

Gap Junctions in Human Synovial Cells and Tissue

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Our objective was to establish the existence of intercellular communication through gap junctions in synovial lining cells and in primary and passaged cultures of human synovial cells. Communication between cells was assessed using the nystatin perforated-patch method, fluorescent dye transfer, immunohistochemistry, transmission electron microscopy, and immunoblotting. Functional gap junctions were observed in primary and passaged cultures and were based on measurements of the transient current response to a step voltage. The average resistance between cells in small aggregates was $300 \pm 150 \text{ M}\Omega$. Gap junctions were also observed between synovial lining cells in tissue explants; the size of the cell network in synovial tissue was estimated to be greater than 40 cells. Intercellular communication between cultured cells and between synovial lining cells was confirmed by dye injection. Punctate fluorescent regions were seen along intercellular contacts between cultured cells and in synovial membranes in cells and tissue immunostained for connexin43. The presence of the protein was verified in immunoblots. Regular 2-nm intermembrane gap separations characteristic of gap junctions were seen in transmission electron micrographs of synovial biopsies. The results showed that formation of gap-junction channels capable of mediating ionic and molecular communication was a regular feature of synovial cells, both in tissue and in cultured cells. The gap junctions contained connexin43 protein and perhaps other proteins. The physiological purpose of gap junctions in synovial cells is unknown, but it is reasonable to anticipate that intercellular communication serves some presently unrecognized function. *J. Cell. Physiol.* 184:110–117, 2000. © 2000 Wiley-Liss, Inc.

Tissues in joints are continuously remodeled and homeostasis is normally maintained despite the complex biochemical environment of joints and the wide range of mechanical loads that they sustain (Pelletier et al., 1993; van den Berg et al., 1998). It is a central problem to understand what accounts for the stability of the system that governs tissue remodeling and the kinds of changes that might cause or predispose toward arthritis or other diseases.

Gap junctions are plaques of aqueous channels that facilitate electrical and metabolic communication between the intracellular compartments of adjacent cells. The plaques are composed of a few to many hundreds of individual channels, each 1–1.5 nm in diameter and capable of passing molecules of up to about 1 kDa (Brink, 1996). The channels are formed by non-covalent linkages between annular hemichannels in the membranes of adjacent cells. Hemichannels are com-

posed of proteins of the connexin family. More than a dozen connexins have been identified, including at least seven human isoforms (Bruzzone et al., 1996).

The functional state of gap-junction channels can be modified dynamically (gated), which results in restriction or augmentation of gap-junction intercellular communication (GJIC). Gating mechanisms include phosphorylation of connexins (Musil et al., 1990; Lau et al., 1991; Oh et al., 1991; Pelletier and Boynton, 1994), elevation of adenosine-3',5'-cyclic monophosphate (cAMP) and guanosine-3',5'-cyclic monophosphate (cGMP) levels (Burt, 1990; DeMello, 1990; Takens-Kwak and Jongsma,

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1992), and changes in intracellular Ca^{2+} and acidification (Lowenstein, 1981; Morley et al., 1995; Bruzzone et al., 1996). Gap-junction channels are thought to have an important role in morphogenesis (Wolburg and Rohmann, 1995), cancer (Ruch, 1994), cardiac physiology (Reaume et al., 1995), certain kinds of peripheral neuropathies (Berghoffen et al., 1993), ocular lens metabolism (Goodenough et al., 1980), and in regulating responses to extracellular signals (Stauffer et al., 1993; Munari-Silem et al., 1995).

Gap junction plaques were observed by electron microscopy between synovial lining cells (SLCs) in normal and arthritic synovia (Dryll et al., 1980; Meek et al., 1991); functional gap-junction channels were found in rabbit synovial fibroblasts (Kolomytkin et al., 1997, 1999). This study was undertaken to find evidence for the existence of functional gap junctions in SLCs and in primary and passaged human synovial cell cultures.

MATERIALS AND METHODS

Tissue and cells

Synovial tissue was obtained from 19 patients who underwent total joint reconstruction or other surgical procedures involving the hip or knee. The group consisted of nine patients with osteoarthritis (Altman et al., 1986), three with rheumatoid arthritis (Arnett et al., 1988), and seven who had traumatic injuries. The tissue was transported in phosphate-buffered saline from the operating room to the laboratory 10–60 min after it was obtained. Neither the extent of disease or injury, nor the intra-articular location from which the specimen studied was obtained, were standardized. Consequently, the results were not analyzed in relation to the presence of disease or injury.

