Pro-inflammatory Cytokine Production in Co-culture of Human Monocytes and Synovial Fibroblasts from the Human Temporomandibular Joint

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Abstract

The synovial membrane is composed of fibroblast-like cells (synovial fibroblasts) and macrophage-like cells. Synovial fibroblasts and monocytes/macrophages are believed to interact and to play a critical role in the development of synovial inflammation. In this study, we investigated the protein production of pro-inflammatory cytokines in co-culture of synovial fibroblasts isolated from the temporomandibular joint (TMJ) and monocytes isolated from peripheral blood. Monocytes that had attached to the plastic surface after culture for 3 days in RPMI containing 10% FBS, exhibited two types of morphologies: “fried egg” and “spindle-like”. Both types of monocytes stained positive for the macrophage-specific markers CD14 and CD68. Synovial fibroblasts were co-cultured with these monocytes for 12 days. Synovial fibroblasts or monocytes were also cultured alone for 12 days. The conditioned media were collected every 3 days, following which fresh medium was added to each culture. The protein concentrations of IL-1β, IL-6, and IL-8 in the conditioned media were measured using ELISA. The protein production of IL-1β, IL-6 and IL-8 was greatly increased in the co-culture of synovial fibroblasts and monocytes compared to each monoculture. These data suggest that interaction between monocytes/macrophages and synovial fibroblasts is likely to contribute to the promotion of, and to increase the inflammatory condition in the TMJ.

Keywords:
interleukin-1β, monocytes, synovial fibroblasts, temporomandibular joint

Introduction

Intracapsular pathological conditions of the temporomandibular joint (TMJ) are characterized by limited jaw motion, as well as by joint sound and pain, and can include disk displacement (DD)/internal derangement (ID) and osteoarthritis (OA). Synovitis, which often accompanies intracapsular pathological conditions, is characterized by chronic inflammatory changes such as hyperplasia of the synovial lining (1) and an increased number of new capillaries and small vessels (2–4).

The synovial membrane, which covers all the intra-articular structures except the articular cartilage of the eminence and fossa, the mandibular condyle, and the articular disc of the temporomandibular joint (TMJ), is composed of fibroblast-like cells (synovial fibroblasts) and macrophage-like cells (5). Synovial fibroblasts are mesenchymal cells that display many characteristics of fibroblasts including vimentin and propyl 4-hydroxylase expression (6). It has been suggested that synovial fibroblasts produce and secrete both synovial fluids and extracellular matrix components such as collagen, as well as a number of putative mediators of inflammation. In contrast, it is plausible that macrophage-like cells are derived from a monocyte-lineage and have high migratory and phagocytic activity. It has been reported that macrophages and fibroblasts communicate via soluble autocrine, paracrine and juxtacrine signals associated with direct cell-to-cell contact, and that both chemical and physical cues exchanged between macrophages and fibroblasts can be important in

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OA and rheumatoid arthritis (RA) (7).

Our hypothesis was that interaction between synovial fibroblasts and macrophages would simulate a disease environment similar to that present in developing OA. In this study, we examined the production of pro-inflammatory cytokines in a co-culture of synovial fibroblasts isolated from a patient with ID of the TMJ and human monocytes.

Materials and Methods

Isolation of synovial fibroblasts and monocytes

Human synovial tissue was obtained from a female patient with ID who underwent arthroscopy of the TMJ (age, 26, female) and provided informed consent for the surgery and for the use of her tissue specimens. Human synovial fibroblasts were isolated from the synovial tissues of the patient using the out-growth method of Ogura et al. (8). The culture medium used was RPMI 1640 (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Technologies, Graveseano, TI, Switzerland), 100 

μg/mL penicillin G (Meiji, Tokyo, Japan), 100 μg/mL kanamycin sulfate (Meiji), and 250 ng/mL Fungizone (Gibco, Grand Island, NY, USA). The medium was changed twice per week. For all experiments, passage 6 to 10 synovial fibroblasts were used.

Monocytes, which were isolated from peripheral blood using positive immunomagnetic selection directed against CD14, were purchased from Lonza (Poetics™ CD14 + monocytes; Lonza, Basel, BS, Switzerland). Monocytes were cultured in RPMI 1640 supplemented with 10% FBS, 100 μg/mL penicillin G, 100 μg/mL kanamycin sulfate, and 250 ng/mL Fungizone.

For co-culture experiments, synovial fibroblasts and monocytes were each seeded at a density of 2.5 x 10^5 cells per well onto a 24-well plate in RPMI medium containing 10% FBS. For monocultures, synovial fibroblasts or monocytes were plated at a density of 5 x 10^5 cells per well. Every 3 days thereafter the medium was replaced with fresh medium. Culture supernatants were collected after 3, 6, 9, and 12 days of culture, and were stored at -80°C until use.

The experiments using synovial fibroblasts were performed according to the guidelines established by the Institutional Review Board of the Nihon University School of Dentistry at Matsudo (Ethics Committee Registration Number: EC15-039).

