

Methods for Evaluation of the Chemotactic Migration of the Cells in 3D Biomimetic Matrix

Kyung Eun You^{1,2}, Dohyun Kim¹, Byeong-Ju Kwon^{1,2}, Barbora Vagaska^{1,2}, Jae Kyeong Kang^{1,2}, Hyok Jin Seo^{1,2}, Mi Hee Lee¹, and Jong-Chul Park^{1,2*}

¹Cellbiocontrol Laboratory, Department of Medical Engineering

²Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, Korea

(Received August 4, 2012/Accepted August 16, 2012)

Cell migration plays a critical role in various biological processes such as embryogenesis, metastasis of tumor cells and wound healing. Therefore, the migratory behavior of the cells has been researched with various influencing factors including chemoattractants. Most researches have done with 2 dimensional (2D) structured stratum to investigate the cell migration, however, this study suggests three different methods for in vitro studies of cell migratory behavior which are performed in biomimetic 3D structural environment by using collagen matrix. In the firstly introduced method, wound model was built up with a silicon insert and the chemoattractant, PDGF-bb was applied. Then, the movement of the cells to the wound area was observed. It was designed for examination of the potential of wound healing effect of certain agents. Other two methods are to study chemotactic migration of the cells in the 3D matrix in the presence of the chemoattractant. The orientation of the cell migration was examined by the under-agarose cell migration assay as the cells were seeded between the control collagen gel and PDGF-bb containing collagen gel. The cells located in the middle of the migrating chamber were affected by the components in both sides and the direction of their movement was determined. The last method was also performed in a 3D collagen matrix and the cell suspension was directly contacted to the chemoattractant. Therefore, the movement behavior of the cells was influenced by the agent. These protocols have different characteristics to evaluate the migration of the cells, and thus an appropriate method would be chosen for various studies of migration.

Key words: Cell migration, Chemotaxis, Chemoattractant, 3D collagen matrix

Introduction

Cell migration plays a critical role in various biological processes such as embryogenesis,¹⁾ metastasis of tumor cells²⁾ and wound healing.³⁾ Therefore, the migratory behavior of the cells has been researched with various influencing factors. As both the physical and chemical properties of the cell vehicles affect the cellular movement behavior,⁴⁾ many researchers have attempted to modify the properties of the stratum and introduce some stimulators to control the movement of the cells. In this research, the collagen was chosen as a movement base for delivery of the cells. The collagen has been widely used for studies of cell movement as it is a major component of the extracellular matrix (ECM)⁵⁾ and it is also expected to provide biomimetic environment with a 3D structure for in vitro studies of the cellular responses. Moreover, agents influencing the cellular locomotion such as platelet derived growth factor-bb (PDGF-bb),⁶⁾ stromal cell-derived factor-1 (SDF-1)⁷⁾ and bone

morphogenetic protein (BMP)-2 and BMP-4⁶⁾ are often introduced to investigate the cell movement behavior with improved locomotion oriented to the agents. Although these various kinds of factors controlling the cell migration are considered to be studied, appropriate technical tools for examination of the hypothesis, quantitative analysis and observable data are essentially required. In this article, three different technical methods were suggested for evaluation of cell movement particularly in the presence of a chemoattractant in 3D structured environment. The chemoattractant used for this research was PDGF-bb which is mitogenic and chemotactic for connective tissue cells during wound repair.^{8,9)} In this study, two different types of cells, Dental pulp cell (DPC) and neonatal human dermal fibroblast (nHDF) were adopted. DPC is a type of stem cell which has a high proliferative potential for self-renewal. Therefore, the DPCs response to dental injury and participate into the wound healing.¹⁰⁾ During the wound healing process, biological activities such as chemotaxis, proliferation, differentiation, neovascularization, and ECM remodeling are importantly interacted. Consequently, effective control of

*Corresponding author: parkjc@yuhs.ac

these activities would contribute to development of wound healing of dental pulp. Each protocol has different pros and cons, and is specifically designed for certain experimental conditions, for example, wound model and directional migration towards the attractant. Therefore, the examiners can selectively take an appropriate method depending on the subject of the research, and also the combined method with some modifications would be possible for investigation of the variety of migration effectors.

