18th CONGRESS OF INTERNATIONAL FEDERATION OF ASSOCIATIONS OF ANATOMISTS (IFAA)

30th CONGRESS OF CHINESE SOCIETY OF ANATOMICAL SCIENCES(CSAS)

AUGUST 8-10, 2014, BEIJING CHINA

CERTIFICATE

This certificate is presented to:

Name: GUSTI REVILLA

Title:

As speaker in 18th IFAA Congress,
30th National Congress of CSAS

Prof. Bernard Moxham
The President of IFAA

Prof. Yunqing Li
The President of CSAS

Synergistic effect of PBMC and BM-MSC on the percentage of integrin α2β1 in full thickness burn in Rats

Abstract

***[1]Gusti Revilla, [2]Eryati Darwin, [1]Yanwirasti and [3]Fedik A. Rantam**

[1]Department of Anatomy Faculty of Medicine, Andalas University, Padang, Indonesia.
[2]Department of Histology, Faculty of Medicine, Andalas University, Padang, Indonesia.
[3]Stem cell Laboratory Institute of Tropical Disease (ITD) and Laboratory Virology and immunology, Department of Microbiology, Vet. Medicine Faculty Airlangga University Surabaya, Indonesia

Burn is a health concern in the community because burn injury will lead to physical disability and even death. Therefore, a proper treatment is needed in burn. Though there are many available treatments for burns, the result remains unsatisfying. So, it is necessary to find another optional treatment for burns, one of which is by using stem cell to repair the anatomy of skin and its functions.In fact , the administration of PBMC and BM-MSC stem cell combination on burns has not yet been carried out.

This study was aimed to analyse the influence of administration of combination of PBMC and BM-MSC stem cells on the expression of integrin α2β1 in burn healing process in rats.

An experimental study with the post test only control design has been carried out on 12 young Wistar rats which were divided into two groups : control group (PBS), and treated group (PBMC and BM-MSC combination). Stem cells were given subcutaneously with dose 2 x 10^6 cell/ml. A full thickness burns was made on their shaved dorsum which has been anesthetized before with xylazine and ketamine. At day 14, the expression of integrin α2β1 from skin tissues was examined by immunohistochemical examination. This study has received approval from the Ethics Committee of the Faculty of Medicine, Andalas University, Padang. The results were analyzed by oneway ANOVA test and further test Tukey.

Immunochemical examination of skin tissues showed that the administration of combination of PBMC and BM-MSC stem cells can increase the percentage of integrin α2β1 and this result statistically shows significant difference.

This study concluded that the administration of combination of PBMC and BM-MSC stem cells on burns can accelerate burn healing process in rats. It is suggested that the stem cell might be working through paracrine effect by affecting growth hormones and cytokines which then will affect micro environment of burn tissues and speed up the migration of cells that play role in wound healing. This situation will accelerate healing process from inflammatory phase to remodelling phase.

Key word : PBMC, BM-MSC, Integrin α2β1 , wound healing.

Introduction

A burn is a serious problem for the community because it can cause physical damage or even death. A burn is a type of injury caused by heat, chemicals, electricity and radiation. The damage and the severity of burns depend on the sites, depth and extent of injuries (Evers et al, 2010).

Temperature and duration of contact have a synergistic effect in depth of burn. Burns are classified as first-degree (superficial), second-degree (partial -thickness) and third-degree (full-thickness) depending on how deep and severe they penetrate the skin's surface. A full-thickness burn destroy epidermis and dermis layer. This condition needs a good and proper treatment to accelerate wound healing. Burn wound healing is a complex process involving several interrelated stages such as inflammation, proliferation (formation of granulation tissues, reepithelization, extracellular matrix formation) and remodelling. All of these phases involving interaction of mediators, cells, and extarcellular matrix proteins (Syamsuhidayat *et al.*, 2003 ; Vern *et al.*, 2001). Cytokines, growth factors and adhesion molecules are chemical responses. Cytokines founded were *interleukin-1* (IL-1), IL-6 dan *tumor necrosis factors-α* (TNF-α) which act as proinflammation (Kumar *et al.*, 2004).

