use of VEGF supplemented media for chondrogenic differentiation of adipose derived mesenchymal stem cells

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ABSTRACT

Aim : to verify the potential of VEGF supplemented DMEM-HG in inducing chondrogenic differentiation of adipose derived MSCs

Experimental : Lipoaspirate derived cells were cultured in 10% human AB serum containing DMEM-HG that was supplemented by VEGF. The culture was observed daily, and when the cells attached on the culture vessel, the medium was changed, and further medium changes were done every 2-3 days. Cell growth was observed, and cell characteristics were identified morphologically with and without staining. After the primary culture (P0) was 70% confluent, the cells were detached and passaged. Passages were done until passage 5. For every passage, seeding number, the day of the first appearance of clones and micromasses and results of alcian blue staining for every passage were noted. Mean and standard deviation values of the day when the cells formed the first clone and when the first micromass appeared were computed.

Result : VEGF supplemented human AB serum containing DMEM-HG yielded first clones on day 4.29 ± 2.05, and first micromass on day 7.69 ± 2.62, which was faster compared to the recently commercially available chondrocyte differentiation medium.

Conclusion : the VEGF supplemented medium can be used as an alternative simple induction media for faster chondrocyte differentiation.

KEYWORDS

VEGF;
Chondrogenic differentiation;
MSC;
Micromass;
Alcian blue.

INTRODUCTION

Lipoaspirate-derived plastic adherent cells were shown to be mesenchymal stem cells (MSCs), which are similar to bone marrow derived MSCs, in term of their morphology, plastic adherent property, and differentiation capacity. However, processed lipoaspirate, and primary culture of lipoaspirate-derived MSCs bear

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CD34, although the $CD34^+$ cell number decreased and finally almost disappeared after several passages[1]. Stem cells that are enriched in CD34 are widely used in regenerative medicine, especially in myocardial infarction to promote angiogenesis[2]. CD34 bearing stem cells are hematopoeitic and endothelial progenitors, and vascular endothelial growth factor (VEGF) containing medium is used to culture and maintain the endothelial lineage cells[3].

Our preliminary study showed that processed lipoaspirate from a patient that was cultured in VEGF supplemented Dulbecco's modified Eagle medium-high glucose (DMEM-HG) formed clones that grew into dome-like structures (micromass), which were stained blue by alcian blue staining[4]. Alcian blue is commonly used to stain cartilage matrix.

Therefore, the aim of this study was to replicate our previous result and to ensure the possible use of VEGF supplemented DMEM-HG in chondrogenic differentiation of lipoaspirate-derived MSCs.

EXPERIMENTAL

This experimental descriptive study was done in the Integrated Laboratory, Faculty of Medicine Universitas Indonesia, from July to December 2012, and has got an approval from the ethical committee of the Faculty of Medicine Universitas Indonesia. Five lipoaspirate samples were obtained by tumescent liposuction from a private clinic in Jakarta, after the patients signed the informed consent form. Before processing, the lipoaspirates were kept in a sterile transport-medium containing bottle at 4°C for no more than 24 hours.

Lipoaspirate processing

Lipoaspirate was filtered and extensively washed in phosphate buffered saline pH 7.4 (Sigma P3813) as described previously. The lipoaspirate was then digested by 0.075% collagenase type I (Gibco 17100-017) at 37°C for one hour, with agitation every 5 minutes. The floating free lipids were discarded, and the infranatant was filtered through a 100 μm mesh filter, and centrifuged at 800g for 10 minutes[5]. The pellet was resuspended and cultured in 10% human AB serum (Gibco 34005-100), penicillin/streptomycin (Gibco 15140-122), and amphotericin B (JR Scientific 50701) containing DMEM-HG (Lonza 15-604D) that was supplemented by VEGF (Invitrogen PHC9394). However, if the pellet appeared red, it was subjected to erythrocyte's lysis buffer for 15 minutes, recentrifuged, resuspended, and cultured.

Culture, passage, staining, and observation

The cultures were done in 12-well plates, and seeding number was variable, depended on the availability of viable cells. For primary cultures 170,000-275,000 viable cells, and for passages 7,000 – 25,000 viable cells were seeded. The culture was observed every day, and when the cells attached on the culture vessel, the medium was changed, and further medium changes were done every 2-3 days. Cell growth was observed under an inverted microscope, and cell characteristics were identified morphologically without staining, and some by haematoxylin eosin and alcian blue staining.

After the primary culture (P0) was 70% confluent, the cells were detached and passaged (subcultured). Passages (subcultures) were done until passage 5. All cultures were done *in duplo*. One culture was used to be passaged, and the other was used to observe clone and micromass formation. However, when the passaged culture got contaminated, the culture that was intended for observation was harvested and passaged.