Primary cell cultures were prepared by treating small pieces of synovial tissue with 1 mg/ml collagenase and 1 mg/ml hyaluronidase in F12 medium at 37°C (Georgescu et al., 1988). After 15 min, cells began detaching from the synovial surface and aggregating on the bottom of the petri dish. Fifteen minutes later (30 min after addition of the enzymes), the tissue was removed and the aggregated cells were incubated in F12 medium for 40 min at 37°C to allow recovery from the enzyme treatment prior to the electrophysiological measurements.

For passage of synovial cells in culture, 3 mg/ml collagenase in F12 medium was used to dissociate pieces of synovial tissue for 30–60 minutes at 37°C, 5% CO_2 . Cells released from the tissue were grown in F12 medium with 10% heat-inactivated fetal bovine serum containing penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). HIG-82 rabbit synovial fibroblasts (American Type Culture Collection (ATCC), Manassas, VA) were also grown in the same medium without antibiotics. Cells were passaged by trypsinizing confluent cultures and seeding 10^6 cells into 4 ml of medium. In the electrophysiological studies, 10^5 cells were added to 35-mm petri dishes and the medium was replaced with bath solution. For in situ measurements on SLCs, pieces of the synovium were fixed to the bottom of a petri dish using a fiberglass net glued to a stainless steel ring (Fig. 1).

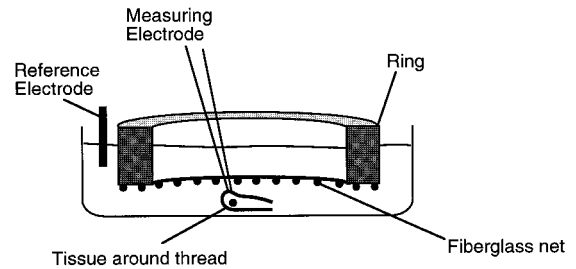


Fig. 1. Chamber for tissue experiments (side view). Tissue (2–5 mm^2), with the synovial surface exposed, was bent around a nylon thread and placed on the bottom of a petri dish. A fiberglass net (1.3 mm gap between fibers) glued to a stainless steel ring (inner and outer diameter of 24 and 32 mm, respectively; height 4 mm) held the tissue in place. The measuring electrode was connected to a cell on the synovial side of the tissue through the gap between the fiberglass fibers.

Electrical measurements

Glass capillaries 1.0 mm in diameter (PB-7, Narashigi, Tokyo, Japan) were pulled in two steps to obtain a tip diameter of $\sim 1.0 \mu\text{m}$ and then fire polished in a microforge (MF-9, Narashigi, Tokyo, Japan). Giga-seals ($\sim 10 \text{ G}\Omega$ unless noted otherwise) were formed under negative pressure (5–10 cm, H_2O), typically within 0.5–5 min; the success rate was greater than 50%. The nystatin perforated-patch method was used to gain electrical access to the cell interior (Horn and Marty, 1988).

Because nystatin interfered with giga-seal formation, the tip of the pipette was filled with a nystatin-free solution prior to the addition of pipette solution containing 0.3 $\mu\text{g}/\text{ml}$ nystatin. The giga-seal was formed during the time needed for the nystatin to diffuse to the tip of the micropipette. The nystatin channels ($40 \pm 20 \text{ M}\Omega$ integral resistance) formed 5–15 min after the negative pressure was removed. Giga-seals and nystatin pores usually remain stable for hours in cultured cells, but only for about 10 min in synovial tissue.

After formation of a perforated patch, a 2-mV step was applied (Axopatch 200B, Axon Instruments, Foster City, CA) and the transient current passing through the electrode was measured using a patch-clamp amplifier (Axopatch 200B, Axon Instruments) connected to a computer (TL-1 DMA Interface, Axon Instruments). Commercial software (pCLAMP 6, Axon Instruments) was used to control the amplifier and collect and analyze the experimental data. Theoretical analysis of typical current responses from aggregates of different sizes established that the time constants of the current decay depended on the resistance between adjacent cells in the aggregate. Thus the existence of gap junctions and junction resistance could be determined by measuring the transient current (Kolomytkin et al., 1997).