Adhesion molecules involved in wound healing are E and P selectin, *intercellular adhesion molecule* (ICAM), *vascular cell adhesion molecule* (VCAM) dan integrin (Lorenz *et al.*, 2003; Kotowict *et al.*, 2000; Nagoka *et al.*, 2000). Adhesion molecules play role in Adhesion molecules play a role to help the migration of neutrophil cells, keratinocytes and fibroblasts. Integrins are transmembrane adhesion molecule receptors consisting of α and β subunits, in which the 2 sub unit join to perform its functions and it has been found 24 types of integrins. The combination of integrins is quite varied and are found in epidermal keratinocytes is particularly α1β1, α2β1, α3β1 and α6β4, and the most common integrin is α2β1. During wound healing, integrin A2β1 roles in hemostatic, cell migration, and reepithelization until wound closure occured, because this integrin plays a role in regulation of keratinocyte (Cavani et al., 1993). This suggested that the α2β1 integrin plays a role in wound healing. For this reason, the study on the influence of combination of PBMC and BM-MSC stem cells on the expression α2β1 integrin needs to be carried out.

PBMC stem cells are hematopoietic cells consisting of monocytes, fibrocytes, and *ephithelial progenitor cell* /EPC. Monocytes and fibrocytes can have character as APC and produce cytokines and growth hormones that play a role in inflammation, formation of collagen and (Abe *et al.*, 2001). Meanwhile, BM-MSC is a non hematopoeitic cells

characterized as multipotent progenitor and is able to differentiate into chondrocytes, osteoblast, fibroblast, epithelial cells, and neuron cells (Pittenger *et al.*, 1999; Liu *et al.*, 2009).

Both PBMC and BM-MSC stem cells expressed similar chemokines and receptors such as SLC/CCR7 , SDF-1α and CXCR4 (Bobis *et al.*, 2006 dan Sasaki *et al.*, 2008) so that these stem cells are able to migrate into damaged tissues. PBMC and BM-MSC stem cells can differentiate into cells needed for refinement of skins , such as fibroblast, keratinocytes, and endothelial cells (Abe *et al.*, 2001; Hedrick *et al.*, 2010; Medina *et al.*, 2000 dan Sasaki *et al.*, 2008).

Previous study showed that the combination of *Hematopoitik stem cells* (HSC) dan BM-MSC from human induced to mice to see the growth of bones , proved that this combination was able to coorperate in forming bone tissues and enhanced the number and diameters of blood vessels (Moioli *et al.*, 2008). Based on previous study and the ability of PBMC and BM-MSC stem cells that were able to differentiate into existing skin cells, then it is necessary to conduct a study on the influence of combination of PBMC and BM-MSC on the expression of α2β1 integrin.

Research Methodology

Peripheral Blood Mononuclear Cell (PBMC) Isolation

Peripheral blood mononuclear cells (PBMCs) used for the research were taken from Wistar rats that were anesthetized with Xylazine and Ketamine (1:1). The blood taken (3.5 ml) from the rat tail was then added with 3.5 ml sterile PBS: the mixture of blood and sterile PBS was then added to the centrifuge tube which was filled with 3.5 ml ficoll isopaque density 0.177. The mixture was centrifuged for 30 minutes on 1600 rpm which then crated the Buffy coat. Next, the Buffy coat was added with sterile PBS 5 ml and then centrifuged for 5 minutes on 1600 rpm to separate the supernatant. This re-suspension was conducted for 2 times to take the granules which were then added with PBS; then the cells could be counted by using hematocytometer. From the result, the number of PBMC was recognized from the sample was 3x107. But the PBMCs used in this research was 1x106 cells/ml.