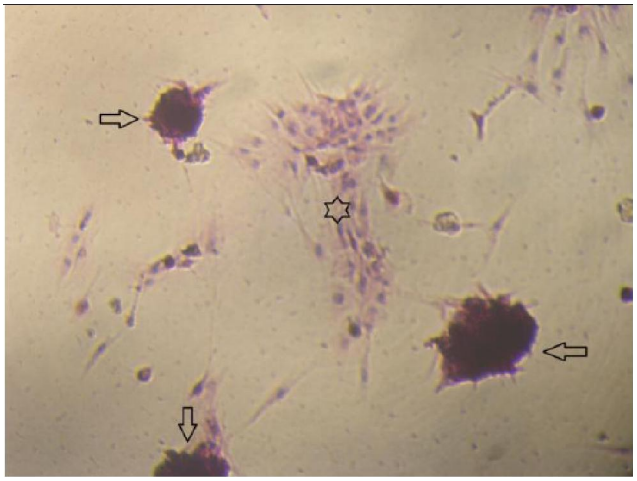
Data collection and analysis

For every passage, seeding number, the day when the cells formed the first clone, the day when the first micromass appeared, and results of alcian blue staining was noted and tabulated. The mean and standard deviation values of available data of the day when the cells formed the first clone and when the first micromass appeared were computed.

RESULTS

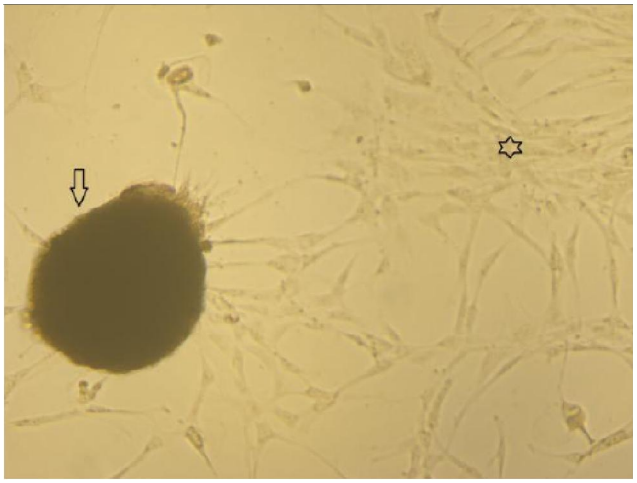
One of the five samples did not grow on primary culture, and for passage-1 of that sample, the cells were collected from a primary culture in MesenCult (from another study). Further, one sample was contaminated at passage-5 (both passages).

Four other samples grew well, and interconnecting clones (Figure 1A) appeared at primary cultures of the four samples, and also at subsequent passages. After



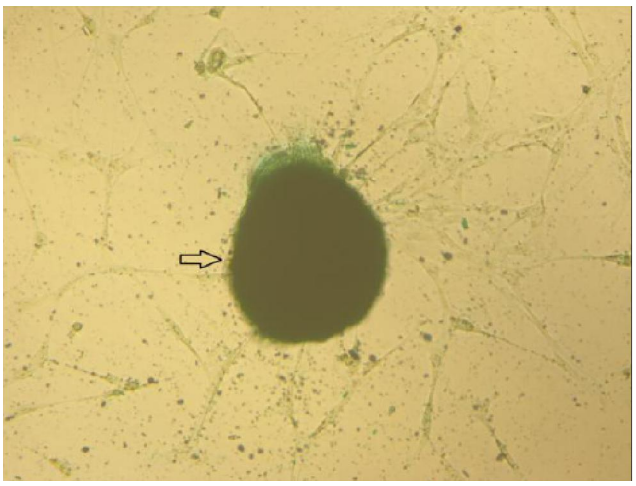
Star= two interconnecting clones, arrow= micromass

Figure 1 : Passage-1, day-4, haematoxylin and eosin staining (100x)



Star= large clone, arrow= large micromass

Figure 2(a) : Primary culture, day-21, unstained (100x)



Arrow= large micromass

Figure 2(b) : Primary culture, day-21, alcian blue staining (100x)

subculture, the passages did not show evenly spreading monolayer growth, but formed clones like in primary cultures. Clones appeared faster when seeding was uneven, which caused a lot of cells gathered in a certain place in the well.

