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Conditioned medium from human amnion-derived mesenchymal stem cells
regulates activation of primary hepatic stellate cells

(羊膜間葉系幹細胞由来培養上清の培養肝星細胞に対する活性化制御)

Background and Aim: Mesenchymal stem cells (MSCs) are stromal cells that exhibit the ability of multilineage differentiation and possess the capacity to self-renew, and reside in almost all organs and tissues. Although MSCs were first reported to be derived from bone marrow, they have been isolated from almost all tissues including adipose tissue, umbilical cord, amnion and dental pulp. Because of advantages that human amnion mesenchymal stem cells (hAMSCs) can be obtained in large amount without invasive procedures and are with enormous proliferative capacity, it has attracted much attention in the fields of cell therapy and regenerative medicine. Researchers have widely demonstrated the anti-inflammatory, anti-fibrotic and anti-apoptotic effects of MSCs in either MSC transplantation or application of conditioned medium obtained from MSCs, and hAMSC transplantation has been reported to ameliorate liver fibrosis in animal models. Hepatic stellate cells (HSCs) are the key contributors to liver fibrogenesis, and following liver injury, HSCs undergo activation which means a transition from quiescent vitamin A-rich cells into proliferative, fibrogenic, and contractile myofibroblasts. As the mechanism by which hAMSCs prevent liver fibrosis is poorly understood, I investigated if conditioned medium from hAMSC cultures (hAMSC-CM) inhibit HSCs activation *in vitro*.

Methods and Results: My experiment mainly consisted of two parts. The first part was the isolation of rat HSCs, and the second part was to explore the effect of hAMSC-CM on primary HSCs. In order to obtain high-purity HSCs, fluorescence-activated cell sorting (FACS) was performed depending on the autofluorescence in primary HSCs, followed by density gradient centrifugation. The sorted cells showed high expression of platelet-derived growth factor receptor beta (*Pdgfrb*) expression, and low expression of C-type lectin domain family 4f (*Clec4f*), and *albumin*, which means that HSCs were efficiently purified but Kupffer cells and hepatocytes were rarely included in the sorted cells. Moreover, the flow cytometry results showed that the sorted cells had high expression of desmin with the rate of 74.6 %, but no expression of CD31 (endothelial cells) and CD163 (Kupffer cells). Furthermore, retinol-based autofluorescence was used to confirm the purity of sorted HSCs, exhibiting a final purity of > 98%. The freshly isolated HSCs were irregularly round shaped, and their cytoplasm was rich in lipid droplets. When excited at 352 nm LASER, the vitamin A-rich lipid droplets emitted blue autofluorescence. Post-culturing for 2 days, HSCs became extended and presented an asteroid phenotype, accompanied by a reduction of lipid droplets. HSCs were further activated by routine culture, and it was difficult to observe autofluorescence post-culturing for 4 days, suggesting that quiescent HSCs were activated by routine culture. Isolated HSCs proliferated well after seeding and long-term culture showed that HSCs proliferated rapidly with good viability. These results fully demonstrated that I obtained HSCs with high purity and activity, and the sorted HSCs could be applied for further experiments. After isolating HSCs, I cultured HSCs in hAMSC-CM or standard medium

