Cadherin-11 Provides Specific Cellular Adhesion between Fibroblast-like Synoviocytes

Xavier Valencia, Jonathan M.G. Higgins, Hans P. Kiener, David M. Lee, Theresa A. Podrebarac, Christopher C. Dascher, Gerald F.M. Watts, Emiko Mizoguchi, Barry Simmons, Dhavalkumar D. Patel, Atul K. Bhan, and Michael B. Brenner

1Department of Medicine, Division of Rheumatology, Immunology, and Allergy, Brigham and Women’s Hospital, and 1Immunopathology Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02115
2Department of Orthopedic Surgery, Brigham and Women’s Hospital, and 1Immunopathology Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02115
4Department of Medicine, Division of Allergy and Immunology, Duke University Medical Center, Durham, NC 27710

Abstract

Cadherins are integral membrane proteins expressed in tissue-restricted patterns that mediate homophilic intercellular adhesion. During development, they orchestrate tissue morphogenesis and, in the adult, they determine tissue integrity and architecture. The synovial lining is a condensation of fibroblast-like synoviocytes (FLS) and macrophages one to three cells thick. These cells are embedded within the extracellular matrix, but the structure is neither an epithelium nor an endothelium. Previously, the basis for organization of the synovium into a tissue was unknown. Here, we cloned cadherin-11 from human rheumatoid arthritis (RA)-derived FLS. We developed L cell transfectants expressing cadherin-11, cadherin-11 fusion proteins, and anti–cadherin-11 mAb. Cadherin-11 was found to be expressed mainly in the synovial lining by immunohistologic staining of human synovium. FLS adhered to cadherin-11–Fc, and transfection of cadherin-11 conferred the formation of tissue-like sheets and lining-like structures upon fibroblasts in vitro. These findings support a key role for cadherin-11 in the specific adhesion of FLS and in synovial tissue organization and behavior in health and RA.

Key words: synovium • synoviocyte • rheumatoid arthritis • cadherin • cell adhesion

Introduction

During development, the differential adhesive properties of various cell populations facilitate the cell rearrangement required for tissue morphogenesis. This cell sorting is directed in part by cadherins such as E-cadherin in epithelia and N-cadherin in the nervous system. Postnatally, cadherins play a major role in maintaining tissue integrity and architecture (1, 2). Cadherins are transmembrane glycoproteins expressed in restricted patterns that mediate homophilic adhesion between cells. In many tissues, adherens junctions are formed by cadherins linked through intracellular catenins to the actin cytoskeleton. These multiprotein complexes can activate intracellular signaling pathways, influence cytoskeletal organization, and orchestrate multicellular arrangements (1, 2). These properties of cadherins are likely to contribute to their role in tumor progression (2). Indeed, changes in cadherin expression have been associated with cell transformation and tumor metastasis (3–5).

The physiological role of the synovium is to balance cartilage remodeling and provide lubricant and nourishment for the synovial fluid that bathes the avascular cartilage surfaces of diarthrodial joints (6). The synovial membrane lining is composed of two major cell types: type A, macrophage-like synoviocyte, and type B, fibroblast-like synoviocyte (FLS). These synoviocytes form cell to cell contacts as well as attachments to an ordered extracellular matrix (ECM). However, the lining lacks a classical basement membrane, and the cellular contacts lack tight junctions and desmosomes (6, 7). Therefore, the lining does not possess the architecture typical of epithelium or endothelium.

The synovium displays marked changes in rheumatoid arthritis (RA) where the lining undergoes striking hyperplasia and the underlying loose connective tissue becomes massively infiltrated with leukocytes. These changes are associ-
ated with activation and condensation of the mesenchymal synovial cells that produce large amounts of matrix-degrading metalloproteinases, inflammatory cytokines, and lipid mediators of inflammation. This mass of cells extends and attaches onto cartilage and becomes locally invasive, damaging cartilage and eroding adjacent bone, resulting in permanent joint destruction.

Surprisingly, despite the central role of the synovial membrane in diarthrodial joint physiology in health and in inflammatory arthritis, little is known about the molecular basis for the organization of this tissue (6, 7). We hypothesized that cadherins might mediate homophilic adhesion between synoviocytes and explain their organization into a tissue. Here, we describe the identification and cloning of a cadherin expressed in the lining of normal synovium, synovium in osteoarthritis, and the hyperplastic rheumatoid synovium. This cadherin mediates adhesion of FLS and confers features of synovial tissue formation upon fibroblasts in vitro.