Immunocytochemistry

Monocytes were fixed with a 10% formalin neutral buffer solution for 30 min at room temperature, were permeabilized in 0.1% Triton X-100, and were blocked with 10% normal goat serum (Thermo Fisher Scientific, Waltham, MA, USA). Primary antibodies were applied for 1 h at room temperature. The cultures were then washed by PBS, following which secondary antibodies were applied. The primary antibodies used were: the anti-CD14 antibody 1H5D8, (Abcam, Cambridge, England), and the anti-CD68 antibody KP1 (Abcam). Secondary antibodies used were: goat anti-mouse/human conjugated to Alexa Fluor® 488(A-11001; Thermo Fisher Scientific). Nuclei were counterstained with 4, 6-diamidino-2 phenylindole (ProLong® Gold Antifade Mountant with DAPI; Thermo Fisher Scientific).

Imaging and image processing

Bright-field images of macrophage differentiation cultures were acquired using an Olympus CKX41 inverted microscope fitted with a DP20 digital camera (Olympus, Tokyo, Japan). Images of neuronal differentiation cultures that were stained were acquired using an Olympus BX51 microscope equipped with a DP72 digital camera (Olympus). All digital images were processed (merge, black balance) using GIMP Portable 2.8. (The GIMP Development team.)

Enzyme-linked immunosorbent assay (ELISA)

IL-1β, IL-6, and IL-8 levels in the conditioned medium were measured using an ELISA kit (R&D Systems, McKinley, MN, USA), according to the manufacturer’s protocol.

Statistical analysis

Data are expressed as mean values ± standard deviations (SD). The statistical significance for multiple comparisons was assessed using one-way ANOVA. Post hoc analyses were carried out using the Student-Newman-Keuls (SNK) Multiple Comparison Test. Statistical significance is indicated in the graphs as P-values.

Results

Characterization of monocytes

First, we examined the morphology of the monocytes isolated from peripheral blood. Twenty-four hours after seeding, several cells had attached to the surface of the culture plate, but had not expanded. Other cells were
suspended (data not shown). Fig. 1a and b shows representative morphologies of monocytes cultured on the culture plate using RPMI containing 10% FBS for 6 days. The monocytes attached to the plastic surface exhibited two types of cell morphology by phase-contrast microscopy: a “fried-egg”-like (Fig. 1a) and a spindle-like (Fig. 1b) morphology.

Expression of the monocyte-/macrophage-specific markers, CD14 and CD68, by these cells were examined using immunofluorescent staining. Both monocytes with a “fried-egg”-like and a spindle-like morphology displayed positive staining for CD14 and CD68 (Fig. 1c-f).

**Co-culture of monocytes and synovial fibroblasts**

Synovial fibroblasts isolated using from the human TMJ had a spindle-like morphology under phase-contrast microscopy. Synovial fibroblasts had expanded by culture day 9 compared to day 3 (Fig. 2a, d). Morphological changes of the cells were not found in the culture after day 12 (data not shown).
In single culture of the monocytes, most of the monocytes that had adhered to the surface of the plate showed a fusiform morphology on culture day 3 (Fig. 2b). The number of monocytes with a cell morphology that had changed from a rounded shape to that of a "fried-egg" - or a spindle-like shape had increased (Fig. 2e).

Synovial fibroblasts in the fibroblast/macrophage co-culture had a more dendritic morphology than the fibroblasts in the monoculture. After 3 and 9 days in co-culture, most of the monocytes were observed to have adhered to synovial fibroblasts (Fig. 2c, f).

**Production of pro-inflammatory cytokines**

To investigate the effect of the interaction between human monocytes and synovial fibroblasts from the human TMJ on the production of pro-inflammatory cytokines, we measured the level of cytokines such as IL-1β, IL-6 and IL-8 in the conditioned media of the cell cultures.

The production of IL-1β, IL-6 and IL-8 was dramatically increased in co-culture compared to the monocultures of monocytes or synovial cells (Fig. 3). The IL-1β level was highest in the conditioned medium of the 3-day co-culture and subsequently decreased in a time-dependent manner. IL-1β was not detected in the medium of the day 12 co-culture (Fig. 3a). In contrast, IL-6 and IL-8 protein production was sustained in the co-culture over 12 days of culture (Fig. 3b, c).

The IL-1β protein level in the media of the monoculture of synovial fibroblasts over 12 days of culture was below the detection limits of the ELISA kit. In contrast, protein production of IL-6 and IL-8 increased in a time-dependent manner in the media of the monoculture, although the levels of these cytokines were significantly lower compared to their levels in the media of the fibroblast/monocyte co-culture.

In the monocultures of monocytes, a small amount of IL-1β was only detected in the conditioned medium of the 3-day culture (Fig. 3a) and IL-6 could not be detected in the conditioned medium at any culture time point (Fig. 3b). IL-8 production increased in a time-dependent manner although IL-8 levels were very low in the media of the monocyte monoculture compared to those in the media of the co-culture even on culture day 3 (Fig. 3c).

