論文の内容の要旨

論文題目　Biological characterization and potential application of umbilical cord Wharton' s jelly-derived mesenchymal stem cells

（臍帯ワルトンゼリー由来間葉系幹細胞の生物学的性状とその応用に関する研究）

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【Introduction】Umbilical cord (UC) Wharton's jelly (WJ) is a rich source of mesenchymal stem cells (MSCs) as well as bone marrow (BM) and adipose tissue. WJ-derived MSCs (WJ-MSCs) exhibit the characteristics of MSCs as defined by the International Society for Cellular Therapy (ISCT) criteria. First, MSCs are plastic adherent when maintained in standard culture conditions; second, they are positive for CD105, CD73, HLA-class I, CD90 and negative for CD45 and HLA-DR surface molecules .The identification of MSCs with the use of specific markers remains discussion. There is no single specific marker can be used to define multipotent MSCs. Stage-specific embryonic antigen (SSEA) 4 has been reported as a stem cell marker in bone marrow-derived MSCs, but whether $SSEA4^+$ cells have growth and differentiation advantages over $SSEA4^-$ cells remains controversial; Third, MSCs have the multipotent ability of various lineages to generate adipocytes, osteoblasts and chondrocytes. Hsieh et al reported that WJ-MSCs were more primitive and more similar to embryonic stem (ES) cells than BM-MSC. Fourth, MSCs have the distinct immunomodulatory effect. Although some conflicting data showed the inflammatory environment might alter the MSCs immunosuppressive effect. My research presentation is consisted of two parts; (1) to investigate the significance of SSEA4 in WJ-MSCs and (2) to study the immunosuppressive effect of WJ-MSCs on activated T cells.

【Methods】WJ-MSCs were collected by the explant (WJe-MSCs) or collagenase methods (WJc-MSCs) and analyzed the characteristics by flow cytometry and reverse transcription polymerase chain reaction (RT-PCR). To study the significance of SSEA4 expression in WJ-MSC, I continued to culture WJ-MSCs until 9 passages (P9) and analyzed SSEA4 expression with gene expression of stem cells markers. In some experiments, I sorted WJe-MSCs by SSEA4 expression using FACS Aria and analyzed the growth and differentiation ability to osteocytes and adipocytes with the induction medium, respectively. To evaluate whether culture conditions influenced the SSEA4 expression, WJe-MSCs were cultured in the medium supplemented with different fetal bovine serum (FBS) concentrations.

Next, I evaluated the WJ-MSCs immunosuppressive effect on activated T cells in vitro and in

xeno-GVHD mice model. Mixed lymphocyte reaction (MLR) was performed on WJ-MSCs or control. CFSE-labeled human peripheral blood (PB) or cord blood (CB) derived mononuclear cells (MNCs) as the responder cells were mixed with irradiated (50Gy) human PB or CB-derived dendrite cells (DCs) at a 10:1 ratio. In the presence of low-dose anti-CD3 antibody, CFSE-labeled responder cells were analyzed by flow cytometry. In order to confirm whether the inhibitory effects of WJ-MSCs on MLR requires the cell-to-cell contact or not, I next performed MLR on separated WJ-MSCs using by transwell chamber, co-cultured MLR with WJ-MSCs supernatant, and evaluated the effect of indoleamine 2,3-dioxygenase (IDO), as a known critical factor of immunosuppression by using the inhibitor. In addition, to evaluate the influence on the immunosuppressive effect by the number of passages, I compared the suppressive effect on MLR by the late passage WJ-MSCs on MLR compared with those of early passage of WJ-MSCs. Although it is still preliminary experiments, I investigated WJ-MSCs immunosuppressive effect in xeno-GVHD mice, which were irradiated and injected with activated T cells transduced luciferase gene. I compared monitored the distribution of human activated T cells by IVIS, weight loss and immunohistological analysis.

