OnLine Journal of Biological Sciences

Original Research Paper

Comparison of Flowcytometric and Immunocytochemistry Analysis of Stem Cell Surface Markers

[1,2]Jeanne Adiwinata Pawitan, [3]Dewi Wulandari, [4]Des Suryani, [1]Lia Damayanti and [2,5] Isabella Kurnia Liem

[1]Departement of Histology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba 6, Jakarta, Indonesia
[2]Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital-Faculty of Medicine Universitas Indonesia, CMU 2 Building, 5th floor, Jl. Diponegoro 71, Jakarta, Indonesia
[3]Department of Clinical Pathology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba 6, Jakarta, Indonesia
[4]Biomedical Science Master Program, Faculty of Medicine, Universitas Indonesia, Jl. Salemba 6, Jakarta, Indonesia
[5]Department of Anatomy, Faculty of Medicine, Universitas Indonesia, Jl. Salemba 6, Jakarta, Indonesia

Article history
Received: 28-12-2014
Revised: 28-01-2015
Accepted: 12-02-2015

Corresponding Author:
Jeanne Adiwinata Pawitan
Departement of Histology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba 6, Jakarta, Indonesia
Email: jeanneadiwip@gmail.com

Abstract: Flowcytometric analysis is widely used in characterization of stem cells. In areas where flowcytometry is not available, characterization of stem cells can be done using immunocytochemistry. This study aimed to compare the results of flowcytometry against immunocytochemistry for the measurement of CD34, CD73 and CD90 in stem cells. Adipose tissue derived stem cells were analyzed for their CD34, D73 and CD90 simultaneously, using a BD FACSCalibur flowcytometer (BD Biosciences) with 10,000 gated events. The same sample was made into spot specimens and checked for CD34, CD73 and CD90 by immunocytochemistry and when available, 100 cells were screened for positive results and percentages of positive results for CD34, CD73 and CD90 were computed. The results of the two methods were compared. Discrepancies between the two methods were noted for all samples. Discrepancies lower than 3, 11 and 11% for CD34, CD73 and CD90 respectively were regarded as agreement between the two methods. Percentages of agreement in CD34, CD73 and CD90 were calculated. Some spot specimens did not yield 100 cells and analyses were done on available cells. The agreement between the two methods for CD34, CD73 and CD90 were 22, 42 and 29% respectively. Agreement between flowcytometry and immunocytochemistry was low for CD34 and CD90 and was moderate for CD73.

Keywords: Flowcytometry, Immunocytochemistry, CD34, CD73, CD90

Introduction

Stem cells are very promising for regenerative medicine, but before the cells can be used to treat patients, they need to be characterized by analyzing their surface markers. Flowcytometry analysis is widely used in characterization of stem cells. Flowcytometry analysis can be done using various numbers of cells. In many instances, 10^5 (Oedayrajsingh-Varma *et al*., 2006; Choudhery *et al*., 2014; Li *et al*., 2013), or 5×10^5 (Mitchel *et al*., 2006), cells were incubated with a fluorophore-conjugated antibody against a certain surface marker. In our study, we used 10,000 cells and 10,000 gated events to simultaneously analyze the percentage of CD90, CD73 and CD34 in Adipose Tissue-derived Mesenchymal Stem Cells (AT-MSCs) (Pawitan *et al*., 2013a).

In some tissue culture labs, flowcytometer is not available. Therefore, in areas where a flowcytometry device is not available, characterization of stem cells can be done using immunocytochemistry, which requires less sophisticated equipments. We have developed a simple spot method that was suitable for immunocytochemistry staining (Pawitan *et al*., 2010), which can be used in stem cells surface marker enumeration. For surface marker enumeration, at least 100 cells should be analyzed and the percentage of positive cells can be calculated. Therefore, to make a simple spot specimen, theoretically less cells are required, though when available we usually use 20,000 cells to make several spots, to be stained by antibodies against various surface markers.

However, there were no data concerning the agreement of stem cell surface marker analysis between

flowcytometry and immunocytochemistry staining. Therefore, in this study we compared the results of flowcytometry against immunocytochemistry for the measurement of CD34, CD73 and CD90 in stem cells.

Materials and Methods

This is an experimental descriptive study, which was approved by the Ethical Committee of the Faculty of Medicine Universitas Indonesia (no. 136/PT 02.FK/ETIK/2012) and was done from September 2012 through May 2013.