Materials and Methods

Cell culture

DPCs were used for both studies of cell migration in a wound model and in a 3D collagen matrix. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin. The cells used in the experiments were the fifth passage. To perform the under-agarose gel migration assay, nHDFs were cultured in DMEM with 10% FBS and 1% antibiotic solution. All the cell culture reagents were purchased from Welgene (Welgene, Korea).

Cell migration in a wound model

To build up the wound model, a silicon insert (Ibidi, Germany) was placed in the middle of a 35 ϕ cell culture dish (SPL, Germany). Then, DPCs were seeded in two wells of the insert at a density of 5×10^3 cells/cm² with DMEM and incubated at 37°C for 24 h. Type I collagen solution (BD biosciences, NJ, USA) was constructed by mixing with 5x media and reconstitution buffer (0.05 M NaOH, 0.26 M Na₂CO₃, and 0.2 M HEPES) in the ratio of 7:2:1, respectively.¹¹⁾ The buffer was used to adjust the pH to 7.3 and the final concentration of the collagen solution was achieved 1 mg/ml. For the experimental solution with PDGF-bb (Sigma, MO, USA), the concentration of PDGF-bb was diluted 50 ng/ml in the collagen solution. After the cells were well attached on the dish, the media and inserts were removed, and then 1 ml of reconstituted collagen solution or 1 ml of collagen solution containing 50 ng/ml of PDGF-bb was added into the dish. The gelation time of the solution was about 30 min. When the gelation was completed, the gel was covered with 2 ml of DMEM to prevent the surface of the gel from being dry. The cell culture dish for wound model was placed in a 5% CO₂ incubator at 37°C for 36 h, and the migration behavior of the cells was observed by the inverted microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and the images were obtained by using Tomoro image capture program at intervals of 12 h.

The Under-Agarose Cell Migration Assay

To investigate directional cell migration towards chemotactic agents, modified under-agarose migration assay developed by

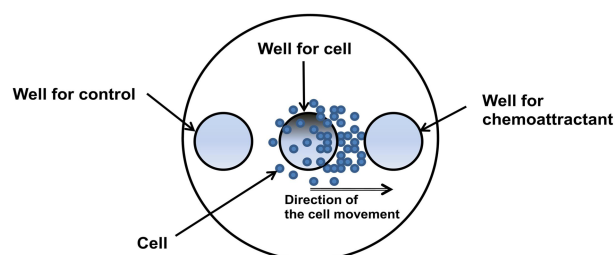


Figure 1. Three identical sized holes were punched on the agarose based chamber for the under-agarose cell migration assay. The cells were seeded into the well in the middle, and the well located in the left hand side was filled with the control collagen solution while the other side of the well was designed for a chemoattractant. The direction of the cell movement was expected towards the well with the chemoattractant in this assay.