【**Results**】Both WJe-MSCs and WJc-MSCs were positive for CD73, CD90, CD105, and HLA-ABC with a small percentage of cells also positive for CD271 and negative for CD34, CD45, and HLA-DR. In addition, both WJe-MSCs and WJc-MSCs expressed the ES cells-related genes, Nanog, Oct4, Klf4, Rex1, and Sox2. I periodically monitored SSEA4, SSEA3, and CD73 expressions in WJe-MSCs and WJc-MSCs during P0–P9. The percentages of $SSEA4^+$ cells at P0 were similar in WJe-MSCs and WJc-MSCs (n=8). However, the percentage of WJe-MSCs $SSEA4^+$cells decreased after the first passage and recovered to the original level by P7, whereas the incidence of WJc-MSCs $SSEA4^+$ cells was relatively stable until P9. In contrast, the percentage of $SSEA3^+$ cells among both WJe-MSCs and WJc-MSCs was highest at P0 that declined and disappeared by P5. I sorted $SSEA4^+$ and $SSEA4^-$ cells from WJe-MSCs at P4 using FACS Aria. The percentage of $SSEA4^+$ cells derived from the sorted $SSEA4^+$ WJe-MSCs decreased rapidly in the first week and then increased gradually until week 4. Interestingly, $SSEA4^+$ MSCs were present in the $SSEA4^-$ WJe-MSCs, and the incidence of $SSEA4^+$ cells in the subsequent cultures was similar to that in the $SSEA4^+$-sorted cells. RT-PCR analysis showed that the sorted $SSEA4^+$ and $SSEA4^-$ WJe-MSCs expressed Nanog, Oct4, and Klf4. Both types of cells showed similar growth rates and could differentiate into adipocytes and osteocytes, although osteogenic differentiation of WJe-MSCs require relatively high concentration of bone morphogenetic protein 2 and a longer culture period up to 5 weeks. I examined SSEA4/3 expression after 1 week in cultures containing various concentrations of FBS (0.1 to 20%). The SSEA4 expression was positively correlated with the FBS concentration, whereas SSEA3 expression was negatively correlated. To see that the increase in SSEA4 expression upon increasing FBS concentration is caused by the change in expression alone but not because of increased WJe-MSCs proliferation, I analyzed SSEA4 expression associated with growth curve with different FBS concentrations. In consistent with the prior data, SSEA4 expression was positively correlated with FBS concentration, while SSEA3 was inversely correlated. The higher FBS

concentration accelerated the proliferation of WJe-MSCs with higher expression of SSEA4. Furthermore, when the sorted SSEA4^{+} and SSEA4^{-} WJe-MSCs were cultured in 1 to 10% FBS for a week, SSEA4 expression in both sorted SSEA4^{+} and SSEA4^{-} WJ-MSCs was positively correlated with FBS concentrations, although the percentage of SSEA4 was higher in SSEA4+ cells than that in SSEA4^{-} cells.

Next, I studied the immunosuppressive effect of WJ-MSCs on activated T cells in mixed lymphocyte reaction (MLR). In MLR, I found that WJ-MSCs efficiently inhibited the responder T cells derived from the same donor of WJ-MSCs, triggered by autologous or allogeneic dendritic cells (DC) (Autologous and allogeneic MLR). The 3rd party MLR also strongly suppressed allogeneic T cells responses triggered by allogeneic DCs indicating that immunosuppressive effect of WJ-MSCs is not restricted by MHC.WJ-MSCs also inhibited T cells proliferation upon PHA stimulation, whereas their inhibitory effects were significantly attenuated by the blockade of cell to cell contact using the transwell chamber. The culture supernatant of WJ-MSCs alone revealed mild inhibitory effects on MLR. These inhibitory effects of the WJ-MSCs were reversed by the addition of indoleamine 2, 3-dioxygenase (IDO) inhibitor, 1-methyltryptophan (1-MT) in a dose-dependent manner, confirming the critical role of IDO reported previously. Concerning the influence of passage number on immunosuppressive effect, I found that WJ-MSCs in the late passage (p10) had the same of immunosuppressive effect compared with that in the early passage (p1).