Isolation of adipose derived stem cells and cell cultures were conducted in the Integrated Laboratory of the Faculty of Medicine Universitas Indonesia as previously described (Pawitan *et al*., 2013b; Suryani *et al*., 2013). Spot specimens was made in the Department of Histology, as previously described (Pawitan *et al*., 2010) and stained by immunocytochemistry, while flowcytometric analysis was done in the Department of Clinical Pathology, Faculty of Medicine, Universitas Indonesia.

Flowcytometric Analysis

Around 10,000 adipose tissue derived stem cells were labelled by three kinds of antibodies i.e., per CP labelled antibody against CD34 (8G12 [BD Biosciences cat. Number BD 340430]), PE labelled antibody against CD73 (BD Biosciences cat. number BD 550257) and APC labelled antibody against CD90 (BD Biosciences cat. Number BD 559869). Another 10,000 cells were labeled by the respective isotypes, i.e., perCP, PE and APC labeled isotypes (BD Biosciences cat. number BD 559425, BD 555749 and BD 555751, respectively). Further the three surface markers were simultaneously analyzed using a BD FACSCalibur flowcytometer (BD Biosciences) with 10,000 gated events.

Immunocytochemistry Staining

The same sample was made into spot specimens and stained for CD34, CD73 and CD90 (Thy-1) (Santa Cruz, Table 1) by immunocytochemistry (SCB, 2014a; 2014b; 2014c), according to manufacturer instruction. When available, 100 cells were screened for positive results and percentages of positive results for CD34, CD73 and CD 90 were computed.

Data Collection and Analysis

The results of the two methods were noted and tabulated and discrepancies between the two methods were noted for all samples. Discrepancies lower than 3, 11 and 11% for CD34, CD73 and CD90 respectively were regarded as agreement between the two methods. Percentages of agreement in CD34, CD73 and CD90 were calculated.

Results

Some spot specimens did not yield 100 cells and analyses were done on available cells. Percentage of CD positive cells for CD34, CD73 and CD90 in flowcytometry and immunocytochemistry can be seen in Table 2.

The agreement between the two methods for CD34, CD73 and CD90 were 2/9 (22%), 5/12 (42%) and 2/7 (29%) respectively.

Discussion

Agreement between flowcytometry and immunocytochemistry in this study was low to moderate. This fact might be due to the primary antibody choices for immunohistochemistry, which epitopes were not the same as those from flowcytometry.

Adipose derived MSCs have rather high CD34 level that decrease upon passages (Mitchel *et al*., 2006) and in our study this trend was better represented by immunocytochemistry result (Table 2). In this study, immunocytochemistry staining of CD34 used mouse monoclonal antibody raised against KG-1 cells (class III CD34 epitope) (Table 1), while flowcytometry used perCP labelled antibody against CD34 (8G12 [BD Biosciences cat. Number BD 340430]), which was derived from 8G12 clone that recognizes two distinct epitopes that are expressed by human KG-1a cell line (BD Biosciences, 2014). CD34 has at least three epitopes, i.e., class I, II and III epitopes. A study showed that different CD34 epitopes gave different results in the detection of CD34 bearing bone marrow and peripheral blood stem cells (Croockewit *et al*., 1998).

Table 1. Primary and secondary antibody for immunocytochemistry staining

	Primary antibody		Secondary antibody	
Surface marker	Source	Cat. number	Kit	Cat. number
CD34 (TUK3)	Mouse monoclonal antibody raised against KG-1 cells (class III CD34 epitope) (SCB, 2014a)	sc-19587	Mouse LSAB Staining Kit	sc-2050
CD73 (IE9)	mouse monoclonal antibody raised against human CD73 purified from placenta (SCB, 2014b)	sc-32299	Mouse LSAB Staining Kit	sc-2050
Thy-1 (K-16)	goat polyclonal antibody raised against a peptide mapping near the C-terminus of Thy-1 of human origin (SCB, 2014c)	sc- 6071	Goat LSAB Staining Kit	sc-2017

Table 2. Flowcytometry and immunocytochemistry percentage discrepancy for CD34, CD73 and CD90