Heit and Kubes (2003) was performed.¹²⁾ The assay was originally based on the Boyden assay (1962).¹³⁾ As a cell delivery vehicle, agarose gel was used for this experiment. The agarose powder (Sigma) was dissolved in sterilized distilled water at elevated temperature to produce 4% w/v agarose solution. Then, 1% gelatin solution was mixed with 4% w/v agarose solution at the same amount, and 2x DMEM was mixed with twofold volume of agarose solution finally to constitute 1% agarose solution. The solution was poured into the gel casting cell culture dish. About 30 min of gelation time was given with air dry in a clean bench. When the gel was well formed with appropriate hardness, uniformed thickness and color, and no bubbles or fissures, two or three identical sized holes were punched by using a glass tube with a diameter of 7 mm. The distance between holes were in the range of 3 mm to 5 mm. Prior to performing the cell migration assay, trypan blue (Welgene) was injected in a hole at the right hand side to ensure that the diffusion was occurred through the gel. As the diffusion was started from the hole filled with trypan blue, the progress was observed at 15, 24, 36 and 84 h thereafter. The analyzing technique of the cell migration was described in Figure 1. For the cell migration assay, three holes were punched and a hole in the middle was set for seeding of the cells. Then, the other hole in the left hand side was filled with the control solution and the collagen solution contained PDGF-bb was loaded into the right hand side of the hole. In this experiment, nHDFs were seeded at a density of 5×10^4 cells/cm², and then the tendency of the cell movement was tracked for 4 days. In order to obtain the visible images, propidium iodide (PI) staining was done. On day 4, the cells in the collagen based matrix were washed twice with phosphate buffered saline (PBS) and pre cooled 70% ethanol was added for fixation of the cells. While the cells were fixed, the culture dish was kept in 4°C. After 30 min of fixation time, the PI (Sigma) was applied to the cells for staining in a light protected condition. Then, the cells were observed by using Olympus fluorescence microscope

(Melville, USA) equipped with DP-71 digital camera (Olympus). The images obtained were corrected with Adobe photoshop 7.0.1 and the image J was used to analyze the data to clearly show the displacement of the cells from the well.

Chemotactic migration in a 3D-collagen matrix

The migration of DPCs in a 3D collagen matrix was examined according to the modified method suggested by Reichardt et al.¹⁴⁾ The migration chamber used in this assay was illustrated in Figure 2. To build up the 3D collagen gel chamber, firstly the paraffin mix was prepared in liquid phase as the 1:8 mixture of petroleum jelly and paraffin (Sigma) was heated to 60°C. To provide a base of the migration, a microscopic slide was used, and readily prepared paraffin mix was applied on the slide by using a 6 mm diameter paint-brush to construct a U-shaped wall. Painting was three times repeated to build up the wall of about 300 µm height, and a cover slip was placed on the top. In other words, one open ended pocket was constructed for cell migration. In this apparatus, the collagen provided the cells with a mediator to move through. For the control, the collagen solution was mixed with 5x DMEM and reconstitution buffer in the same manner as the experimental

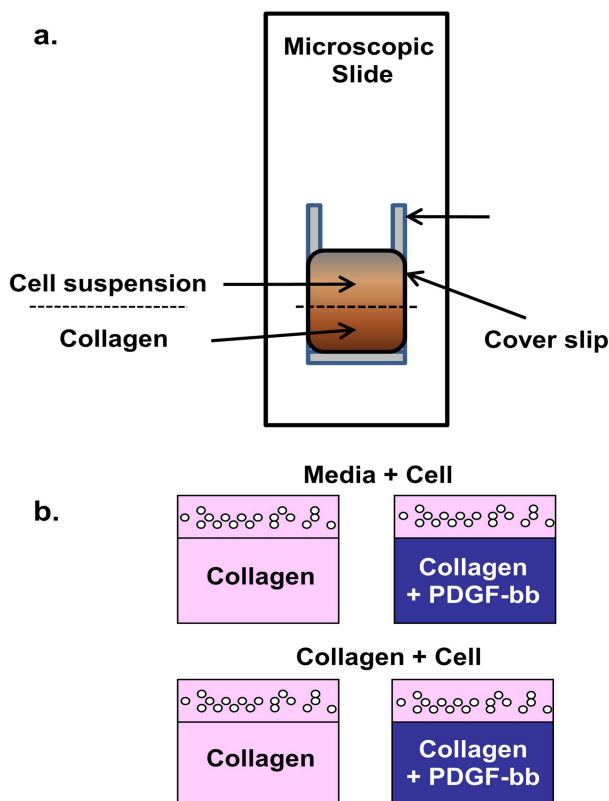


Figure 2. For the chemotactic migration assay in a 3D-collagen matrix, a U-shaped chamber was made of three sided paraffin walls (a). In the well, the collagen or the mixture of the collagen with PDGF-bb was loaded, and cell suspension in media or in collagen was added on both types of collagen gel pre-loaded (b).