Materials and Methods

Isolation and Culture of FLS. Synovial tissues from RA patients (American College of Rheumatology criteria; reference 8) were discarded tissue from synovectomy or joint replacement procedures, obtained with approval of the Brigham and Women’s Hospital Institutional Review Board. Synoviocyte cell suspensions were prepared from synovial tissues by mincing; treatment with 1 mg/ml collagenase (type I; Worthington Biochemicals), 0.15 mg/ml DNase I (Sigma–Aldrich), and 2 mM CaCl₂ in HBS (20 mM Hepes, 137 mM NaCl, and 3 mM KCl, pH 7.4); and rocking at 37°C for 1 h. The cell suspension was passed through a 40-mesh sieve and cultured in DMEM, 10% FBS (Hyclone), 2 mM MgCl₂, pH 6.9, for 30 min; blocked; and incubated with primary antibodies for 1 h at room temperature followed by Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and Alexa 488-conjugated phalloidin (Molecular Probes) for 1 h at room temperature.

For flow cytometry, cultured cells were released essentially as described for adhesion assays and stained with primary antibodies in HBS, 1 mM CaCl₂, 2% FBS for 1 h at 4°C followed by FITC-conjugated secondary antibody for 1 h at 4°C, and analyzed on a FACScan (Becton Dickinson). For multicolor flow cytometric analysis, freshly isolated RA synovial cells were disaggregated essentially as described before and resuspended in HBS, 2 mM Ca²⁺, 5% FBS, exposed to the following primary antibodies for 1 h at 4°C: IgG1-FITC, CD45-FITC, IgG1–biotin, or cadherin-11–SH6–biotin followed by Cychrome–conjugated streptavidin (BD Biosciences) for 1 h at 4°C and analyzed on a FACScan flow cytometer.

In Vitro Cadherin-induced Multicellular Organization. L cells transfected with cadherin-11 or vector control were plated at 5 × 10⁵ cells/ml (5 × 10⁶ cells/75 cm² surface area). Cellular organization was examined after 4 d by phase-contrast microscopy. For in vitro lining formation experiments, distinct areas of tissue culture flasks (Becton Dickinson) were coated with fibronectin (GIBCO BRL) by applying fibronectin drops (0.1–10 µg/ml) and incubation overnight at 4°C, followed by blocking of the entire surface with 1% BSA in HBS with 1 mM CaCl₂ overnight at 4°C. Cells were released essentially as described for adhesion assays and, after washing twice, were plated at 5 × 10⁴ cells/ml in serum- and ECM-free media (X-VIVO 15; Cambrex BioScience). Photographs were taken by phase microscopy after 2 d at 37°C.

Online Supplemental Material. The online supplemental material includes descriptions of cloning of the synovial cadherin, generation of L cell transfectants, production and characterization of cadherin-11–Fc fusion protein, and anti–cadherin-11 monoclonal antibodies, including Fig. S1, and of other antibodies and cell lines used in this work. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20041545/DC1.

Results and Discussion

Cloning of the Synoviocyte Cadherin. To determine if there is a cadherin that might function to mediate homophilic adhesion among synoviocytes, we performed PCR amplification of human synovial fibroblast cDNA using degenerate oligonucleotides based on the highly conserved cytoplasmic domains in human cadherins, as de-
scribed in the online supplemental material (available at http://www.jem.org/cgi/content/full/jem.20041545/DC1).

Six out of eight clones obtained matched the canonical cadherin-11 sequence. Northern blot analysis confirmed that the 4-kb cadherin-11 mRNA is present in total RNA of cultured FLS derived from RA synovial tissue, but not in RNA from epithelial cells (16E6.A5) or Jurkat T leukemia cells (Fig. 1). PCR amplification of the entire coding region of human cadherin-11 from FLS cDNA and nucleotide sequencing further confirmed the presence of bona fide cadherin-11 mRNA within cultured human FLS (see online supplemental text).

**Cadherin-11–Fc Supports Adhesion of Synoviocytes.** To test the adhesive capacity of human cadherin-11, we produced cadherin-11–Fc fusion protein and monoclonal anti–cadherin-11 antibodies (see online supplemental text and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20041545/DC1) and performed cell to substrate adhesion assays. Both cultured FLS derived from RA synovium, and cadherin-11–transfected L cells adhered to cadherin-11–Fc in a concentration-dependent fashion (Fig. 2 A). In a typical experiment, ~30–40% of FLS adhered to cadherin-11–Fc. In contrast, only 5% of FLS adhered to control E-cadherin–Fc (Fig. 2 A). Similarly, when the cadherin-11 gene was transfected into L cells (L/cad-11), these cells bound efficiently to cadherin-11–Fc–coated plates, whereas empty vector-transfected L cells (L/vector) bound only at background levels (Fig. 2 B). The binding of cadherin-11 L cell transfectants to cadherin-11–Fc–coated wells was blocked 80% by anti–cadherin-11 mAbs cadherin-11–2G4 and cadherin-11–5H6 (Fig. 2 C). The cadherin-11–3H10 mAb caused partial inhibition of adhesion (unpublished data). In contrast, neither the isotype matched P3 mAb nor the cell-binding mouse anti–MHC class I mAb (36–7–5) blocked the adhesion of cadherin-11 L cell transfectants to cadherin-11–Fc. Together, these findings are in agreement with other reports of the ability of cadherin-11 to support homophilic cell aggregation (13–15) and demonstrate for the first time that cadherin-11 mediates homophilic adhesion of cultured human RA-derived FLS.

