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Effects of PBMC and Combined-PBMC with BM-MSCs to the Expression of α2β1 Integrin Level on Full Thickness Rat' Burn Skin

Gusti Revilla[a*], Eryati Darwin[b], Fedik A. Rantam[c], Yanwirasti[d]

[a,d]*Department of Anatomy, Faculty of Medicine, Andalas University. Jl. Proklamasi Kemerdekaan No.94 PO BOX 49 Padang, 25127, West Sumatra, Indonesia*

[b]*Department of Histology, Faculty of Medicine, Andalas University. Jl. Proklamasi Kemerdekaan No.94 PO BOX 49 Padang, 25127, West Sumatra, Indonesia*

[c]*Department of Microbiology Veterinary Medicine Faculty and Stem cell Laboratory, Institute of Tropical Disease (ITD), Faculty of Medicine, Airlangga University, 60115, Surabaya, Indonesia*

[a]*Email: gustirevilla@med.unand.ac.id;* [d]*Email: yanwirasti@med.unand.ac.id*

[b]*Email: eryatidarwin@med.unand.ac.id*

[c]*Email: fedik.ar@gmail.com*

Abstract

Bone marrow mesenchymal stem cells (BM-MSCs) increases the percentage of integrin α2β1 expression on rat' wound healing process, but the study which is related to PBMC (peripheral blood mononuclear cell) and PBMC combined with BM-MSCs towards burn healing process has not been conducted yet. The aim of the study was to determine the expression of integrin α2β1 as a result of PBMC and PBMC combined to BM-MSCs administrations towards rat' wound healing process. Experimental research with the post test only control design was conducted on 12 Wistar rats, divided into three experimental groups: PBS (Phosphate Buffer Saline) as control group, PBMC, and PBMC combined with BM-MSCs. Stem cells were subcutaneously injected with 2x106 cells/ml. A full-thickness burn was made on the dorsal side (back). Skin tissues were collected to investigate the expression of integrin α2β1 using immunohistochemistry method on day-14.

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* Corresponding author.

Administration of only PBMC (2.972), and PBMC combined with BM-BSCs (3.297) elevated the percentage of integrin α2β1 compared with control group (2.340), but significant result was only in combination group (p=0,05). The administration of only PBMC and PBMC combined with BM-MSCs elevated the expression of integrin α2β1, which showed that both of stem cells accelerated the migration of keratinocyts that will expedite the re-epithelialization phase in burn wound healing.

Keywords: PBMC; BM-MSC; integrin- α2β1; wound healing.

1. Introduction

PBMC (Peripheral Blood Mononuclear Cell) is hematopoietic cells consisting of monocyte, fibrocytes, and Epithelial Progenitor Cells (EPC). Monocyte and fibrocyte act as Antigen Presenting Cells (APC) and produce cytokines and growth factors, play an important role in inflammation, collagen formation and angiogenesis [1, 2]. While, BM-MSCs (Bone Marrow Mesenchymal Stem Cells) are non-hematopoietic cells, multi-potent progenitor, and can differentiate into other cells, such as chondrocytes, osteoblasts, fibroblasts, epithelial cells, endothelial cells, and neurons [3, 4].

Previous study of BM-MSCs has investigated the expression of type-1 collagen and α2β1 integrin in healing of rat' burns. The results showed that BM-MSCs are able to increase the thickness of type-1 collagen, and showed a significant difference with control group. In addition, BM-MSCs also increase the percentage of α2β1 integrin expression level but it was not statistically significant [5]. PBMC showed its effect on the increase of thickness of type-1 collagen and showed the similar results with BM-MSCs [6].

BM-MSCs and PBMC are potential in wound healing, and express similar chemokines and receptors; SLC/CCR7, SDF-1α and CXCR4. Both of them are able to migrate to damaged tissues [7, 8], differentiate into some cells needed in skin wound healing, including fibroblasts, keratinocytes, and endothelial cells [9, 10]. Therefore, the investigation is needed to identify the combination of BM-MSCs with PBMC for therapy of burn wound healing.

The combination of Hematopoietic Stem Cells (HSC) and human BM-MSCs which implanted in mice to monitor bone growth showed their cooperation in formating of bone tissue and increase number and diameter of blood vessel [11]. Administration of PBMC and BM-MSCs improved the thickness of type-1 collagen and showed significant result in rat's burn wound.

Burns is a serious health problem in developing country, causes physical damage even death. Burns is caused by thermal temperature, chemical compound, electrical, and radiation. The damage depends on the location, depth, and extent of burns [12].