**Discussion**

Synovial inflammation is a process characterized by synovial thickening and cell infiltration. Histological analysis of synovium with OA shows an increased number of
infiltrating cells, mainly consisting of macrophages (9). Monocytes/macrophages and synovial fibroblasts are in intimate contact in the synovium and are believed to play a critical role in the development of rheumatoid arthritis (10). It has also been reported that CD68 positive cells were found in synovial tissue with OA in TMJ (11). However, cell-cell interactions inside the synovial tissue are still poorly understood in the pathological conditions of TMJ. Using synovial fibroblasts from the TMJ and human peripheral blood monocytes, we investigated cytokine production during the interaction between these two cell types.

The human peripheral blood monocytes were found to display two types of cell morphology: a “fried egg” and a spindle-like morphology, on culture day 3. Both types of monocytes stained positive for CD14 and CD68 using immunocytochemistry. CD68 positive staining is mainly found on synovial macrophages located in the OA synovial membrane (9). We consider that these findings suggest that the monocytes used in this study can differentiate into macrophages by monolayer culture and that these monocytes are useful as an in vitro synovitis model.

Next, we examined the protein production of inflammatory cytokine such as IL-1β, IL-6, and IL-8 in monoculture of synovial fibroblasts or monocytes, and in co-culture of synovial fibroblasts and monocytes. The production of IL-1β, IL-6, and IL-8 was significantly increased in the co-culture of synovial fibroblasts and monocytes compared to in the monoculture of each cell type.

IL-1β protein was detected in the media of the co-culture at the early culture days of day 3 and day 6. IL-1β is a potent pro-inflammatory cytokine that is produced in response to infection or injury. IL-1β is synthesized as an inactive precursor molecule (pro-IL-1β, 31 kDa) by several cell types such as macrophages upon the activation of pattern recognition receptors by pathogen- or damage-associated molecular patterns (12). Activated macrophages then require an additional stimulus to trigger the assembly of a multimolecular complex called the inflammasome that results in the activation of caspase-1 and the processing of pro-IL-1β to an active, secreted IL-1β molecule (13). In our previous study, IL-1β protein could not be detected in the conditioned medium of synovial fibroblasts treated with inflammatory cytokines such as IL-1β, IL-17, and TNF-α, although the gene expression of IL-1β was significantly increased in synovial fibroblasts by treatment with these cytokines (14–16). Although it is unclear what signal (s) activated IL-1β and led to the production of IL-1β protein by synovial fibroblasts and/or monocytes in the present study, the co-culture of synovial fibroblasts and monocytes did lead to the processing and maturation of pro-IL-1β into the active form of IL-1β. It is well established that synovial fibroblasts and macrophages are stimulated by IL-1β, and produce several inflammatory factors and tissue degradation enzymes (6, 8, 14–15). It is hypothesized that a transient increase in IL-1β production by the interaction between synovial fibroblasts and monocytes/macrophages may enhance inflammation in synovial tissue of the TMJ.

Likewise, high levels of IL-6 and IL-8 protein production were observed in the fibroblast/monocyte co-culture compared to production in the individual monocultures. IL-6 has an important role in inflammation and tissue destruction in joint diseases such as RA (17), and its concentration is elevated in the synovial fluids of arthritic patients (18, 19). IL-6 was shown to have an important role in inflammation-evoked osteoclast formation and bone erosion (20). It was recently demonstrated that IL-6 can promote Th17 cell differentiation in effector CD4+ T cell subsets (21). This function of IL-6 is thought to play a major role in the development of RA (22). In the TMJ, the IL-6 level was also increased in synovial fluid from patients with ID and/or OA (23, 24). It has been reported that IL-6 production increased in co-cultures with monocytes cell line U937 and synovial fibroblasts from patient with rheumatoid arthritis compared to monoculture (25). Therefore, the interaction between monocytes and synovial fibroblasts is likely to associate with the progression in inflammation. In contrast, IL-13 was not detected in both co-culture and monoculture in this report (25). We consider that the ability for IL-13 production may be different between monocyte cell line U937 and CD14 positive monocyte isolated human blood.

IL-8 is a member of a chemokine superfamily that is chemotactic for neutrophils (6) and T-cell subsets (26), and that activates neutrophil functions including calcium mobilization, degranulation, and respiratory burst (27). Neutrophils and T cells that infiltrate into synovial tissue are believed to play pathological roles in the development and continuation of RA via their abilities to release degradative enzymes and various products of oxidative metabolism (28), although chemotaxis is a necessary function of homeostasis. It has been reported that IL-8 was detected in the synovial fluid from patients with TMJ disorders (29), and that inflammatory cells were present in the fluid from patients.
with TMJ disorders but not from healthy individuals (30).
The excessive production of IL-6 and IL-8 in the interaction between synovial fibroblasts and monocytes/macrophages thus appears to be related to the pathological condition in the TMJ.

In this study, our findings demonstrated that the coculture of monocytes/macrophages and synovial fibroblasts strongly increased the protein production of IL-1β, IL-6 and IL-8, which are pro-inflammatory cytokines that appear to be related to abnormalities associated with intracapsular pathological conditions of the TMJ. We suggest that this interaction between monocytes/macrophages and synovial fibroblasts is likely to contribute to the promotion of, and to increase the inflammatory condition in the TMJ.

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