solution of wound model. The 5 ng/ml PDGF-bb in collagen solution was also prepared. Before the cell migration assay was executed, trypan blue was used to ensure the diffusion was occurred in the collagen medium. Then, the control collagen solution or the collagen solution containing PDGF-bb was injected into the U-shaped chamber through the open ended side. The cell suspension for this assay was ready in two different types. One of the suspensions was produced as the cells were mixed with media, but another mixture of the cells was with collagen solution. Each type of suspension was loaded on the collagen or collagen with PDGF-bb solution in the chamber. After filling the collagen chamber with cell suspension, the opened end was sealed with paraffin mix. Then, the chamber was incubated for 3 days at 37°C with 5% CO₂, and the migration was observed by the inverted microscope and the image was photographed 3 days after seeding the cells.

Results and Discussion

Cell migration in a wound model

In order to evaluate the cell migration in the collagen containing PDGF-bb, a wound model was applied and the migration in the collagen without any agents was used as a control. As represented in Figure 3, the cells in the collagen with PDGF-bb travelled longer distance for a certain period time compared to the control. It indicates the migration was more actively done in PDGF-bb containing gel. This phenomenon was also able to be observed in the photographs of the cells. According to Figure 4, the cells moving in both collagen-based fields tended to direct towards the wound site but the cells in the collagen with PDGF-bb were more rapidly moved into the space, which was shown in the serial images based on time. As the time went by, cells moving through the PDGF-bb containing collagen were appeared in the wound site with increasing number compared to the number of the cells in the control collagen. PDGF-bb is known not only for a chemoattractant

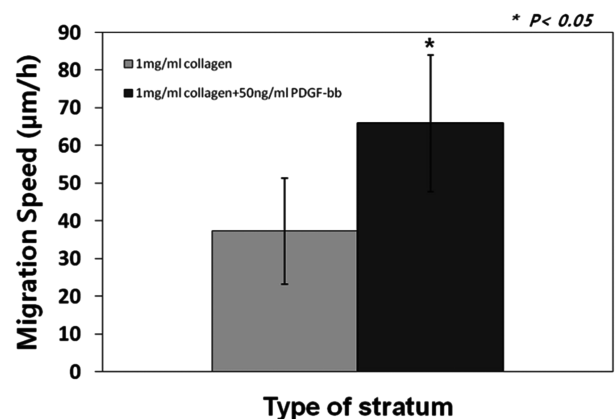


Figure 3. Migration speed of DPCs through the different collagen stratum.

