The New Strategy for Fracture Healing by Ex-vivo Expanded Bone Marrow CD34 Positive Progenitor Cells
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INTRODUCTION

Failures in fracture healing are mainly caused by a lack of neovascularization. We have previously demonstrated that local transplantation of G-CSF-mobilized peripheral blood (GM-PB) CD34+ cells, an endothelial/hematopoietic progenitor enriched cell population,(1) contributed to fracture healing via vasculogenesis and osteogenesis. (2),(3) However, the scarcity of CD34+ cells in PB and the biological side effect of G-CSF administration and apheresis still remain to be possible problems in clinical settings. In terms of expanding clinical application, we postulated the hypothesis that local transplantation of same number of not only bone marrow (BM) CD34+ cells but also culture expanded BM CD34+ cells as that of GM-PB CD34+ cells might exhibit similar or more potent therapeutic potential for fracture healing without increasing the original number of cells for transplantation. In this study, we performed a series of experiments to compare the therapeutic effects of local transplantation of expanded BM CD34+ cells, BM CD34+ cells and GM-PB CD34+ cells using a rat unhealing fracture model.

MATERIALS AND METHODS

Ex-vivo BM CD34+ Cell Expansion: BM CD34+ cells (LONZA) were cultured in specific medium “Stemspan” with 5 growth factors (VEGF, SCF, IL-6, Flt-3lig, TPO) for 1 week. After the expansion, these culture expanded BM CD34+ cells (cEx-BM CD34+ cells), BM CD34+ cells and GM-PB CD34+ cells were used for the following experiments.

In-vitro Study: To assess the ability of angiogenesis and osteogenesis, we performed colony forming assay, tube formation assay and osteoinduction assay with the cells.

Animal Fracture Model: A reproducible model of femoral fracture was created in nude rats (F344/nude rat, 8-12 wk old) with periosteum cauterization. All animal procedures were performed in accordance with the Japanese Physiological Society Guidelines for the care and Use of Laboratory Animals.

Experimental Groups: Rats received local administration of the following cells (1x10^6 cells) or PBS alone (control) with atelocollagen (KOKEN) after fracture creation: (1) Culture expanded BM CD34+ cells (cEx-BM CD34 group), (2) BM CD34+ cells (BM CD34 group), (3) GM-PB CD34+ cells (GM-PB CD34 group) or (4) PBS. (n=20 in each group)

RESULTS AND DISCUSSION

Characterization of Expanded Cells: Total cell number of BM CD34+ cells was increased 22.7±7.8 times after culture expansion for one week. FACS analysis demonstrated 90.3±6% and 60.8% positivity of CD34 expression in BM CD34+ cells and cEx-BM CD34+ cells, respectively. cEx-BM CD34+ cells were positive for CD31, CD44, CD133, CD90, CD105, and CD166, and negative for c-kit and STRO-1.

BM-derived CD34+ cells show high capacity of colony/tube formation and osteogenic differentiation in vitro: After 15 days in culture with methylcellulose- or Matrigel-based medium, number of small and large colonies was significantly great, and formed tubes were also frequently observed in cEx-BM CD34 group compared with the other groups. When cells were cultured in osteogenic differentiation medium for 3 weeks, calcium deposit assessed by Alizarin red staining was striking and mRNA expressions of osteocalcin and collagen I A1 were significantly high in both cEx-BM CD34 and BM CD34 group but not in GM-PB CD34 group by osteogenic induction. These data suggest that BM-derived CD34+ cells have high differentiation potential into osteoblasts.

cEx-BM CD34+ cells exhibit potent therapeutic potential in fracture healing: Fracture healing was assessed radiographically 8 weeks after surgery. Fractures healed with bridging callus formation in 100% animals of cEx-BM CD34 group, 80% animals of BM CD34 group and 50% animals of GM-PB CD34 group, while fracture sites in all animals receiving PBS showed no bridging callus formation and finally fell into non-unions. In micro-computed tomography (μCT) analysis, callus volume of cEx-BM CD34 group and BM CD34 cell group exhibited great values among all groups significantly. (Fig1) In histological evaluation, cEx-BM CD34 group showed the best fracture healing score assessed by Allen’s classification among all groups.

Figure 1: Radiographical assessment 8 weeks after operation

BM-derived CD34+ cell transplantation leads to functional bone healing in fracture: To confirm physiological functional recovery of the fractured bone, biomechanical evaluation by a three-point bending test was performed at week 8 in all groups. cEx-BM CD34 group and BM CD34 group showed significantly high values of stress test ratio (Percent ultimate stress in fractured femur to that in contralateral intact femur) compared with the other two groups.

Enhancement of intrinsic vasculization and osteogenesis: Vascularity in peri-fracture sites was assessed by immunohistochemical staining with isoelectin B4 at week 2. Capillary density was significantly great in cEx-BM CD34 group compared with the other groups. Osteoblast staining with OC at week 2 also revealed that Osteoblast density was great in both cEx-BM CD34 group and BM CD34 group compared with the other groups. (Fig2)

Figure 2: Vascularity with isoelectin B4 staining

BM-derived CD34+ cells promote blood flow recovery in sites of fracture: Laser Doppler perfusion imaging was serially performed at week 0, 1, 2 and 3 in each group. There was no significant difference in the blood flow ratio (fractured site/contralateral site) 1 hour after fracture creation among each group, while the ratio was significantly higher in cEx-BM CD34 group than the other groups at week one, and similar trend could be observed until 2 weeks after surgery. (Fig3)

Figure 3: Serial improvement of blood flow at fracture site

DISCUSSION AND CONCLUSION

Our 7-day culture expansion technique allowed us to obtain around 20 times of BM CD34+ cells maintaining 60% purity of CD34 positivity. Moreover, the culture expanded BM CD34+ cells exhibited striking therapeutic efficacy for unhealing fracture promoting neovascularization and osteogenesis in sites of fracture even in the same number of freshly isolated BM CD34+ cells or GM-PB CD34+ cells. BM CD34+ cells can be obtained from the fracture site at the time of primary operation and preserved for further use. Autologous culture expanded BM CD34+ cell transplantation therapy would be simple but powerful therapeutic strategy for unhealing fracture.

REFERENCES

(3) Mifune Y. et al. Stem Cells,26,1395-1405, 2008