Burn wound healing is a complex process involving several interrelated stages: inflammation, proliferation (granulation tissue formation, reepithelization, extracellular matrix), and remodeling, involves the interaction of several mediators, extracellular matrix protein cells, and adhesion molecules [13].

E and P selectin, *Intercellular Adhesion Molecule* (ICAM), *Vascular Celladhesion Molecule* (VCAM) and integrin are adhesion molecules involved in wound healing [14, 15, 16], adhesive molecules plays a role to help the migration of neutrophil, keratinocyte, and fibroblast cells. Integrin is an adhesion molecule as transmembrane receptors, consists of α- and β-subunits, combines in performing their functions, and there are 24 types of integrins which has been found. Combination of integrin varies, and α1β1, α2β1, α3β1, α6β4 is found in keratinocytes, and α2β1 is predominant integrin. In wound healing process, α2β1 integrin has an important role in the process of homeostasis, cell migration during proliferation phase, such as reepithelization. Thus, integrins regulates the migration of keratinocytes [17], so wound healing can be processed fastly.Therefore, PBMC and PBMC combined with BM-MSCs administrations was conducted to investigate the expression of α2β1 integrin.

2. Material and Methods

2.1. Isolation of PBMC in rat

Approximately 3.5 ml blood was collected from the rat tail and 3.5 ml sterile PBS was added. The mixtures were transferred into new tube filled with 3.5 ml ficol isopaque density 0.177. Blood was centrifuged at 1600 rpm for 30 minutes to obtain buffy coat contained mononuclear cells. Five milliters of sterile PBS was added, blood was resuspended twice to obtain pellet. Pellet was added with sterile PBS to obtain PBMC, cells were counted using hemocytometer. The concentration of PBMC used in this study was 2x106 cells/ml.

2.2. Mesenchymal Stem Cells (MSC) originated from rat bone marrow

MSC was provided in Laboratory of Stem Cell, Airlangga University, Surabaya. MSC was placed in monolayer tube, cells were separated and calculated before application. Each rat was injected with 2x106 cells/ml of MSC.

2.3. Preparation of rats for burns

Skin burns of rat were made by [18]. Rat was anesthetized using xylazine and ketamine (ratio 1:1) and fur on rat back was shaved. Full-thickness burns were made by heating a plate in the boiling water for 30 minutes and patched on rat back for 20 seconds. Rat in control group was injected with sterile PBS, and treatment groups were injected with PBMC and PBMC combined with BM-MSCs. Then, the burns were covered with tegaderm film and elastomult haft. Antalgin as an analgetic was injected to rats during observation process. Skin tissue of rats was taken at day 14 to determine α2β1 integrin expression (R&D system) by using immuno histo chemistry method.

2.4. Immunohistochemical

Deparaffinization was conducted by dipping the slides into xylol solution, three times for five minutes, rehydrated by dipping the slides into xylol twice for 3-5 minutes, into ethanol 70% twice for two minutes, and washed with distilled water three times. Slide was immersed into 3% H2O2 in methanol for five minutes and washed with distilled water and PBS three times. The cleaned slides were immersed in anti-integrin α2β1 (mouse anti-rat 1:50) for 30 minutes at ambient temperature, washed with PBS three times for two minutes.

Slide were marked by using Pap pen, and dipped into secondary antibody (rabbit anti-mouse biotinylated antibody label) for 30 minutes and washed using PBS three times for two minutes.

Slides were transferred into Horseradish Peroxide (HRP) conjugated-streptavidin for 30 minutes and washed with PBS three times for two minutes. Slides were immersed in chromogenic substrate for 3-10 minutes, washed with PBS three times for two minutes and washed with distilled water. Last, slides were immersed in Mayer's hematoxylin solution for 6-15 minutes, washed using tap water, and mounted.

2.5. Research Ethics

This research was already passed the ethics clearance and has been approved by the Ethics Committee of the Faculty of Medicine, Andalas University, Padang.

2.6. Data Analysis

The effect of combination between PBMCs and BM-MSCs to the percentage of α2β1 integrin was analyzed by using Oneway ANOVA test, with Tukey test as an advanced test.

3. Result

Fig.1 shows the result of isolation to obtain PBMC from blood preripher, and mesenchimal stem cells from bone marrow.