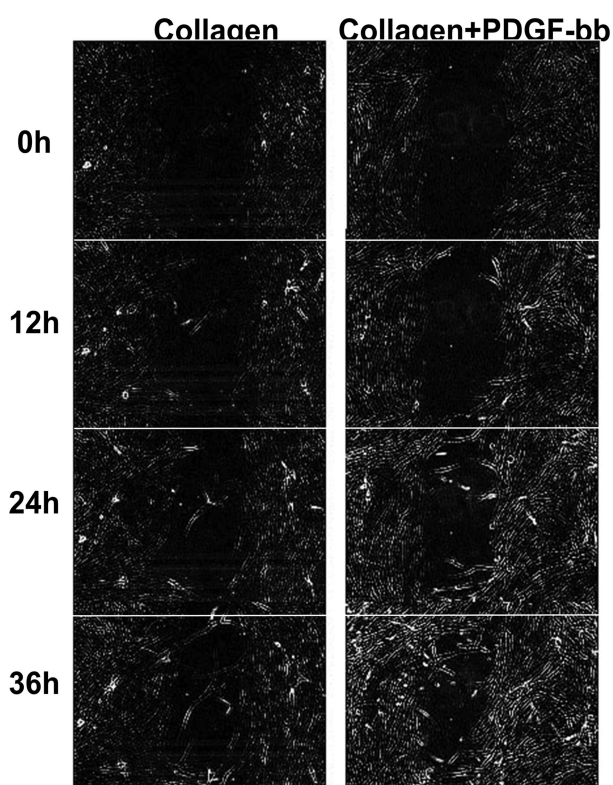


Figure 4. The cell migratory behavior towards the wound area in a 3D collagen gel. Cell movements in the collagen only matrix and the matrix containing PDGF-bb were observed every 12 h for 36 h.

which stimulates chemotactic migration of the cells⁶⁾ but also for a proliferation inducer of DPCs.¹⁵⁾ In addition, cell proliferation and migration may be influenced by collagen.^{16,17)} Therefore, the method of the wound model introduced above has some limitations to clarify the chemotactic activity of PDGF-bb. The cells observed in the middle of the cell culture dish where the wound site might be derived by proliferation and spread of the cells, and the cells already have the behavior to spread to the empty space as shown in the control.¹⁸⁾ For these reasons, alternative methods were considered to explain chemotactic migration of the cells. However, this method for examination of the cell migration is still a useful tool as it provides the evidence of the wound healing effect of the some chemoattractants such as PDGF-bb.

The Under-Agarose Cell Migration Assay

As the under-agarose cell migration assay was carried out, the directional migration towards a chemoattractant was able to be illustrated. Figure 5 shows the diffusion of trypan blue through the agarose gel. The dye was initially loaded into the hole at the right hand side and spreading out around the hole. After 15 h, the dye was reached the hole at the left hand side, and finally all over the collagen gel was occupied by trypan blue in 84 h. As the molecular size of trypan blue is smaller than PDGF-bb, longer time period was required so that PDGF-bb could affect the cell migration from one well to the other side of the well. In Figure 6, displacement of the nHDFs from the well up to day 4 was represented. The cells were migrated to all the direction around the seeding chamber, but the circular area occupied by the cells was shifted towards the PDGF-bb. Put differently, the larger amount of the cells was spread out towards the PDGF-bb and more cells were found at further area from the seeding chamber. Consequently, the modified protocol of the under-agarose cell migration assay is relatively simple to evaluate the directional cell movement. The seeding chamber and the chambers for a control and a chemoattractant are made in the same agarose gel, which means that the cells used for both conditions are identical and has been evenly influenced by both treatment condition as the distance from the seeding chamber is the same. However, the cells would be more sensitively responded to one side and determine their direction of the movement between the control and the chemoattractant. Thus, the movement of the cells is more accurately compared with reduced errors as not separated seeding chambers for each condition are used. Furthermore, the migration tendency of the cells could be directly detected by using this method.

Chemotactic migration in a 3D collagen matrix

The migration assay in a 3D collagen matrix is another tool for the study of the chemotactic migration. While the under-agarose cell migration assay is one of easy methods to examine the directional migration, the cell suspension in media diffuses through the agarose gel. However, the use of collagen gel for a cell vehicle is more mimicked the ECM as it allows the collagen based gel directly to contact with the cell suspension. For the pre-test of diffusion with trypan blue, the dye in the col-

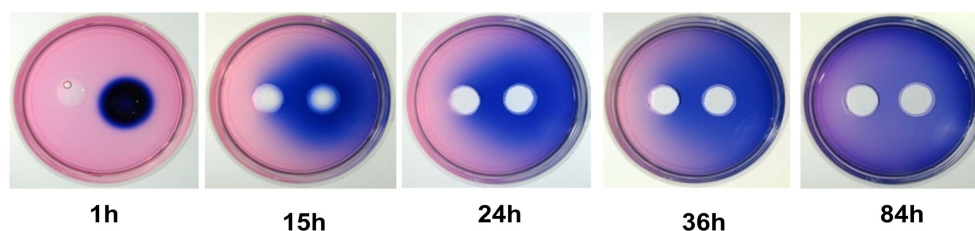


Figure 5. Diffusion of Trypan blue through the agarose gel in the under-agarose cell migration assay. The dye was spread out all over the agarose gel in 84 h.