Bone Marrow Mesenchymal Stem Cells

Mesenchymal stem cells were derived from bone marrow of the rats. Rats BM-MSC that are used in this research were gotten from Stem Cell Laboratory, Institute of Tropical Disease (ITD) Airlangga University Surabaya. Prior to be used, the MSC in monolayer tube petridish would be separated, so the number of cells which will be used for this research can be counted. The number of BM-MSCs cells that are equal for 1 rat is 1 x 10^6 cells/ml.

Experimental to Animal's Burn

The Wistar rats used in this research were divided into 2 groups. Group 1 served as the control group while group 2 was treated by using the combination of BM-MSC and PBMCs scale 1:1. Burn-making on rats were applied by using method from (Paramonov D. and Cheboterev cit Shuid et al., 2008) method. Rats were anesthetized with xylazin and ketamine (ratio 1: 1), then the fur of the rats were sheared. To make a thick burn mark on the rats, the plate was heated on boiling water for 30 minutes which is then sticks to the back of the rats for 20 minutes. After the burn making procedure, the PBS was injected to the control group; while the combination of BM-MSC and PBMCs was injected into the treatment group. The burn wounds were wrapped by using tegaderm film and gauze elastomull haft; antalgin as an analgesic was also injected to the rat. On the 3rd and 7th day, the blood was taken via intracardiac to measure the growth factor of TGF-β 1 from serum and collagen type I of the tissue

The Wistar rats that were used in this research were divided into 2 groups. Group 1 served as the control group while group 2 was treated by using the combination of BM-MSC and PBMCs scale 1:1. Burn-making on rats were applied by using method from (Paramonov D. and Cheboterev cit Shuid et al., 2008) method. Rats were anesthetized with xylazin and ketamine (ratio 1: 1), then the fur of the rats were sheared. To make a thick burn mark on the rats, the plate was heated on boiling water for 30 minutes which is then sticks to the back of the rats for 20 minutes. After the burn making procedure, the PBS was injected to the control group; while the combination of BM-MSC and PBMCs was injected into the treatment group. The burn wounds were wrapped by using tegaderm film and gauze elastomull haft; antalgin as an analgesic was also injected to the rat. On the 3rd and 7th day, the blood was taken via intracardiac to measure the growth factor of TGF-β 1 from serum and collagen type I of the tissue.

Immunohistochemistry α2β1 Integrin

The preparations were de-parafinisation by dipping it into xylol 3 for times; it was then rehydrated, by using xylol, which was then followed in absolute ethanol and 70% ethanol. To finish the rehydrated; we need to rinse it with distilled water. Slides were then dipped in 3% H2O2 and then in methanol for 5 minutes; the slides were then rinsed with distilled water and PBS for 3 times. The cleaned slides were put in anti α2β1 Integrin (mouse anti-rat 1:50) for 30 minutes on room temperature and then rinsed with PBS 3 times, each processes is done for 2 minutes. The daubed tissues were marked with Pap pen. Slides were then incorporated on the secondary antibody (Rabbit anti-mouse antibody biotinylated label) for 30 minutes and rinsed with PBS solution 3 times; similar with the previous procedure, the rinsed process with PBS Solution was done for 2 minutes. Put the slides into streptavidin HRP labeled for 30 minutes, then rinsed in a solution of PBS 3 times, each of them was also done for 2 minutes. Put slides into the substrate chromogen for 3-10 minutes, and rinse with a solution of PBS for 2 minutes that needs to be repeated 3 times and finally it need to be rinsed with aquadestilata. After that, the slides were put into Mayer haematoxylin for 6-15 minutes; to end the process we need to rinse the slide with running water and mounting. Microscope was used to measure the expression of α2β1 Integrin after immunohistochemical staining.

Analysis of Data

ANOVA test and further Tukey test were applied to analyze the effect of PBMCs stem cells on the expression α2β1 integrin.

Results

Immunohistochemical examination on the expression of α2β1 integrin performed on day 14 by counting number of cells that gave a positive reaction to anti- α2β1 integrin. Number of α2β1 integrin expression performed by counting expressed cells divided by the total number of cells/15625μm^2 on rats burn tissues. Skin tissues were examined in each group, then counted cells-integrin expressed divided by visible total cells. The average percentage of α2β1 integrin expression can be seen in table 1 and figure 1.