**Expression of Cadherin-11 in the Rheumatoid Synovium.** To determine if cadherin-11 protein was expressed in RA, we performed immunohistochemistry of frozen human synovial tissue sections from RA patients. First, we stained RA synovium with anti–DAF (CD55), a marker expressed by FLS in the synovial lining layer (Fig. 3 A). For comparison, we stained RA synovium with mAb against CD68, which is expressed by synovial macrophages. As expected, anti-CD68 labeled cells in both the lining and sublining re-
regions (Fig. 3 B). The cadherin-11–3H10 mAb showed prominent staining of the lining in RA synovium (Fig. 3 C). In addition, rare, strongly cadherin-11–reactive cells were noted in the sublining region. A similar staining pattern was noted in synovia from patients with osteoarthritis (Fig. 3, D–F) and in normal synovium (Fig. 3, G–I), although reactivity was of weaker intensity. These findings contrast with results in skin and colonic tissues where no cadherin-11–reactive cells were seen, whereas the expected CD68 staining of tissue macrophages in all tissues and E-cadherin in skin was observed (Fig. 3, J–N). We found no evidence of E-cadherin expression in RA synovium (see online supplemental text). In addition, we find that cadherin-11 is expressed in normal mouse synovial tissue, but not in mouse skin (unpublished data).

Next, we tested whether RA-derived FLS expressed cadherin-11 on their cell surfaces. In vitro–cultured, RA-derived FLS stained with anti–cadherin-11–3H10 mAb by flow cytometry (Fig. 3 O). Thus, all the data obtained, including molecular cloning, Northern analysis, and flow cytometry of cultured FLS, were consistent with cadherin-11 expression on FLS. To confirm that FLS cadherin-11 expression did not result from tissue culture artifact, we performed multicolor flow cytometry on disaggregated fresh ex vivo RA synovial tissue using anti–cadherin-11–5H6 mAb and anti-CD45 mAb (a lineage marker of bone marrow–derived cells). Cells expressing cadherin-11 predominantly lacked CD45, and cells expressing CD45 mainly lacked cadherin-11, although a small population (~5%) of CD45–expressing synovial cells was stained by the mAb 5H6 (Fig. 3 P). Together, the immunohistology and the flow cytometry results indicated that within the inflamed rheumatoid synovium cadherin-11 expression is found predominantly on the FLS lineage.

Cadherin-11 Mediates Tissue Sheet and Lining Formation In Vitro. Because classical cadherins mediate homophilic adhesion that can result in cell sorting into aggregates and tissue morphogenesis, we examined the potential of cad-
cadherin-11 to mediate the association of cells into tissue-like sheets in vitro. L cells transfected with cadherin-11–containing vectors (L/cad-11) or with empty pCEP4 vectors (L/vector) were plated at equal numbers in tissue culture flasks. After 4 d of culture, cadherin-11–expressing L cells formed tight aggregates that grew as tissue-like sheets, whereas empty vector–containing L cells grew as random cells without specific cell–cell interactions (Fig. 4 A). To examine the formation of cadherin-11–mediated intercellular junctions in human FLS, we plated cultured FLS at 50% confluence where cells are not crowded. After overnight culture, the cells were well spread and contained actin stress fibers (Fig. 4 B, left). Additionally, the FLS developed numerous filopodial processes with the greatest number of filopodia at sites of close cell–cell contact. Specifically, at sites of intimate cell–cell contact with numerous interdigitating filopodia, the anti–cadherin-11 antibody labeled a ladder-like series of lines corresponding to intercellular junctions (Fig. 4 B, middle, right, and schema). No cadherin-11 staining was seen at sites without cell–cell contact.