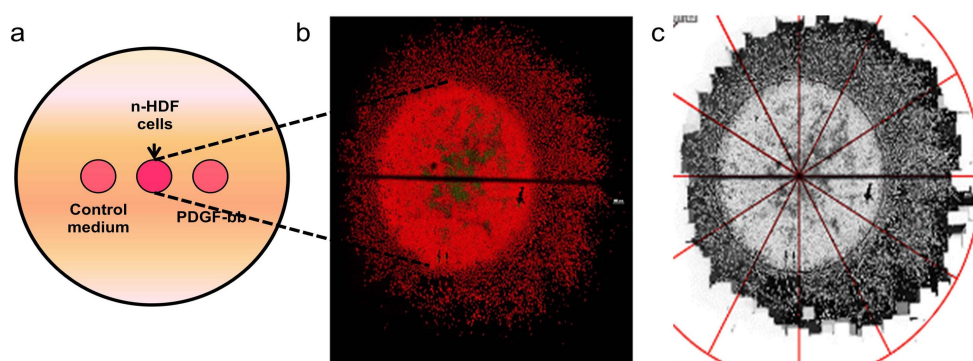


Figure 6. Displacement of the cells towards the PDGF-bb containing well. Design of the under-agarose cell migration assay (a), PI stained image of cell movement (b), Analysis of displacement of the cells using image J (c).

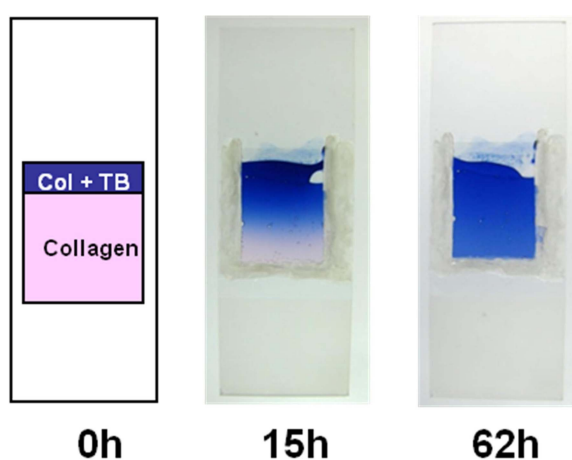


Figure 7. Diffusion of Trypan blue through the collagen gel. The collagen matrix was occupied by trypan blue in 62 h.

lagen mixture was well diffused into the collagen matrix and all over the matrix was dyed with blue in 62 h (Figure 7). Once it was ensured that the collagen gel was able to deliver a substance by the trypan blue diffusion experiment, occurrence of the diffusion of PDGF-bb was supposed. The experiment with DPCs was carried out with two different types of cell suspension. Firstly, when the cell suspension prepared in DMEM was applied in the control collagen or the collagen with PDGF-bb, the cells were not noticeably moved towards the PDGF-bb compared to the movement of the cells seeded on the control collagen gel even on day 3 after seeding of the cells (Figure 8a, 8b). Secondly, another type of cell suspension with media was used for the experiment, and the result is shown in Figure 8c and Figure 8d. The cell movement towards the collagen gel mixed with PDGF-bb was able to be seen more clearly in these images. As shown in figure 8c and figure 8d, the cells in media migrated further in the collagen gel containing PDGF-bb than the cells in the control collagen gel. The result might be caused by that PDGF-bb diffusion encouraged the infiltration into the 3D gel matrix. Furthermore, the density of the cell around a boundary between collagen-PDGF-bb gel and cell