P	Med	Flowcytometry (%)	Immunocytochemistry (%)	Screened cell s	Discrepancy (cut off 3%)
CD34					
3	Mes	46.6	16.7	6	29.9
5	Mes	0.4	0.0	46	0.4
1	P10	1.1	20.0	100	-18.9
2	P10	2.8	5.0	100	-2.2
1	P5	1.9	47.1	34	-45.2
2	P5	8.2	21.4	28	-13.2
4	P5	21.3	2.0	100	19.3
2	VE	1.2	30.0	100	-28.8
5	VE	7.8	0.0	57	7.8
CD73					
					Discrepancy (cut off 11%)
1	Mes	65.2	72.7	77	-7.5
2	Mes	70.2	79.2	96	-9.0
5	Mes	60.9	90.9	11	-30.0
1	P10	54.0	86.0	100	-32.0
2	P10	60.5	70.6	17	-10.1
4	P10	76.1	39.1	23	37.0
1	P5	54.6	53.3	32	1.3
2	P5	58.6	59.1	22	-0.5
4	P5	72.3	86.7	15	-14.4
0	VE	38.4	77.8	9	-39.4
3	VE	34.0	82.7	75	-48.7
5	VE	21.7	95.7	23	-74.0
CD90					
					Discrepancy (cut off 11%)
3	Mes	91.0	88.9	9	2.1
5	Mes	96.4	70.1	83	26.3
1	P10	96.2	95.6	90	0.6
2	P10	91.4	71.4	7	20.0
1	P5	96.8	75.0	8	21.8
2	P5	58.6	25.0	4	33.6
5	VE	95.3	53.3	15	42.0

P = passage, Med = medium, Mes = complete MesenCult® medium (Stem Cell technologies basal medium 05401, Stem Cell technologies stimulatory 05402), P10 = 10% Platelet Rich Plasma (PRP) containing high glucose DMEM (DMEM-HG [Lonza C15-604 F]), P5 = 5% PRP containing DMEM-HG, VE = final 10 ng mL^{-1}VEGF (Invitrogen PHC9394) and 10% human AB serum (Gibco 34005-100) containing DMEM-HG

Mitchel *et al*. (2006) in a flowcytometry study on AT-MSCs that was cultured in 10% Fetal Bovine Serum (FBS) containing Dulbecco's modified Eagle medium/Ham's F12 (DMEM/Ham's F12) used CD34 antibody that was derived from 8G12 clone, as was our CD34 flowcytometry antibody. However, we used different fluorophore, i.e., per CP, while Mitchel *et al*. (2006) used PE. Mitchel *et al*. (2006) showed that in early passages (P0-P2), CD34 percentage was high, with high variability especially in passage-2, where the standard deviation was greater than the mean value. CD34 tended to decrease upon passages and the percentage in passage-4 was 1.7+1.0 (Mitchel *et al*., 2006). Our flowcytometry study showed variable results for CD34, that was in line with Mitchel's results (Mitchel *et al*., 2006).

In this study, agreement between flowcytometry and immunocytochemistry for CD73 was moderate, while immunocytochemistry results showed better agrrement to AT-MSCs in term of CD73 level, except for one case in passage-4 that showed CD73 level of 39.1% (Table 2). Mitchel *et al*. (2006) used the same CD73 antibody as that was used in this study to analyze CD73 expression in AT-MSCs. Their results were in accordance with our results for AT-MSCs that were cultured in MesenCult® and 5 and 10% PRP containing DMEM-HG (Table 1). However, CD73 percentages in adipose derived stem cells that were cultured in VEGF and 10% human AB serum containing DMEM-HG showed much lower percentage compared to those in Mitchel *et al*. (2006) study. Our study showed that adipose derived stem cells that were cultured in VEGF and 10% human AB serum containing DMEM-HG were not MSCs, but had differentiated into chondrogenic cells, which was shown by Alcian blue staining (Pawitan *et al*., 2013c).

Immunocytochemistry staining of CD73 used mouse monoclonal antibody (IgG3) raised against human CD73 purified from placenta (Table 1), while flowcytometry used PE labelled antibody against CD73 from AD2 clone (BD Biosciences cat. number BD 550257), which was purified from tissue culture supernatant or ascites (BD Pharmingen, 2014a). The epitope that was recognized by immunocytochemistry was FL(h) (SCB, 2014d), while by flowcytometry was whole CD73, which is ecto-5'-nucleotidase, a 70 kDa, Glycosyl Phosphatidylinositol (GPI)-anchored glycoprotein (BD Pharmingen, 2014a).

Table 2 showed that for CD90, all flowcytometry results were higher compared to immunocytochemistry, thus were more appropriate for AT-MSC. Immunocytochemistry staining of CD90 used goat polyclonal antibody raised against a peptide mapping near the C-terminus of Thy-1 of human origin (Table 1), while flowcytometry used monoclonal antibody against whole human CD90 from 5E10 clone that was purified from tissue culture supernatant or ascites (BD Pharmingen, 2014b).

Mitchel *et al*. (2006) used FITC labelled CD90 antibody from 5E10 clone as our antibody and showed that CD90 expression in AT-MSCs was high and became higher upon passages. This finding was corroborated by our results in AT-MSCs that were cultured in MesenCult®, which is a special commercial medium for MSCs and 10% PRP containing DMEM-HG.