The pipette solution contained (in mM) 125 K-aspartate, 30 KCl, 4 NaCl, 1 CaCl_2 , 10 N-2-hydroxyethylpiperazine- N' -2-ethanesulfonic acid (HEPES)-KOH, pH 7.2. The bath solution contained (in mM) 145 NaCl, 5.4 KCl, 1.5 CaCl_2 , 1.0 MgCl_2 , 5.0 HEPES-NaOH, 5.0 glucose, pH 7.3. The resistance of the electrodes was

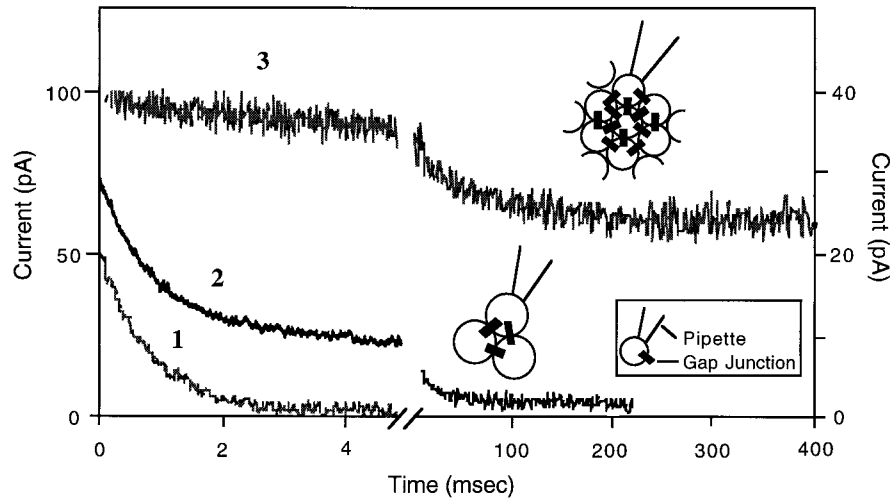


Fig. 2. Transient current responses in human synovial cells to a voltage step (passage 4 cells). After perforated patch formation, a 2 mV step was applied to the electrode at zero time. Curve 1, patch of an isolated cell; curve 2, patch of a cell that was aggregated with 2 other cells; curve 3, patch of a cell aggregated with more than 20 cells.

Individual records were filtered with a 10 kHz low-pass 4 pole Bessel filter. Each curve was produced by averaging the results of 10 voltage steps. Scale for curve 1 is shown on the left side, and for curves 2 and 3 on the right side.

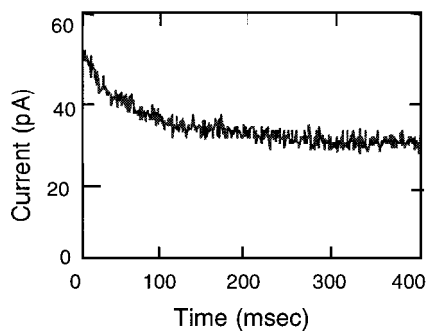


Fig. 3. Transient current responses of a human synovial lining cell to a voltage step. After perforated patch formation, a 2 mV step was applied to the electrode and the curve was produced by averaging the transient current for 10 voltage steps. Individual records were filtered with a 10 kHz low-pass 4 pole Bessel filter.

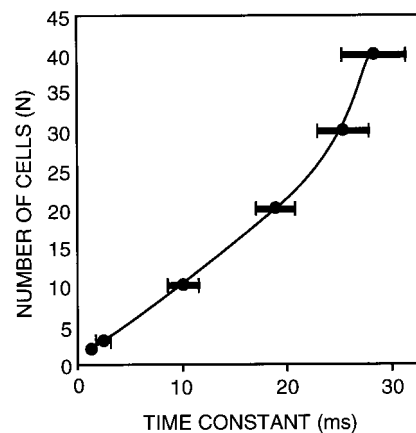


Fig. 4. Relation between the time constant for current decay in aggregates of HIG-82 cells and the number of cells in the aggregate (N).

7–9 M Ω . All electrical measurements were made at room temperature in bath solution.

Dye transfer

Cells were seeded on coverslips in petri dishes and raised to confluence. The coverslips were washed with bath solution and placed in a chamber filled with 1 ml of bath solution for observations of dye transfer (Paterostro et al., 1995; Spray and Dermietzel, 1996). For measurements on SLCs, the synovial tissue was bent over nylon thread and held on the bottom of the chamber ($40 \times 10 \times 1$ mm) with a platinum weight so that the synovial surface was accessible. All observations were made under a slow flow of bath solution (20 μ l/s).