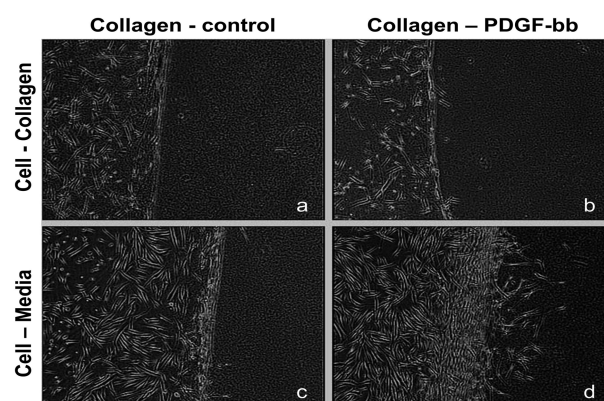


Figure 8. Cell movement in a 3D collagen matrix. The cell suspension in collagen was used (a,b) and the cell suspension in DMEM was also used (c,d). Cells were migrated in collagen only matrix or collagen with PDGF-bb.

suspension was relatively higher than the cell density at the boundary of the control gel and cell suspension (Figure 8c, 8d). These results indicate that the PDGF-bb actively played the role as a chemoattractant during movement of the DPCs. However, the cells in the collagen solution could not apparently represent the activity of PDGF-bb, and so the difference between two groups was unable to be detected (Figure 8a, 8b). Although the role of PDGF-bb as a chemoattractant was proved in the result of the experiment using cell-media suspension, with different cell suspension the result was not correspondent. The delivery of PDGF-bb by diffusion in media was faster than the delivery in the collagen media. Thus, the collagen mixed with the cells might negatively affect the cell migration. These different results between those different types of cell suspension could be also induced by the different gradient of rigidity of the cell suspension. According to Sheetz et al., the interaction between the cells and ECM protein determines the stiffness of the substratum.¹⁹⁾ In our experiment, the collagen might improve the interactions of the cells and the matrix while the interactions in media are relatively weak. For this reason, the cell movement within the suspension in media

might be easier and more cells could move towards the chemoattractants in the same period of time, compared to the movement of the cells in the collagen gel. Therefore, the cell suspension in media would be better to be taken to design the experiment for evaluation of the role of a chemoattractant during the cell migration as one negatively affected factor of the migration is eliminated. In the cell migration method in a 3D collagen matrix, the cells infiltrate into and move in the collagen matrix, which mimics the in vivo environment and makes possible to investigate the cell migratory behavior while conventional methods of migration were mostly performed in 2D environment.

Conclusion

In this article, three different methods to study cell migration with chemoattractants such as PDGF-bb were proposed. The first protocol was designed for wound model, which is functional to investigate the potential wound healing effect of certain chemoattractants. In contrast, it is difficult to declare that the appearance of the cells in wound area is entirely caused by cell migration as some chemoattractants affect migration as well as proliferation of the cells. The other two methods established are adequate to examine the chemotactic migration of the cells with a chemoattractant. Both methods are simple and easy to be performed, and make the observation of the cell migratory behavior available. Those three methods provide not only technical advantages but also the ECM like structure with 3D collagen gel. Therefore, these experiments in biomimetic environment would contribute to obtain the meaningful results for in vitro studies of the cell migration.

Acknowledgments

This work was supported by National Research Foundation (NRF) of Korea Grant funded by the Korean Government (MEST) (Grant number 2012-0006172).