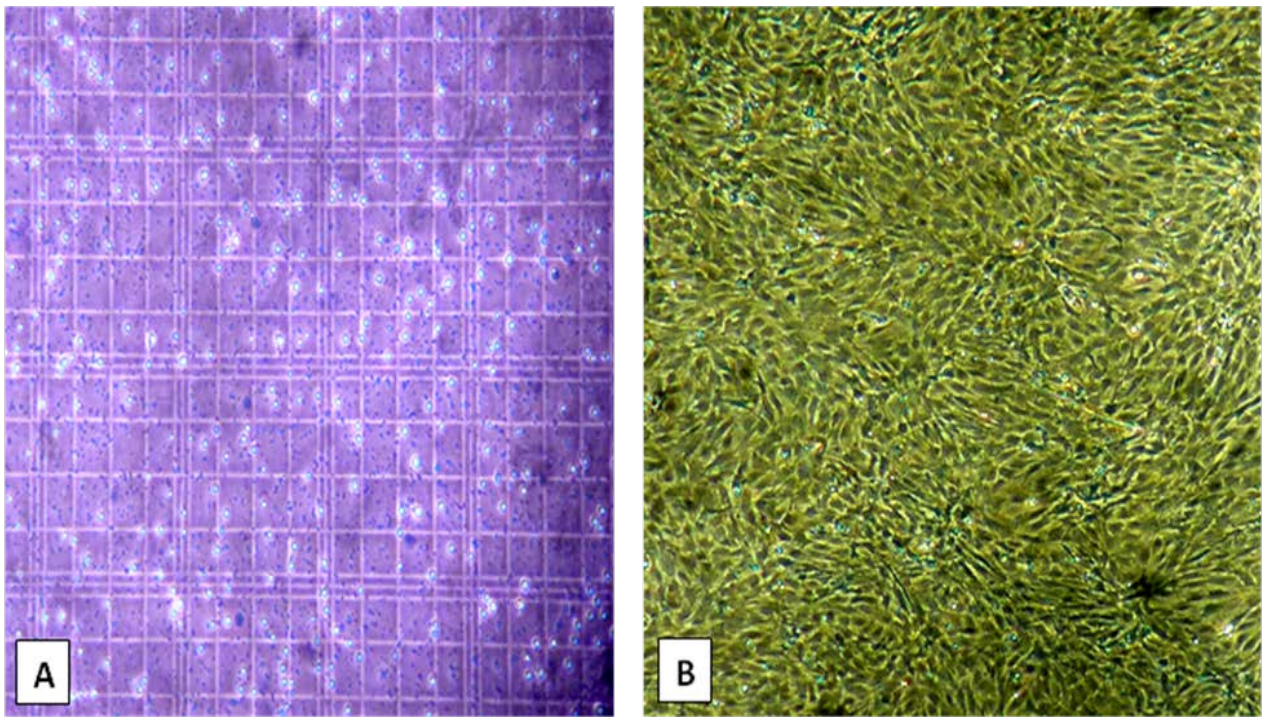

Figure 1: The isolation result of PBMC from peripheral blood (A) and (B) mesenchimal stem cells from bone marrow of rat

The administration of both stem cells showed the acceleration of burns healing on rat skin compared with control group (Fig.2).

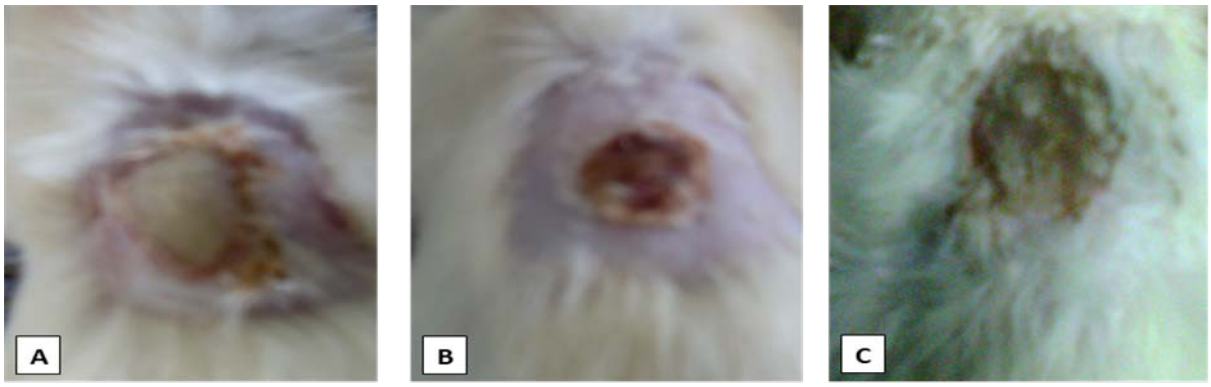

Figure 2: The healing process of burns after day-14in control group with PBS administration (A), PBMC administration (B) and combination of PBMC and BM-MSCs (C)

3.1. The xpression of Integrin α2β1

Immunohistochemical assay was conducted to determine the expression of α2β1 integrin by calculating the number of cells which positively reacted to the anti-integrin α2β1. Positive cell was counted by dividing number of cell expressing integrin with total cell/15625 μm2 in burns tissue. The average percentage of α2β1 integrin was presented in Table 1 and Figure 3.