It can be seen in table 1 and figure 1 that on day 14, the average percentage of cells that expression α2β1 integrin in burn tissues of rats treated with the combination of PBMC

and BM-MSC stem cells was 3.297% higher than control namely 2.340%. The results statistically showed significant difference (p=0.037). Analyzing the IHC, it shows that the expression of α2β1 integrin on the control group only has a shadow of a thin brownish color (400 x). While the treatment group showed the positive dark brown can be seen in Fig. 2

Table 1. Average percentage of cells that gave a positive reaction on α2β1 integrin in burn tissues in control and treated group on day 14.

Group	Average expression of α2β1 Integrin (%) on day 14	P
control	2.340 ± 0.329	0.037
Treated	3.297 ± 1.039	

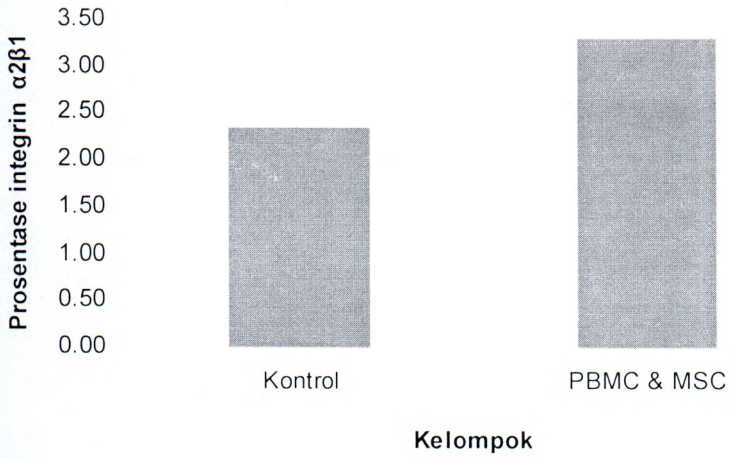

Figure 1. average level of α2β1 integrin expression in burn tissues in control and treated group on day 14.

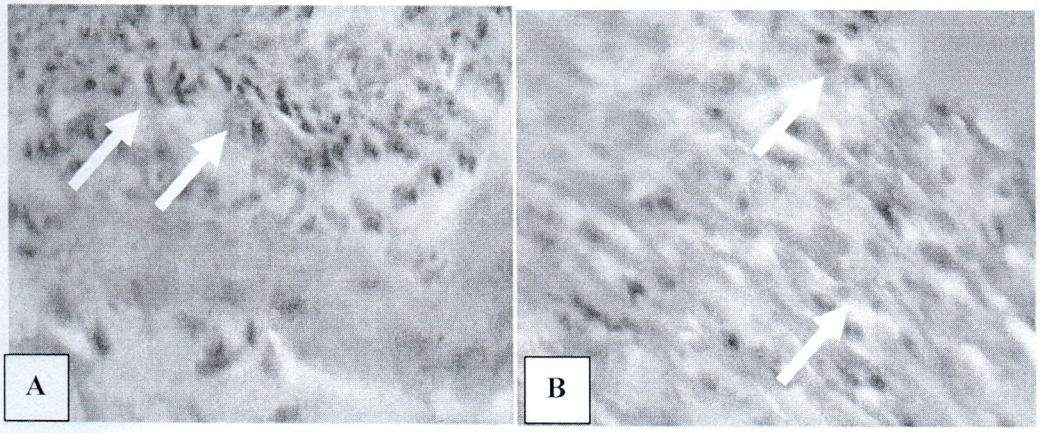

Figure 2. Staining IHC of α2β1 integrin on Staining Positive Control Group (A) and Combine BM-MSC and PBMCs (B)

Discussion

α2β1 integrin is an adhesion molecule taht plays a role in binding cells and extracellular matrix. As an attacher of cells, integrin will connect to extracellular matrix such as collagen, laminine, and fibronectin that will cause migration of cells.

