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Flow Cytometry Analysis Of Adipose Tissue-Derived Stem Cells That Were Cultured In Various Media

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Abstract: Adipose tissue derived stem cells (ASCs) are very promising for regenerative medicine. However, before the stem cells can be used to treat patients, they need to be propagated in animal serum-free materials and should be characterized. Therefore, to know the characteristics of ASCs that were grown in various animal serum-free medium, we cultured the ASCs in various media, i.e. 5% or 10% human AB platelet rich plasma (PRP), or combination of vascular endothelial growth factor (VEGF) and human AB serum containing Dulbecco's modified Eagle medium (DMEM), and conducted flow cytometry analysis; then, we compared them with those that were grown in the fetal bovine serum (FBS)-derivate containing commercial medium (MesenCult®). Our results showed that the grown cells in the four kinds of medium had different proportions of CD34, CD73 and CD90 positive cells. Cells that were grown in 5% and 10% PRP containing media were comparable to those that were grown in MesenCult®, and could be regarded as adipose tissue-derived mesenchymal stem cells (AT-MSCs), while VEGF and human AB serum containing medium might preserve and promote the CD34 positive cells, but at the same time differentiated a proportion of AT-MSCs into chondrogenic cells. In conclusion, flow cytometric analysis showed that different media caused different proprortions of surface marker expressions in AT-MSCs

Key words: stem cells, regenerative medicine, PRP, VEGF, human AB serum, CD34, CD73, CD90, flow cytometry.

Introduction

Adipose tissue-derived stem cells (ASCs) are very promising for regenerative medicine, due to the easier sampling of adipose tissue and fewer side effects that occurred after sampling compared to bone marrow sampling. Moreover, ASCs were shown to be mesenchymal stem cells (MSCs), which have similar characteristics with bone marrow-derived MSCs.[1] Another advantage of ASCs is the fact that adipose tissue can be obtained from clinics, which perform liposuction to reduce obesity.

However, before ASCs can be used to treat patients, they should be characterized.[2] One of the various characterization procedures is the analysis of their surface markers using flow cytometry. Further, to be used in patients, a lot of stem cells are needed. Therefore, the stem cells should be propagated by *in vitro* culture. To avoid serious adverse effect due to rejection, the culture condition should avoid the use of animal-derived materials, as they contained xenoproteins that can be incorporated into the cells. These xenoproteins are difficult to be completely eliminated.[3]

Therefore, in this study, we cultured the ASCs in various media, i.e. various xenofree supplements containing Dulbecco's modified Eagle medium (DMEM) and conducted flow cytometry analysis on them, and compared the results to those that were cultured in fetal bovine serum (FBS)-derivate containing commercial medium.

Materials And Methods

This experimental descriptive study was conducted in the Integrated Laboratory and Department of Clinical Pathology of Faculty of Medicine Universitas Indonesia, from July through December 2012. The ethical approval was got from the Ethical Committee of the Faculty of Medicine Universitas Indonesia.

Materials

Four lipoaspirate samples (S1-4) were obtained by tumescent liposuction from Espour skin care clinic in Jakarta, after the patients signed the informed consent form. Materials that were used in isolation and culture were: phosphate buffered saline pH 7.4 (PBS, Sigma P3813), collagenase type I (Gibco 17100-017), human AB serum (Gibco 34005-100), penicillin/streptomycin (Gibco 15140-122), amphotericin B (JR Scientific 50701), DMEM (Lonza 15-604D), VEGF (Invitrogen PHC9394), MesenCult® (STEMCELL Technologies), and TrypLE Select (GIBCO 12563-011). Further, materials used for flow cytometry analyses were monoclonal antibodies against surface antigens, i.e. CD34 antibody linked to perCP (BD 340430), CD73 antibody linked to PE (BD 550257), and CD90 antibody linked to APC (BD 559869), and their respective isotypes i.e. perCP (BD 559425), PE (BD 555749), and APC (BD 555751) linked isotype.