Micropipettes (200 M Ω , P87, Sutter Instruments, Novato, CA; Sigma, St. Louis, MO; Neurodata Instruments, Delaware Water Gap, PA; Chroma, Brattleboro, VT) were pulled from glass capillaries and filled overnight with a 4% solution of lucifer yellow (dilithium

salt, L-0259, Sigma). After the cell was impaled, the dye was injected using 100-ms pulses, 0.3–0.7 nA (IR-283, Neurodata Instruments). To prevent cell damage and photo-bleaching, cells were illuminated (425 nm) for only the time needed to record the results (about 15 sec). Emission was measured at 540 nm using an upright microscope, lucifer-yellow filters (Chroma), and a digital camera, and the results were stored on a computer for post-processing.

Immunocytochemistry

Tissues were frozen in 2-methylbutane/acetone/solid CO₂ and prepared as described previously (McCarthy et al., 1989). Cultured cells were fixed in Clarke's solution. Affinity-purified rabbit anti-human polyclonal antibody directed against specific peptide sequences of connexin43 (Zymed, San Francisco, CA; Jackson Im-

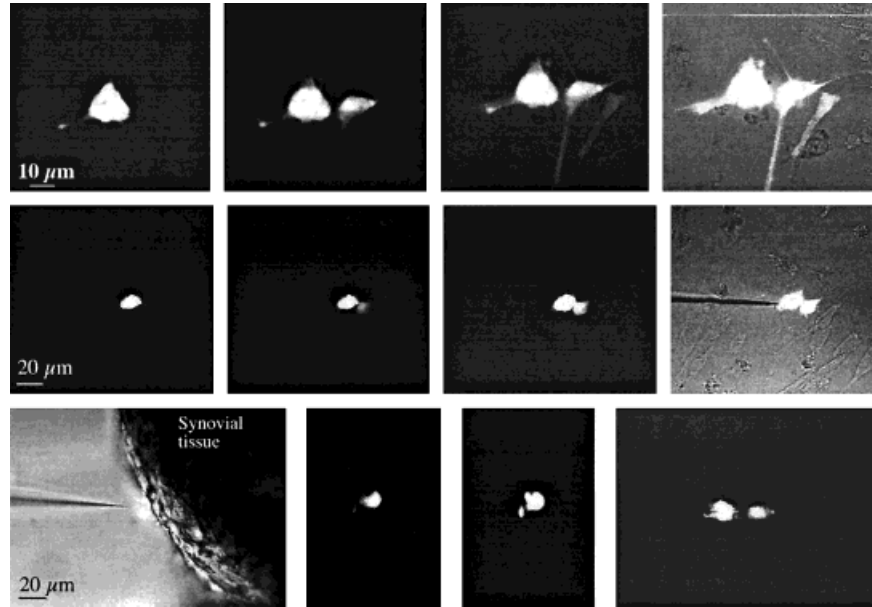


Fig. 5. Sequential images taken over a few minutes showing lucifer-yellow transfer through gap-junction channels between HIG-82 cells (**top**), human synovial cells (**middle**) and SLCs (**bottom**). Some pictures (rows 1 and 2, right; row 3, left) were taken using both ultraviolet and visible light to identify the location of coupled cells.

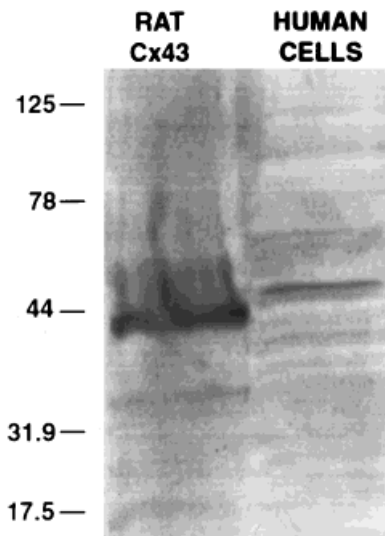


Fig. 6. SDS-polyacrylamide gel and Western blot analysis of a synovial membrane preparation using anticonnexin43 antibody. **Lane 1:** connexin43 standard (rat brain). **Lane 2:** cell lysate. The positions of molecular mass standards (kDa) are indicated.

munoresearch, West Grove, PA; Olympus, Melville, NY; Sensys Photometrics Ltd., Tucson, AZ) was used to stain sections and cells. The specificity of the antibody was established previously (Nagy et al., 1997, 1998). Isotype-specific secondary antibodies were conjugated to Texas Red (Jackson Immunoresearch). Negative controls (secondary antibodies) and positive controls (heart connexin43) were stained in parallel. Images were acquired on an inverted microscope (Olympus

IX-70) that was equipped for epifluorescence illumination and phase contrast optics and interfaced to a digital camera (Sensys, Photometrics Ltd.) with a readout rate of 1 megapixel/sec.