(SM) to investigate the effect of hAMSC-CM on HSC activation in routine culture. I found that hAMSC-CM inhibited the expression of α smooth muscle actin (α -SMA) both at gene level and protein level. Moreover, the expression of matrix metalloproteinases (*Mmps*) including *Mmp2*, *Mmp9*, and *Mmp13* was markedly increased by hAMSC-CM, but hAMSC-CM did not affect the synthesis of collagen type I $\alpha 1$ (*Colla1*). Even though the expression of tissue inhibitor of metalloproteinase 1 (*Timp1*) was also up-regulated by hAMSC-CM, the extracellular matrix (ECM)-associated genes were down-regulated by hAMSC-CM, which was evaluated by the ratio of *Mmp13/Timp1*, and the interstitial collagen I which was measured by ELISA was decreased as well. In addition, I also found that the inhibitory effect of hAMSC-CM on HSC activation was concentration-dependent. Next, I examined if hAMSC-CM affect proliferation of HSCs. Gene expression of G2/M-associated proteins such as cyclin B1 (*Ccnb-1*) and B2 (*Ccnb-2*) were inhibited by hAMSC-CM, and cell proliferation assay using CCK-8 also showed that HSC proliferation was inhibited in culture with hAMSC-CM. Besides routine culture, I cultured HSCs with transforming growth factor- β (TGF β), the most efficient collagen synthesis factor, to investigate whether hAMSC-CM antagonize the pro-fibrogenic effect of TGF β . As a result, hAMSC-CM reduced the gene expression of α -Sma promoted by TGF β , indicating that hAMSC-CM inhibited TGF β -induced HSC activation. Although hAMSC-CM did not affect the expression of *Colla1* in routine culture, the up-regulated expression of *Colla1* induced by TGF β was significantly suppressed by hAMSC-CM. Along with up-regulation of *Colla1*, TGF β inhibited expression of *Mmps* to promote ECM accumulation. hAMSC-CM remitted this inhibition and significantly increased the expression of *Mmps*. The ratio of *Mmp13/Timp1* was obviously upgraded by hAMSC-CM as well, which implied that hAMSC-CM promoted ECM degradation even in the presence of TGF β . Overall, hAMSC-CM exhibited the capacity of inhibiting the accumulation of ECM at gene expression level. Interestingly, the gene expression of TGF β receptor 1 (*Tgfb1*) was increased more by hAMSC-CM than by TGF β . Furthermore, given that hAMSCs are likely to be contaminated with fibroblasts by using current isolation scheme and in order to investigate whether the suppressive effect on HSCs is specific to hAMSC-CM, I cultured HSCs with conditioned medium obtained from skin fibroblasts (fibroblast-CM). Although fibroblast-CM significantly enhanced *Colla1* expression and suppressed *Timp-2* expression in HSCs, it increased the expression of *Mmps* and *Timp-1*, and decreased the expression of α -Sma and *Ccnbs* as well.

Discussion: In the present study, I obtained highly purified HSCs and I demonstrated the anti-activation effect of hAMSC-CM on HSCs *in vitro*. Followed by density gradient centrifugation, additional autofluorescence-based FACS greatly improved the purity of HSCs. Moreover, given the specificity of HSC markers is still questionable, in order to accurately confirm the purity of isolated HSCs, autofluorescence was used instead of using those markers. α -SMA is the activation marker of HSC, and the decreased α -SMA expression at both gene and protein levels indicated HSC activation was inhibited by hAMSC-CM. When HSCs are activated, large amounts of COL1 are secreted, leading excessive ECM accumulation. In this study, I showed that hAMSC-CM decreases COL1 accumulation. On the basis of that hAMSC-CM does not influence *Colla1* expression but has a positive effect on *Mmps* and *Timps*, I believe that instead of inhibiting COL1 synthesis in routine culture of HSCs, hAMSC-CM reduces ECM accumulation by promoting COL1 degradation. HSC activation is accompanied by massive cell proliferation, and proliferation assay indicated that hAMSC-CM reduces HSC proliferation in this study. Inhibited expression of *Ccnbs* suggested that this process is performed by regulating the cell cycle. TGF β is the most efficient fibrogenic factor and HSCs can be activated further by TGF β . In this study, I observed that hAMSC-CM inhibits TGF β 1-induced HSC activation. In addition, I found that hAMSC-CM contains TGF β 1, and hAMSC-CM induced *Tgfb1* up-regulation is most likely caused by additional exogenous TGF β 1. The similar effects shown by hAMSC-CM and fibroblast-CM suggest that hAMSCs and fibroblasts have something in common in certain functions, but their common mechanism is unclear.

Conclusions: The major findings in my study were (1) hAMSC-CM inhibited the activation of HSCs, (2) hAMSC-CM regulated the accumulation of ECM during the activation of HSCs, (3) hAMSC-CM suppressed the proliferation of HSCs. These findings demonstrate that hAMSC-CM can modulate the function of HSCs via secretory factors and provide a plausible explanation for the protective role of hAMSCs in liver fibrosis.