Isolation and Characterization of Synovial Mesenchymal Stem Cells in Charcot Neuroarthropathy

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Introduction: Charcot neuroarthropathy (CNA) is a late complication of diabetes and difficult to manage. Bone and joint destruction caused by CNA in a diabetic foot often leads to foot deformity, ulceration and infection. The pathology of CNA is the interplay peripheral neuropathy, chronic disorder of glucose metabolism and inflammation. Fibroblast-like synoviocytes in the synovium have been identified playing an important role in the development of CNA. Similarly resided in the synovial stroma are mesenchymal stem cells (MSCs), which participate in tissue repair and immune modulation. Synovial MSCs may as well play a role in the development of CNA and be critical for repairing of the damaged bone and cartilage in a CNA joint. It has been shown that, in diabetes, the number of bone marrow MSCs is decreased and MSCs have impaired functions in proliferation and differentiation. Under a pathological condition as complicate as in CNA, synovial MSCs endure intriguing influence and their functionality becomes uncertain. The functional state of MSCs is hard to define but can be partially assessed in several ways. In tissue culture, the first recognizable sign of MSCs is the colonies they formed. Those MSC colonies, however, are not uniform, indicating the inherent heterogeneity and giving a hint of the state of MSCs. The goal of this study was to investigate the quantity and colony-forming features of synovial MSCs, as a part of the pathology of CNA joints.

Methods: Synovial samples were collected during reconstructive surgery for CNA joints (n=4) and trauma or osteoarthritis (n=4) in the foot. After enzymatic digestion, stromal cells in the synovium were cultured. After one passage, the cells from each donor were plated in triplicate in 10-mm tissue culture plates at a density of 3,000 per cm2. After two weeks, the cells were fixed with cold ethanol and stained with Crystal Violet for colony formation. The stained plates (n=12) were imaged and all the images were adjusted to a standard threshold (8,250). A colony (> 50 cells) was defined on image as >2,000 pixels2. Only half of a plate was processed to avoid glare noises. The Analyze Particles function in ImageJ program (NIH) was used for automatic tracing and numbering of colonies. Following the circling of an individual colony, the Analyze Particles function was used to obtain the “% area” output. This gave the percentage of the area in a colony that had been turned to black in the binary process, as MSC coverage or density of the colony.

For data analysis, all the individual colonies of the CNA and non-CNA groups were inputted into the R programming language environment, with the input containing each colony’s size and corresponding density. Using R, the colonies were sorted by size. The colonies were then stratified by size with every additional 5,000 pixels2 being a new subgroup. These CNA subgroups’ colony densities were then compared to the corresponding non-CNA subgroups, using an unpaired T-Test, with p<0.05 being the determinant of being statistically significant. To further analyze colonies in the CNA and non-CNA groups, the colonies were ordered by size or density and divided into thirds. The small, medium and large sizes of colonies, and low, medium and high densities of colonies were compared between the CNA and non-CNA groups, using unpaired T-test.

In addition, synovial MSCs from CNA joints and non-CNA joints were characterized by the expression of MSC cell surface markers using flow cytometry. Synovial MSCs of the CNA and non-CNA groups were cultured in osteogenic, chondrogenic and adipogenic medium. Osteogenic, chondrogenic and adipogenic differentiation of the MSCs were assessed with Alizarin Red, Safranin O and Oil Red staining, respectively.

Results: MSCs isolated from both CNA and non-CNA synovium expressed comparable CD73, CD90 and CD105. Both groups of MSCs differentiated into osteogenic, chondrogenic and adipogenic lineages. The number of colonies formed by CNA-MSCs was 6 ± 3.5 per (1/2) plate, while it was 43 ± 21.6 by the non-CNA MSCs (p < 0.05; Fig 1). The average size (pixels) of colonies was smaller in the CNA MSCs than the non-CNA MSCs (4780±1232 vs. 7960±1687; p < 0.05) but the average colony densities of CNA MSCs and non-CNA MSCs were not significantly different. According to the size, colonies of both CNA MSCs and non-CNA MSCs were stratified into thirds, i.e. the large, middle and small subgroups. In the corresponding subgroups, CNA MSCs formed smaller colonies than non-CNA MSCs did (p <0.05; Fig 2). When the colonies were similarly stratified according to density, in the high density subgroup, formed by the CNA MSCs had a reduced density compared with the non-CNA MSCs (30±6 vs. 35±10; p < 0.05). The other two subgroups, with low and medium densities, were not different between CNA and non-CNA groups in colony density.

Discussion: This study revealed a significantly reduced population of MSCs in CNA synovium. Unlike in diabetes, where the reduced number of MSCs is likely the result of disorder of glucose metabolism, the depletion of synovial MSCs in CNA attributes to a combination of diabetic pathology, peripheral nerve neuropathy and inflammation. The reduced number of MSCs in Charcot tissues could negatively impact the healing of the damaged joints and adds another layer of difficulty to the treatment of Charcot joints.

Although CNA MSCs possess similar properties as non-CNA MSCs, such as the expression of common MSC surface markers and the capability of differentiation, they formed colonies with distinct features. In general, the colonies formed by MSCs vary in size, shape, number of cells and the density of cells. This study demonstrated that CNA MSCs formed smaller size of colonies than non-CNA MSCs. This may be due to slower proliferation of CNA MSCs because the density of the colonies formed by CNA MSCs was virtually the same as the colonies formed by non-CNA MSCs.

The varied colony sizes and densities are features of heterogeneous MSCs. Changes of these features may mean shifting of subpopulations or properties of MSCs. After colonies were stratified, according to colony size and density, or colonies formed by CNA MSCs were smaller than the ones formed by non-CNA MSCs in each category. The density of the colonies formed by CNA MSCs were the same as, or lower than, the colonies of non-CNA MSCs. Image analyses employed by this study were based on a single stain of colonies. It is convenient to perform and suitable for high throughput screening MSCs. The biological meaning of the colony analysis, however, are to be determined.

In summary, detailed analyses of colonies formed by CNA MSCs suggest that synovial MSCs in CNA are reduced in quantity, with altered biological property.

Significance: CNA represents a complex pathology at the late stage of diabetes. Understanding the biology and pathological role of synovial MSCs is fundamental to develop novel strategies of managing CNA.