Table 1: The average of positive reactions of cell to integrin α2β1 on burns skin tissue after administrating PBMC, and PBMC + MSCs on day-14

No	A number of cells reacted positively to integrin (%) in each treatment		
	Control	PBMC	Combination of PBMC and BM-MSCs
1	2.16	3.99	3.60
2	1.97	2.62	3.76
3	2.82	2.12	3.22
4	2.18	3.81	4.88
5	2.24	2.32	2.16
6	2.67	2.97	2.16
Averages	2.34	2.972	3.297

The results showed that on day-14, the average percentage of cells expressing α2β1 integrin after administering

with PBMC was not significantly different with control group (p=0,191). Whereas, in combination group, PBMC showed significant result, and advanced test was discontinued.

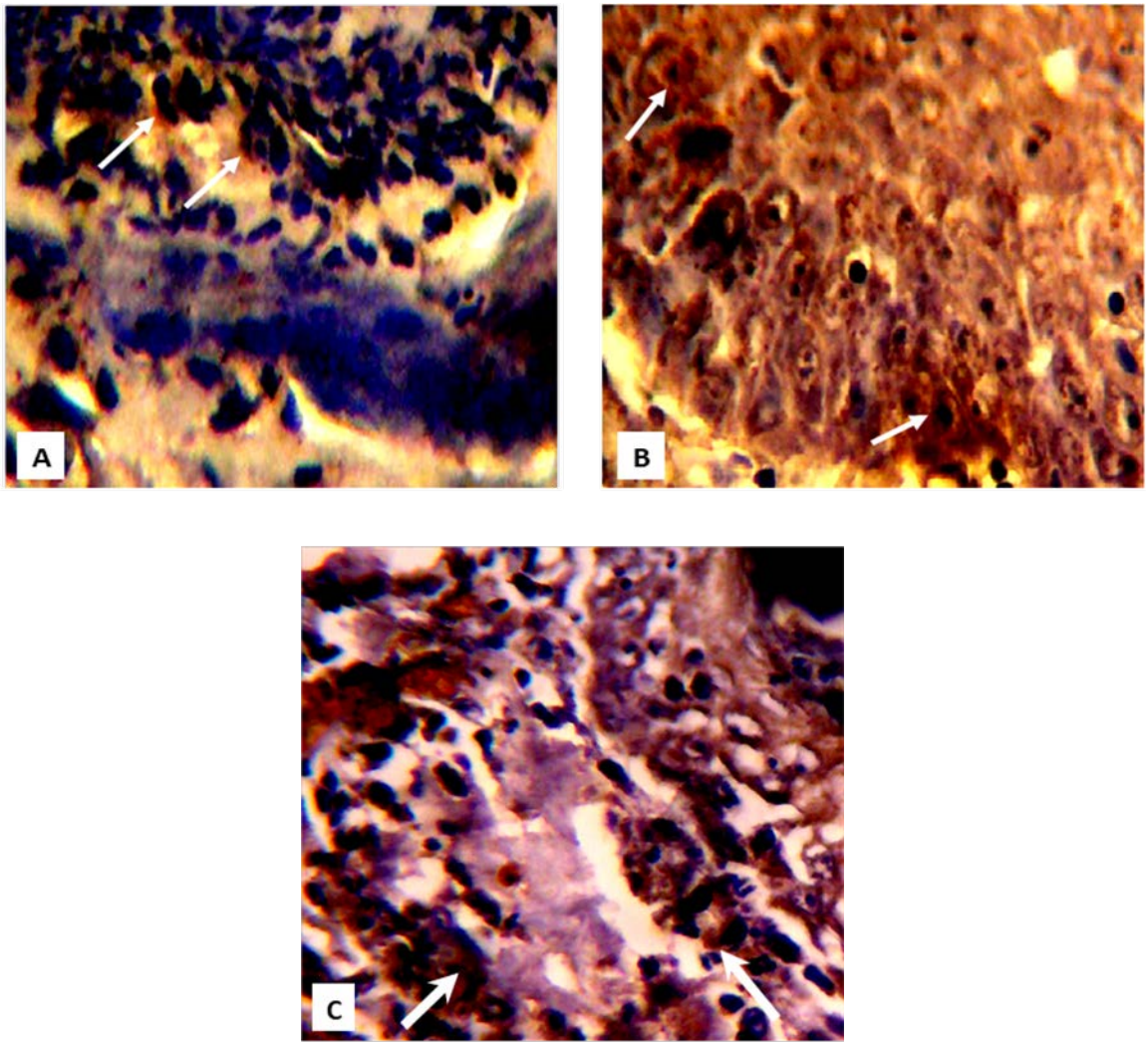

Figure 3: Immunohistochemical staining of α2β1 integrin expression in (a) control group (b) PBMC group, and (c) combination group (M=400x)