Conclusion

Agreement between flowcytometry and immunocytochemistry was low for CD 34 and CD90 and was moderate for CD73. The difference might be due to different epitopes that were used to generate the antibodies.

Acknowledgement

This study was funded by the research grant from Ministry of National Education Republic of Indonesia (PUSNAS 2014).

Author Contributions

Jeanne Adiwinata Pawitan: Development of research idea, supervising research work, analysis of whole data, writing draft of whole manuscript and final approval of manuscript.

Dewi Wulandari: Running flowcytometry, analysis of flowcytometry data, reading draft, correcting and final approval of manuscript.

Des Suryani: Preparing cells for flowcytometry and making spot specimens for immunocytochemistry, reading draft, correcting and final approval of manuscript.

Lia Damayanti: Immunocytochemistry staining and analysis of immunocytochemistry data, reading draft, correcting and final approval of manuscript.

Isabella Kurnia Liem: Compiling and analysis of whole data, reading draft, correcting and final approval of manuscript.

Ethics

All authors declare that there is no conflict of interest

References

BD Biosciences, 2014. Monoclonal antibodies detecting human antigens. BD Biosciences.

BD Pharmingen, 2014a. Technical data sheet PE mouse anti-human CD73. BD Pharmingen.

BD Pharmingen, 2014b. Technical data sheet APC mouse anti-human CD90. BD Pharmingen.

Choudhery, M.S., M. Badowski, A. Muise, J. Pierce and D.T. Harris, 2014. Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. J. Transl. Med., 12: 8-8. PMID: 24397850

Croockewit, A.J., R.A. Raymakers, F.W. Preijers, G. Vierwinden and T.J. de Witte, 1998. The role of the different CD34 epitopes in detection and positive selection of CD34+ bone marrow and peripheral blood stem cells. Scand J. Immunol., 47: 82-90. PMID: 9467663

Li, T.X., J. Yuan, Y. Chen, L.J. Pan and C. Song *et al*., 2013. Differentiation of mesenchymal stem cells from human umbilical cord tissue into odontoblast-like cells using the conditioned medium of tooth germ cells *in vitro*. Biomed. Res. Int. PMID: 23762828

Mitchel, J.B., K. McIntosh, S. Zvonic, S. Garrett and Z.E. Floyd *et al*., 2006. Immunophenotype of human adipose-derived cells: Temporal changes in stromal-associated and stem cell-associated markers. Stem Cells, 24: 376-385. PMID: 16322640

Oedayrajsingh-Varma, M.J., S.M. van Ham, M. Knippenberg, M.N. and J. Klein-Nulend *et al*., 2006. Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure. Cytotherapy, 8: 166-177. PMID: 16698690

Pawitan, J.A., L. Damayanti, A. Arleni and N.M. Swantari, 2010. Cytology technique: Development of a simple spot method for cultured cell suspension. Med. J. Indones., 19: 26-31. DOI: 10.13181/mji.v19i1.378

Pawitan, J.A., D. Wulandari, D. Suryani, L. Damayanti and R.Y. Purwoko *et al*., 2013a. Flow cytometry analysis of adipose tissue derived stem cells that were cultured in various media. Int. J . Pharm Tech. Res., 5: 1301-1306.

Pawitan, J.A., I.K. Liem, D. Suryani, A. Bustami and R.Y. Purwoko, 2013b. Simple lipoaspirate washing using a coffee filter. Asian Biomed., 7: 333-338.

Pawitan, J.A., D. Suryani, J. Lilianty, R.Y. Purwoko and I.K. Liem, 2013c. The use of VEGF supplemented media for chondrogenic differentiation of adipose derived mesenchymal stem cells. Biotechnol. Ind. J., 7: 169-173.

Suryani, D., J.A. Pawitan, J. Lilianty, R.Y. Purwoko and I.K. Liem *et al*., 2013. Comparison of fetal bovine serum and platelet-rich plasma on human lipoaspirate-derived mesenchymal stem cell proliferation. Med. J. Indones., 22: 146-151. DOI: 10.13181/mji.v22i3.583

SCB, 2014a. CD34 (TUK3): sc-19587. Santa Cruz Biotechnology.

SCB, 2014b. CD73 (IE9: sc-32299. Santa Cruz Biotechnology.

SCB, 2014c. Thy-1 (K-16): sc-6071. Santa Cruz Biotechnology.

SCB, 2014d. CD 73 antibodies. Santa Cruz Biotechnology.