Some clones became thicker and formed dome-like structures that were covered by matrix (micromass) (Figure 1, 2a, and 2b), such as happened in chondrocyte differentiation medium. When the chondrocyte-like cells were passaged, some of the cells did not attached,

TABLE 1 : Chondrocyte differentiation stages

Sample-passage number	Seeding number	Presence of clones on day	Presence of micromasses on day
S1-PC	171,200	4	7
S1-P1	24,700	Unnoticed	3
S1-P2	16,800	Unnoticed	5
S1-P3	7,200	1	9
S1-P4	13,200	2	2
S1-P5	18,000	8	15
S2-PC	176,400	6	Harvested day-6
S2-P1	24,000	4	6
S2-P2	21,280	Unnoticed	7
S2-P3	13,200	4	10
S2-P4	20,800	4	8
S2-P5	11,200	Unnoticed	8
S3-PC	270,400	Unnoticed	8
S3-P1	23,600	2	9
S3-P2	21,600	Unnoticed	6
S3-P3	12,250	7	9
S3-P4	22,000	2	9
S3-P5	22,800	Contaminated day-3	
S4-PC	209,600	5	7
S4-P1	20,800	Unnoticed	9
S4-P2	9,600	4	10
S4-P3	17,500	4	8
S4-P4	19,800	8	9
S4-P5	19,800	2	7
S5-PC	240,000	Did not grow	
S5-P1	20,400	3	4
S5-P2	22,400	3	8
S5-P3	9,600	7	Harvested day-17
S5-P4	20,400	6	11
S5-P5	20,280	4	6
Mean ± SD		4.29 ± 2.05	7.69 ± 2.62

S= sample, PC= primary culture, P= passage, SD= standard deviation

but the attached cells were still able to grow and form micromasses even until passage 5.

All samples, which grew and did not get contaminated, formed micromasses, except for two cases; one was harvested before the micromass was formed (primary culture on day 6 due to contamination of the culture that was intended to be passaged), and the other was passage-3 that was harvested at day 17, which was regarded as unable to form micromass TABLE 1. The individual values, means, and standard deviations of the seeding number, the day when the cells initially formed clones, the day when micromass appeared for the 5 samples can be seen in TABLE 1. All micromasses appeared blue on alcian blue staining.

DISCUSSION

Alcian blue staining is a special staining for sulphated glycosaminoglycans, such as keratan sulphate that was abundant in cartilage matrix, and will not stain mesenchymal stem cell matrix[6]. As the micromasses were stained blue with Alcian blue staining, the cells in the micromasses were shown to produce cartilage matrix. Thus, they must be chondrocyte lineage cells.

However, it is not known, whether the VEGF containing medium caused differentiation of lipoaspirate derived mesenchymal stem cells into chondrocyte-like cells or benefited the chondrocyte progenitors that might be present in the lipoaspirate, as lipoaspirate contains myriads of stem cells in different stages of differentiation[7]. Therefore, further detailed study in this topic should be performed.

The property of VEGF containing medium to induce chondrogenic differentiation may be useful in cartilage engineering for patients who need cartilage replacement due to cartilage damage, such as in osteoarthritis. In addition, it can be used for cosmetic purposes, to engineer cartilage structures to enhance patient's appearance. However, before using VEGF supplemented medium to provide chondrogenic cells for patient's purposes, further research to validate this method is needed.

In using MSCs for regenerative medicine, the MSCs should be characterized, including testing their differentiation capacity. Capacity to differentiate into cartilage cells is usually tested by micromass culture in chondrocyte differentiation medium[8], which contains various substances, which causes media preparation to be cumbersome. Further, the micromass needs around 21 days to grow, and it should be either frozen[9] or histologically processed[8], followed by cutting by a cryo or ordinary microtome, and finally stained[8,9]. This method is laborious, and needs a long time to prove the differentiation capacity.

To date, chondrogenic differentiation medium is commercially available either for micromass culture in a conical tube for histological staining[9], or in a multi-well plate for direct staining[10,11]. The time needed to grow the micromass[10] or cartilage spheroids[11] is more than 14 or 21 days, respectively[10,11].

In our study, the micromasses developed between day-3 and -15 (7.69 ± 2.62). Therefore, using VEGF supplemented human AB serum containing DMEM-HG is a faster alternative to the recently commercially available chondrocyte differentiation medium to develop the micromass. The time needed to develop the micromass can be reduced, if the seeding is done unevenly (by making spots of concentrated cells). Further, when it is combined with direct staining of the micromass on the culture vessel, such as was done in our study, it is a faster and simpler method compared to the histological staining method.

CONCLUSION

VEGF-supplemented human AB serum containing DMEM-HG can be used as an alternative simple induction media for faster chondrocyte differentiation. Further detailed study to elucidate whether the VEGF containing medium induces differentiation of lipoaspirate derived mesenchymal stem cells into chondrocyte-like cells or benefits the chondrocyte progenitors in the lipoaspirate should be performed.

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