Isolation and culture of stem cells

Before isolation, the lipoaspirate samples were extensively washed in PBS, using a coffee filter and porcelaine bowls. Further, the adipose tissue was subjected to collagenase type I treatment, processed, and cultured as described previously,[4] in various kinds of media, i.e. 5% or 10% human AB platelet rich plasma (PRP), or combination of vascular endothelial growth factor (VEGF, final 10 ng/ml) and 10% human AB serum containing Dulbecco's modified Eagle medium (DMEM) compared to fetal bovine serum (FBS)-derivate containing commercial medium (MesenCult). All media were supplemented with penicillin/streptomycin and amphotericin B to prevent bacterial and fungi contamination.

Cultures were done for primary cultures (P0, with and without fibronection coating), and passage-1 (P1) until passage-5 (P5). Before passages, the cells were detached using TrypLE Select, when the cultures had become confluence. Cell seeding was done as described previously.[5]

Flow cytometry analysis

Flow cytometry analyses of harvested cells from the various media were done for P0-P5, when available, on a BD FACSCalibur flow cytometer (BD Biosciences), simultaneously for CD34, CD73, and CD90, using 10,000 gated events.

Data collection and analysis

Flow cytometry data of the cells, which were cultured in the various media for P0-P5, were analyzed to get the percentage of CD34, CD 73, and CD90 that were present on the cell suface. Individual data of P0 with and without fibronectin from two samples that were cultured in the various media were tabulated. Further, available data of P1-P5 from all samples that were cultured in various media were tabulated, and the means and standard deviations (SDs) of the values were computed, and tabulated.

Table 1. The percentage of CD34, CD 73, and CD90 of primary cultures in various media with and without fibronectin

Medium	Sample	Culture duration	Fibronectin coating	Percentage		
				CD34	CD73	CD90
MesenCult	No.2	5 days	-	58.57	36.81	80.48
	No.2	12 days	-	41.27	32.48	83.58
	No.3	6 days	-	60.00	35.84	93.75
	No.3	6 days	+	29.03	19.17	63.90
DMEM-PRP 10%	No.2	12 days	-	10.62	9.82	91.71
	No.3	6 days	-	56.06	32.30	89.62
	No.3	6 days	+	49.11	30.21	85.82
DMEM-PRP 5%	No.2	12 days	-	12.05	21.78	90.54
	No.3	6 days	-	63.12	29.91	84.71
	No.3	6 days	+	38.26	24.68	78.86
DMEM-huAB-VEGF	No.3	6 days	+	62.46	38.39	92.28

Results And Discussion

The results of flow cytometry analysis of P0 with and without fibronectin in various media can be seen in Table 1. The means and SDs of the percentage of the various surface markers in various media for P1-P5 can be seen in Table 2.

International Society for Cellular Therapy (ISCT) has set a standard for the surface markers of bone marrow mesenchymal stem cells (BM-MSCs), i.e. CD34 should be negative or less than 2%, while CD73 and CD90 should be more than 95%.[6] However, there is no consensus for adipose tissue-derived mesenchymal stem cells (AT-MSCs). AT-MSCs are similar to BM-MSCs in term of their morphology and characteristics that are adherent to plastics, but a little bit different in term of their surface markers, as primary cultures of AT-MSCs bear more CD34,[7, 8] and less CD 73 and CD90 compared to BM-MSCs.[8] Further, upon passage, CD34 tended to decrease, while CD 73 and CD90 tended to increase.[8]

Table 1 shows that overall, primary cultures in 5% and 10% PRP containing media, and MesenCult® have higher number of CD34, CD73 and CD90 positive cells in fibronectin non-coated compared to coated plates, when harvested on the same day, and the number of CD34 positive cells declined when the culture was prolonged. Our results in fibronectin non-coated plates showed similar results to Lin et al, and Mitchel et al,[7, 8] thus, the cells that were grown in 5% and 10% PRP containing media, and MesenCult® in this study can be regarded as AT-MSCs.