【Discussion】 To gain insight into the role of SSEA3 and SSEA4 in UC WJ-MSCs, I examined the SSEA3 and SSEA4 expressions on WJ-MSCs obtained by different methods and compared the differentiation abilities of SSEA4^{+} and SSEA4^{-} cells. I did not find any significant differences between WJe-MSCs and WJc-MSCs in cell numbers, MSC surface markers, or ES cell-related gene expressions at P0. In addition, the incidence of SSEA4^{+} and SSEA3^{+} at P0 was similar between the two types, although SSEA3 disappeared rapidly in the early culture passages. The role of SSEA3 and SSEA4 in MSCs remains controversial. Gang et al. (Blood, 109:1743, 2007) reported that SSEA4^{+} cells proliferated predominantly when the culture was initiated from primary BM cells, which were mostly hematopoietic cell. But in my study, both sorted SSEA4^{+} and SSEA4^{-} WJ-MSCs eventually differentiated into osteocytes and adipocytes in a similar manner, and there were no differences in ES-marker gene expression between the SSEA4^{+} and SSEA4^{-} MSCs. Interestingly, SSEA4^{+} cells appeared even from the SSEA4^{-} MSCs, and the incidence of SSEA4^{+} cells derived from the SSEA4^{-} MSCs demonstrated a similar transition pattern as those derived from the SSEA4^{+} MSCs. These results suggested that the culture medium might include the substrate for SSEA4. Finally, I demonstrated clearly for the first time that SSEA4 expression was positively correlated with FBS concentration, while SSEA3 expression appeared to be negatively correlated with FBS concentration, regardless of cell growth or cell concentration. These results indicate that SSEA4 may display altered expression profiles in response to culture medium including FBS and may not be an essential marker of WJ-MSC pluripotency.

Concerning the immunomodulatory effects of MSCs, MSCs have the ability to migrate to inflammatory tissues and suppress adverse immune reactions. In fact, BM-derived MSCs are already

applied for the patients with acute graft versus host disease (aGVHD) with promising efficacy. However, the mechanism by which WJ-MSCs exert their immunosuppressive effects is not completely understood. Here, I demonstrated that WJ-MSCs have the immunosuppressive effect on the stimulated T cells in autologous, allogeneic and 3rd party MLR setting, indicating that immunosuppressive effect of WJ-MSCs is not restricted by MHC. In addition, I observed the inhibitory effect of separated WJ-MSCs on MLR using transwell chamber or co-cultured MLR with WJ-MSCs supernatant, although the immunosuppressive effect was less than that of cell to cell contact. The immunosuppressive effect of WJ-MSCs on MLR was reversed by the addition of IDO inhibitor, 1-MT in a dose dependent manner. It suggests that soluble IDO may play an important role in immunosuppressive effect of MLR. In previous papers, BM-derived MSCs secreted soluble factors such as HLA-G, TGF-β, pGE2, IDO, IL6. I think I need to detect the additional factors to IDO, in cell-to-cell contact between WJ-MSCs and the responder T cells. Furthermore, I am now exploring to administer WJ-MSCs for the treatment of xenogeneic GVHD in mice. But as reported previously in BM-MSCs, WJ-MSCs suppressed GVHD and prolong the survivals, but in some conditions, they stimulated the GVHD. Recently, some reports suggest that the inflammatory environment might alter the MSC from immunosuppression to immunostimulation. To make the steady suppressive function of MSCs, primed MSCs with IFN-γ with or without TNF-α were recommended recently. I thought I need to treat the WJ-MSCs before injection to mice.

【Conclusion】WJ-MSCs could be efficiently obtained by explants method. WJ-MSCs are feasible alternative source of other MSCs for regenerative medicine and immunotherapy. In order to make the effects of WJ-MSCs more effective and safety, further investigations are necessary on the mechanisms of multipotency and immunomodulatory effect.