In Figure 1(A), cells expressed less of α2β1 integrin, demonstrated as a positive brownish-like color, while in Figure 1(B) and (C), cells expressed a more brownish color.

4. Discussion

Administration of PBMC and PBMC combined with BM-MSCs showed to accelerate the burns healing on rat, compared with control group. The acceleration of burns healing on rat skin may show one of molecular mechanism to burns healing, by investigating the expression of α2β1 integrin after PBMC stem cells administration, and the combination of PBMC and BM-MSC. These results were supported with our previous study about the implantation of hematopoietic stem cells (HSC) combined to human BM-MSCs on rat, in observing to the bone growth and blood vessel formation. Apparently, the combination was able to cooperate in

the formation of bone tissue and increased the number and diameter of blood vessels [19].

Integrin α2β1 is an adhesion molecule, play a role in inter-binding cells and with extracellular matrix. As the adhesion molecule, integrin associates to extracellular matrix, such as collagen, laminin, and fibronectin, to initiate cell migration.

The percentage of α2β1 integrin in rat' skin burns tissue increased after administering of PBMC, but the results was not significant compered with control group. The expression of α2β1 integrin naturally increases in wounded condition, it's due to α2β1 integrin affects the migration function and proliferation of inflammatory cells; keratinocytes, and fibroblasts. Zhang and his colleagues [20] reported that keratinocyts need integrin α2β1to bind with collagen in order to initiate migration process.

Insignificant result of only PBMC administration was caused by the characteristics of PBMC as hematopoietic cell, by initiating wound healing phase. In that phase, several adhesion molecules are needed, such as integrin, E and P selectin, ICAM, and VCAM. In addition, in order to develop from a stem cell niche, the tissue requires integrin, so that endogenous or exogenous stem cells will be able to replace the damaged tissue [21, 22]. In wound healing phase, especially in re-epithelialization, several types of integrins were found on the surface of keratinocyte, such as α2β1, α3β1, α6β4, αvβ6, and α5β1. Integrins in keratinocytes have an important role in keratinocyte migration from the margin's wound and in proliferation process, initiating re-epithelialization phase [23]. Therefore, further study related to the expression of other integrins in burns healing process is strongly needed. The results demonstrated that the administration of PBMC combined with BM-MSC elevated significantly the percentage of α2β1 integrin expression compared with control group. It may be caused by a synergistic relation between PBMC and BM-MSC. PBMC initiates wound healing phase, while BM-MSC works from the initial until remodeling phase. In addition, BM-MSCs secrete several chemical mediators in contributing of homing and differentiating process of PBMC into cells needed in wound healing, such as epithelial cells (keratinocytes) and fibroblasts. Keratinocytes derive from trans-differentiated PBMC, affect the function of fibrocyte during wound healing by increasing the expression of MMP. The increased expression of α2β1 integrin is needed by keratinocytes to bind with collagen, in order to α2β1 integrin provides transduction signal to initiate migration of cells [24, 25, 26]. In addition, the increasing of α2β1 integrin was followed by collagen thickness by combining PBMC with BM-MSCs, results showed significant elevation of type-1 collagen thickness compared with control group. Both of stem cells express chemokine with similar receptors; SLC/CCR7 and SDF-1α and CXCR4, so the increase of integrin accelerates the migration of both stem cells, indicates a synergistic effect in wound healing. Integrin cooperates with growth factors by activating and regulating the signals, such as TGF-β [27], and assists proliferation phase in wound healing [28, 29]. Gusti and his colleagues [30] suggested that the administration of PBMC combined to BM-MSCs significantly enhanced the level of TGF-β in burned rats on day-3 and day-5. Integrin also contributes in apoptosis, angiogenesis, and neural function [31].

4. Conclusion

The result will become a basis for further research and be applied as a cell therapy using the combination of

PBMCs and BM-MSCs for tissue regeneration, especially for skin tissue.

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