Primary culture in VEGF and human AB serum containing medium yielded the highest number of CD34, CD73 and CD90 positive cells even in coated plates (Table 1). For primary culture in VEGF and human AB serum containing medium, we did not get the data for fibronectin non-coated plates, thus we could not compare the results; this fact is the limitation of our study. Further, in prolonged primary culture (Table 1) and on passages until passage 5, the CD34 positive cells tended to be preserved compared to those that were cultured in 5% and 10% PRP containing media (Table 2). CD34 cells may represent endothelial progenitor, and VEGF was known to be able to differentiate CD133 cells, which are more primitive stem cells into CD34 bearing endothelial

cells.[9] In this study, VEGF final concentration (10 ng/ml) was lower compared to that used by Gehling et al (50 ng/ml).[9] Therefore, our result suggests the preservation of CD34 positive cells, which constitute a certain proportion in AT-MSCs.[7, 8] However, primary culture and subsequent passages in VEGF and human AB serum containing medium showed that parts of the cells underwent clonal cell growth, which developed into micromasses. These micromasses were stained blue on alcian blue staining, which implied that the cells were chondrogenic cells.[5]

Table 2. The percentage of CD34, CD 73, and CD90 of P1-P5 in various media

Medium-passage	Sample number (n)	Culture duration (days)	CD34 percentage		CD73 percentage		CD90 percentage	
			Mean	SD	Mean	SD	Mean	SD
MesenCult-P1	S2-3 (2)	7-9	24.67	7.53	63.28	1.93	86.74	7.95
MesenCult-P2	S1-2-3 (3)	9-12	10.83	6.95	62.40	5.20	90.49	2.29
MesenCult-P3	S2-3(2)	9	23.63	22.92	59.08	14.78	92.21	1.24
MesenCult-P4	S1-2(2)	7	13.34	12.97	55.74	5.15	92.24	4.17
DMEM-PRP 10%-P1	S2-3-4(5)	4-9	35.64	27.81	63.18	9.81	95.85	0.58
DMEM-PRP 10%-P2	S1-2-3-4 (6)	7-14	26.95	27.22	58.89	8.53	89.35	5.82
DMEM-PRP 10%-P3	S1-2-3 (5)	7-9	22.28	25.08	60.41	14.12	96.64	1.33
DMEM-PRP 10%-P4	S1-2-3-4 (5)	4-9	24.47	25.63	57.06	12.15	87.25	5.04
DMEM-PRP 10%-P5	S1-2(3)	5-10	4.18	4.82	46.48	8.86	92.01	5.45
DMEM-PRP5%-P1	S2-3-4 (5)	4-9	29.94	28.85	55.67	12.42	92.77	4.18
DMEM-PRP5%-P2	S1-2-3-4 (6)	7-14	27.22	30.43	43.53	21.70	76.49	26.15
DMEM-PRP5%-P3	S1-2-3 (5)	7-11	18.28	16.41	54.78	18.18	84.94	14.67
DMEM-PRP5%-P4	S1-2-3-4 (5)	4-9	19.92	12.02	60.39	14.13	83.81	8.09
DMEM-PRP5%-P5	S2 (2)	5-7	0.40	0.00	81.92	5.59	81.81	5.63
DMEM-huAB-VEGF-P1	S2-3(2)	7-15	49.42	3.95	53.32	2.17	91.85	5.93
DMEM-huAB-VEGF-P2	S1-2-3 (3)	9-19	26.20	16.68	56.01	4.89	92.20	2.20
DMEM-huAB-VEGF-P3	S2-3 (2)	8-14	29.09	27.85	51.51	17.47	94.94	2.11
DMEM-huAB-VEGF-P4	S1(1)	15	10.54	-	35.82	-	95.77	-
DMEM-huAB-VEGF-P5	S1(2)	8-14	10.04	2.23	28.46	6.78	93.80	1.53

Prolonged primary cultures (until 12 days) in 5% and 10% PRP containing media, and MesenCult® tended to decrease CD34 and CD73, but increase CD90 positive cells (Table 1). A study showed that in growth medium, bovine BM-MSCs tended to differentiate spontaneously into chondrogenic cells in prolonged condensate cultures.[10] It was not clear which event caused the decrease in CD34 and CD73, and increase in CD90 positive cells, but we supposed that AT-ASCs might undergo spontaneous differentiation in prolonged culture. Nevertheless, the actual causal event needs to be further elucidated.