A perinuclear granular cadherin staining demonstrated that FLS contained intracellular cadherin-11, consistent with similar findings for E-cadherin (Fig. 4 B and reference 16). In vivo, the synovial lining exists as a condensed cell layer, one to three cells thick, overlying and imbedded in the ECM of the synovial sublining that also contains small blood vessels and scattered fibroblasts. The other side of the lining layer faces the synovial space (Fig. 5 A). The basis for the formation and organization of the synovial lining is unknown. To study this process, we set up a culture system in vitro to simulate synovial lining formation. To mimic the in vivo setting of a tissue layer adjacent to the joint cavity, we coated the ECM component fibronectin (representing synovial sublining ECM) onto discrete regions of a tissue culture flask. The surrounding plastic surface was blocked with BSA (representing the joint cavity) and FLS were added in serum- and ECM-free medium. After 2 d of culture, the FLS formed a lining-like structure at the fibronectin–BSA interface and formed visible interconnections with cells below the interface, in a distribution resembling that in the synovium (Fig. 5 B). To confirm that this phenomenon could be accounted for by cadherin-11 expression, we compared L cells expressing or lacking cadherin-11. When L cells were plated in the same culture system, they adhered randomly to the fibronectin–coated surface and did not form a lining layer (Fig. 5 C). Strikingly, after culture for 2 d, L cells expressing cadherin-11 formed a lining-like structure at the fibronectin–BSA interface similar to that obtained with FLS (Fig. 5 D). Although these studies do not attempt to incorporate all of the cellular elements of the synovial tissue, they support the hypothesis that cadherin-11 mediates intercellular adhesion that imparts morphologic characteristics of the tissue in vivo. These include the formation of tissue sheets and a lining-like layer at a matrix interface, analogous to the interface between the loose connective tissue matrix of the synovium and the joint space.

Implications for Synovial Lining Formation and RA. Based on our findings and the fact that cadherins are widely recognized to mediate tissue morphogenesis in development and contribute to tissue architecture in the adult (2), we propose that cadherin-11 may serve this function for the synovium. Cadherin-11 may allow the homophilic adhesion between FLS that is required for the process of cell rearrangement responsible for the organization of the synovial lining layer. Additional interactions including those between FLS and macrophage–like synoviocytes, possibly
herins, such as cadherin-11. Unlike E-cadherin, and mesenchymally expressed cad-
herins, such as cadherin-11, may exist between the functions of epithelial cadherins,
and in embryonic tissues, such as developing limb buds and branchial arches, indicates a role in outgrowing or extend-
ing enzymes (MMPs), cytokines (IL-6), growth factors (FGF), and angiogenic factors (VEGF) during inflammatory arthri-
tis (29), and fibroblast-like cells are the major population in
the invasive pannus that ultimately leads to joint destruc-
tion. Many of the processes that are key to rheumatoid syn-
ovitis, such as cellular condensation, tissue extension, and invasive behavior, have been linked to cadherin function.

Therefore, the synovial cadherin may have a determining
role in regulating cell to cell adhe-
sion, cadherins also modulate other cell functions via intra-
cellular signaling pathways (2). Thus, cadherin-11 likely
plays a role in regulating the behavior of RA FLS that are
able of foci formation, anchorage-independent growth in soft agar, and invasive tumor-like behavior in SCID mice (24, 25). It is also intriguing that cell contact between
fibroblasts mediated by cadherin-11 induces up-regulation
of endothelial growth factor VEGF-D gene expression
(26), and that in embryonic lung, only cadherin-11–express-
ing cells appear to produce VEGF-D (27). Therefore, it is
possible that synovial cadherin-11 expression also contrib-
utes to the increased angiogenesis in RA (28).

Our identification of a synovial cadherin and its expres-
sion in normal and RA FLS offer a previously unrecognized
opportunity to understand the structural basis for this tissue
and the biology of the synovium. FLS of the synovial lining
are of great interest in understanding both the normal phys-
iology of the joint and how it is damaged in synovitis. In
the normal synovium, FLS participate in the active process
of synovial homeostasis via production of matrix components,
matrix remodeling enzymes, and enzyme inhibitors. In
pathological states such as RA, it remains unclear whether synovial lining hyperplasia results from underlying inflam-
mation in the synovium or from primary abnormalities of
FLS. Nevertheless, FLS are key producers of matrix-damag-
ing enzymes (MMPs), cytokines (IL-6), growth factors (FGF),
and angiogenic factors (VEGF) during inflammatory arthri-
tis (29), and fibroblast-like cells are the major population in
the invasive pannus that ultimately leads to joint destruc-
tion. Many of the processes that are key to rheumatoid syn-
ovitis, such as cellular condensation, tissue extension, and
invasive behavior, have been linked to cadherin function.

Therefore, the synovial cadherin may have a determining
role in the mesenchymal tissue response to chronic inflam-
mation. A new focus on the role of cadherin-11 in FLS bio-
logy will provide new insights into the aggressive behavior
of the synovial tissue in conditions such as RA.

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