References

1. J. B. Turpen, Cynthia M. Knudson, "Ontogeny of hematopoietic cells in *Rana pipiens*: Precursor cell migration during embryogenesis," *Dev. Biol.*, **89**(1), 138-151 (1982).
2. L. R. Bernstein, and L. A. Liotta, "Molecular mediators of interactions with extracellular matrix components in metastasis and angiogenesis," *Curr. Opin. Oncol.*, **6**, 106-113 (1994).
3. P. Martin, "Wound healing: aiming for perfect skin regeneration," *Science*, **276**, 75-81 (1997).
4. S. B. Carter, "Principles of cell motility: the direction of cell movement and cancer," *Nature*, **208**, 1183-1187 (1965).
5. E. J. Miller, "The collagen of the extracellular matrix," in *Cell and Tissue Interactions*, J.W. Lash and M.M Burger (Ed.), Raven Press, New York, 1977, pp71-86.
6. J. Fiedler, G. Roderer, K. Gunther, and R. E. Brenner, "BMP-2, BMP-4, and PDGF-bb Stimulate Chemotactic Migration of Primary Human Mesenchymal Progenitor Cells," *J. Cell Biochem.*, **87**, 305-312 (2002).
7. A. Aiuti, I. J. Webb, C. Bleul, T. Springer, and J.C. Gutierrez-Ramos, "The Chemokine SDF-1 Is a Chemoattractant for Human CD34⁺ Hematopoietic Progenitor Cells and Provides a New Mechanism to Explain the Mobilization of CD34⁺ Progenitors to Peripheral Blood," *J. Exp. Med.*, **185**(1), 111-120 (1997).
8. G. R. Grotendorst, G. R. Martin, D. Pencev, J. Sodek, and A. K. Harvey, "Stimulation of granulation tissue formation by platelet-derived growth factor in normal and diabetic rats," *J. Clin. Invest.*, **76**, 2323-2329 (1985).
9. M. S. Agren, M. Haisa, and G. R. Grotendorst, "Differential expression of platelet-derived growth factor receptors in porcine fibroblasts cultured from skin and granulation tissue," *Wound Repair Regen.*, **4**, 288-296 (1996).
10. A. J. Sloan and A. J. Smith, "Stem cells and the dental pulp: potential roles in dentine regeneration and repair," *Oral Diseases*, **13**, 151-157 (2007).
11. H. Zhou, K. Kimura, T. Orita, T. Nishida, and K. Sonoda, "Inhibition by female sex hormones of collagen degradation by corneal fibroblasts," *Mol. Vis.*, **17**, 3415-3422 (2011).
12. B. Heit and P. Kubers, "Measuring Chemotaxis and Chemokinesis: The Under-agarose Cell Migration Assay," *Sci. STKE*, **170**, PL5 (2003).
13. S. Bouden, "The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes," *J. Exp. Med.* **115**, 453-446 (1962).
14. P. Reichardt, F. Gunzer, and M. Gunzer, "Analyzing the Physicodynamic of Immune Cells in a Three-Dimensional Collagen Matrix," in *Immunological Tolerance: Methods and Protocols*, P. J. Fairchild (Ed.), Humana Press Inc, Totowa, 2007, pp. 253-269.
15. M. S. Agren, H. H. Steenfors, S. Dabelsteen, J. B. Hansen, and E. Dabelsteen, "Proliferation and Mitogenic Response to PDGF-BB of Fibroblasts Isolated from Chronic Venous Leg Ulcers is Ulcer-Age Dependent," *J. Invest. Dermatol.*, **112**, 463-469 (1999).
16. R. L. Ehrmann, and G. O. Gey, "The growth of cells on a transparent gel of reconstituted rat tail collagen," *J. Natl. Cancer Inst.* **16**, 1375-1390 (1956).
17. K. G. Stenn, J. A. Madri, and F. J. Roll, "Migrating epidermis produces AB₂ collagen and requires continual collagen synthesis for movement," *Nature*, **277**, 229-232 (1979).
18. B. L. Comber and A. I. Gotlieb, "In Vitro Endothelial Wound Repair. Interaction of Cell Migration and Proliferation," *Arteriosclerosis*, **10**, 215-222 (1990).
19. M. P. Sheetz, D. P. Felsenfeld and C. G. Galbraith, "Cell migration: regulation of force on extracellularmatrix-integrin complexes," *Trends Cell Biol.*, **8**, 51-54 (1998).