Passages until P4 or P5 showed that CD34 positive cells were gradually reduced in 5% and 10% PRP containing media, and these results were in line with the result of Mitchel et al and Astori et al, which showed that upon passages, AT-MSCs became more similar to BM-MSCs, in term of the CD34 positive cells. [8, 11] However, in MesenCult®, CD34 in passage 4 was still relatively high (13.34 $\pm$ 12.97, [Table 2]), which was unexpected, as MesenCult® contains FBS derivate that is supposed to be similar with the media in the studies of Mitchel et al and Astori et al.[8, 11]

Further, passages in MesenCult®, 5% and 10% PRP containing media caused an increase in CD73 and CD90 positive cells compared to primary cultures (Table 1, Table 2), and in MesenCult®, CD90 positive cells tended to increase upon further passages, which is in line with the findings of Mitchel et al and Astori et al. [8,11] However, in 5% and 10% PRP containing media, CD90 positive cells showed fluctuation in percentage, though they remained relatively high. Further, in MesenCult®, and 10% PRP containing medium, CD73 positive cells tended to decrease with further passages, while 5% PRP containing medium showed fluctuation in percentage of CD73 positive cells (Table 2).

Therefore, our study showed that different media caused different proportions in surface marker expressions. The cells that were grown in 5% and 10% PRP containing media, and MesenCult® can be regarded as AT-MSCs, as AT-MSCs contains myriads of stem cells at different stages of differentiation,[1] while VEGF and human AB serum containing medium might preserve and promote the CD34 stem cells in AT-MSCs, but at the same time differentiate a proportion of AT-MSCs into chondrogenic cells.

Conclusion

Flow cytometric analysis showed that different media caused different proprortions of surface marker expressions in AT-MSCs.

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References

1 Pawitan JA. Prospect of adipose tissue derived stem cells in regenerative medicine. Cell &tissue transplantation &therapy 2009:2;7-9.

2 Placzek MR, Chung IM, Macedo HM, Ismail S, Blanco TM, Lim M, Cha JM, Fauzi I, Kang Y, Yeo DCL, Ma CYJ, Polak JM, Panoskaltsis PN, Mantalaris A. Stem cell bioprocessing: fundamentals and principles. J R Soc Interface. 2009;6: 209–232.

3 Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ, Hsu SC, Smith J, Prockop DJ. Internalized antigens must be removed to prepare hypoimmunogenic

mesenchymal stem cells for cell and gene therapy. Mol Ther. 2004;9:747–756.

4 Pawitan JA, Liem IK, Suryani D, Bustami A, Purwoko RY. Simple lipoaspirate washing using a coffee filter. Asian biomedicine 2013; 7(3): in press.

5 Pawitan JA, Suryani D, Lilianty J, Purwoko RY, Liem IK. The use of VEGF supplemented media for chondrogenic differentiation of adipose derived mesenchymal stem cells. BioTechnology: An Indian Journal 2013; 7(5):169-173.

6 Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ, Horwitz EM. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8(4): 315-317.

7 Lin G, Garcia M, Ning H, Banie L, Guo YL, Lue TF, Lin CS. Defining stem and progenitor cells within adipose tissue. Stem cells and development 2008;17:1053–1064

8 Mitchel JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Halvorsen YD, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM. Immunophenotype of Human Adipose-Derived Cells: Temporal Changes in Stromal-Associated and Stem Cell–Associated Markers. Stem Cells 2006;24:376–385.

9 Gehling UM, Ergün S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schäfer B, Hossfeld DK, Fiedler W. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000 ;95(10):3106-3112

10 Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumur M, Fujinag T.Gene expression profile of bovine bone marrow mesenchymal stem cell during spontaneous chondrogenic differentiation in pellet culture system. Jpn J Vet Res. 2006;53(3-4):127-139.

11 Astori G, Vignati F, Bardelli S, Tubio M, Gola M, Albertini V, Bambi F, Scali G, Castelli D, Rasini V, Soldati G, Moccetti T."In vitro" and multicolor phenotypic characterization of cellsubpopulations identified in fresh human adipose tissue stromal vascular fraction and in the derived mesenchymal stem cells. Journal of Translational Medicine 2007; 5:55.

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