The measurement of percentage of α2β1 Integrin in burn tisssues showed increased in group treated with combination of PBMC and BM-MSC compared to control, statistically showed significant difference compared to control. The increase of α2β1 Integrin expression caused by PBMC containing hematopoeitic cells consisting of monocytes, fibrocytes and *endothelial progenitor cell* (EPC) which will express the integrins. The result of this study also suported by other studies that the expression of α2β1 Integrin increase in wound because of its function in migration and proliferation of inflammatory cells, keratinocytes and fibroblast.

In platelet, α2β1 Integrin will interact with type I collagen, so that the next binding of platelet will regulate platelet agregation to secrete growth factors that play a role in inflammation phase of wound healing. α2β1 Integrin is essential for keratinocytes to bind to collagen, so that α2β1 integrin can give transduction signal to give phenotype to initiate cells migration (Zhang *et al.*, 2006). Both stem cells also expressing chemokine with the same receptors namely SLC/CCR7, SDF-1α and CXCR4 (Bobis *et al.*, 2006 and Sasaki *et al.*, 2008) hence, with the increase of integrin, migration of both stem cells might be accelerated.

This situation showed that both tested-stem cells provide synergistic effect on wound healing and can be seen that BM-MSC did not affect the effect of PBMC.

The result of this study also suported by previous study showed that the combination of *Hematopoitik stem cells* (HSC) dan BM-MSC from human induced to mice to see the growth of bones , proved that this combination was able to coorperate in forming bone tissues and enhanced the number and diameters of blood vessels (Moioli *et al.*, 2008). The same result also gained the increase of integrin expression in immunohistochemical examination in burn treated with combination of PBMC and BM-MSC.

The increase of α2β1 integrin in burn tissues treated with combination of PBMC and BM-MSC stem cells may be due to the α2β1 integrin expression needed to regulate migration and proliferation of platelet and keratinocytes cells (Pilcher *et al.*, 1997).

In wound healing phase, particularly in reepithelization phase, many types of integrins were founded on the keratinocytes surfaces such as α2β1, α3β1, α6β4, αvβ6, dan α5β1. Integrin on this keratinocytes plays a role in migration of keratinocyte cells from the edge of wound and proliferation of keratinocyte so that reepithelization occured (Larjava *et al.*, 1993 and Watt, 2002).

Integrin with its function is able to cooperate with growth factors by means of activating and regulating growth factor signal in order to support proliferation phase in wound healing (Steffensen *et al.*, 2001) for example TGF-β (Reynold *et al.*, 2005). Others role of integrin are in apoptosis, angiogenesis and neural function (Tsuji, 2004).

Result of study on influence of combination of PBMC and BM-MSC stem cells suggested that this combination was able to accelerate wound healing in rats by increasing percentage of α2β1 integrin . Even so, the influence of this combination stem cells on other adhesion molecules has not yet been carried out. For that reason, further investigation on the influence of combination of PBMC and BM-MSC stem cells on adhesion molecules such as ICAM, VCAM and selectin needs to be developed..

REFERENCES

Abe R, Donnely SC, Peng T, Bucala R, Metz CN (2001). Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. J. Immunol. (166): pp. 7556-62.

Bobis S, Jarocha D and Majka M. 2006. Mesenchymal stem cells: characteristics and clinical applications. Folia Histochemica Et Cytobiologica Vol. 44, No. 4, 2006 pp. 215-230

Cavani A Zambruno G, Marconi A, Manca V, Marchetti M and Giannetti A. 1993. Distinctive Integrin Expression in the New^ly Forming Epidermis During Wound Healing in Humans. J Investigative Dermatology 101:600-604.

Evers LH, Bhavsar D, Maila¨nder P (2010). The biology of burn injury. Experi. Dermatol. (19): pp. 777–783.

Herdrich BJ, Lind RC, Liechty KW (2008). REVIEW Multi-potent adult progenitor cells: their role in wound healing and the treatment of dermal wounds. Cytotherapy 10 (6): pp. 543-550.

Kotowict KGL, Dixon JN, Klein NJ, Peter MJ and Callard RE. 2000. Biological function of CD 40 on human endothelial cells, costimulation with CD 40 ligand and IL-4 selectively induces expression of VCAM and p-selectin resulting in preferential adhesion of lymphosites. Journal immunology. 100. 441-448

Kumar S, Peng FW, David JL (2004). What is new in wound healing. Turkish J. Med. Sci. (34): pp. 147–160.

Larjava H, Salo T, Haapasalmi K, Kramer RH and Heino J. 1993. Expression of integrins and basement membrane components by wound keratinocytes. J Clin Invest ;92:1425–1435

Liu ZJ, Zhuge Y, Velazquez OC (2008). Trafficking and differentiation of mesenchymal stem cells. J. Cellul. Biochem. (106): pp. 984–991.

Lorenz HP and Longaker M T. 2003. Wounds: Biology, Pathology, and Management. Dalam Essential Practice of Surgery Basic Science and Clinical Evidence. Edited by Jeffrey A. Norton, MD. Springer-Verlag New York Berlin Heidelberg

Medina A, Erin B, Nicholas C, Aziz G (2000). Circulating monocytes have the capacity to be trans-differentiated into keratinocyte-like cells. Wound repair and regeneration 17, pp. 268–277 by the Wound Healing Society.

Moioli EK, Clark PA, Chen M, Dennis JE, Erickson HP. Gerson SL and Mao JJ. (2008). Synergistic actions of hematopoietic and mesenchymal stem/progenitor cells in vascularizing Bioengineered Tissues. Plos One . 3 (12): pp. 1-11.

Nagoka A, Yuko K, Yushito H, Minoru H, Kazuhiko T, Douglas AS, Thomas FT and Shinichi S. 2000. Delayed wound healing in the absence of intercellular adhesion molecule-1 or L-selectin expression. American journal of phatology. 157. 237-247

Pilcher BK, Dumin JA, Sudbeck BD, Krane SM, Welgus HG and Parks WC. 1997. Collagenase -1 is required for keratinocyte migration on the type I collagen matrix. J. Cell Biol. 137: 1445-1457.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999). Multi-lineage potential of adult human mesenchymal stem cells. Science (284): pp. 143–147.

Reynold LE, Conti FJ, Lucas M, Groose R, Robinson S, Slone M, Sauderns G *et al.* 2005. Accelarated re-epithelization in β integrin deficient mice is associated with enhanced TGF-β1 signaling. Natural medicine. Volume 11. Number 21 February 167-174

Sasaki M, R Abe, Y Fujita, S Ando, D Inokuma, Hiroshi Shimizu (2008). Mesenchymal stem cells are recruited into skin cell type repair by trans-differentiation into multiple wounded skin and contribute to wound. J. Immunol. 2008; (180): pp. 2581-2587.

Steffensen B, Hakkinen L and Lariava H. 2001. Proteolitic events of wound –healing coordinated interactions among matrix metalloproteinases (MMPs), integrin and extracellular matrix molecules. Critical Reviews in Oral Biology & Medicine 12(5): 373-398

Syamsuhidayat R (1997). Buku ajar ilmu bedah. Edisi Revisi, Penerbit Buku Kedokteran EGC. Jakarta Vern AK, BA Latense (2001). Specimen collection and analysis burn wound. Methods in molecular medicine wound healing. Edited by Luisa D & Aime LB. 78. Human Press Inc.

Vern AK and Latense BA. 2001. Specimen collection and analysis burn wound. Methods in molecular medicine wound healing. Edited by Luisa D and Aime, L.B. Human Press Inc. Totowa, N.J Vol. 78.

Zhang ZG, Bothe I, Hirche F, Zweers M et al. 2006. Interactions of primary fibroblasts and keratinocytes with extracellular matrix proteins: contribution of α2 β1 integrin. Journal of Cell Science 119, 1886-1895