Transmission electron microscopy

Specimens were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 37°C for 2 h and post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 h. Lanthanum nitrate (1%) was added to the osmium tetroxide for some specimens to better visualize the gap junctions. The specimens were infiltrated in Polybed812 (Polyscience, Inc., Niles, IL), cut at 50–90 nm, stained with uranyl acetate and lead citrate, and observed and photographed with an electron microscope (Hitachi H-300, San Jose, CA) operating at 80–100 kV.

Immunoblotting

Membrane fractions from confluent monolayers of human synovial cells were run on 4–20% linear-gradient sodium dodecyl sulphate (SDS) polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with antibody to connexin43 (Zymed). Rat brain connexin43 (Zymed) was used as a positive control. Immunoreactive bands were detected with peroxidase-labeled anti-rabbit immunoglobulin G (IgG) antibody.

RESULTS

Patch clamping

Typical results for the transient current response from aggregates of cultured synovial cells after the application of a 2-mV step are shown in Figure 2. Curve 1 shows the response from an isolated synovial cell in a passage-4 culture. After the voltage step was

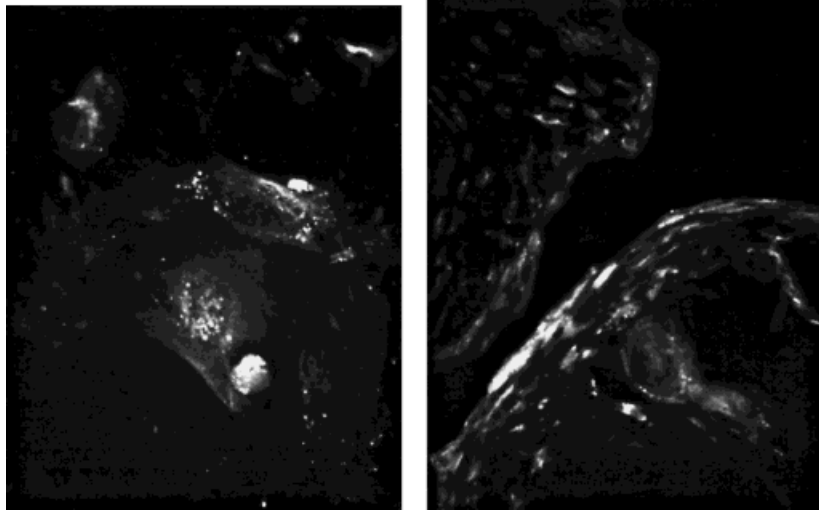


Fig. 7. Immunofluorescence for connexin43 in cultures of synovial cells (**left**) and synovial tissue (**right**). Connexin43 staining of cultured cells was found randomly located on cell surfaces in areas where cells were not confluent. In areas of confluency (shown) where contact between cells was occurring, connexin43 staining appeared to be localized in a punctate manner at the region of cell–cell contact. (Final

magnification 400 \times). Right: Connexin43 staining of synovial membrane showed labeling of individual synovial cells, with staining prominently below the free margin of the tissue. In some cells, punctate staining of the connexin could be readily seen. (Final magnification 400 \times).

applied, the current charged the capacitance of the cell membrane (about $1 \mu\text{F}/\text{cm}^2$) and then decayed with a time constant (τ) of about 1 msec. Curve 2 shows the transient current for the case where the patched cell was aggregated with two other cells. The current decay was slower ($\tau \approx 4$ msec), indicating that the capacitance of the patched cell was in parallel with that of the other cells and, therefore, additive to it. This could occur only if the cells were connected to each other by gap junctions (Kolomytkin et al., 1997). Curve 3 was obtained from a patched cell aggregated with more than 20 cells. The decay was even slower ($\tau \approx 47$ msec), as would be expected if the patched cell was part of an extensive network of gap junctions.

The results shown in Figure 2 were observed in cultured cells from all patients (passages 2–10). More than 250 cells in aggregates were patched and, in each instance, the time constant of the current decay lengthened as the number of cells in the aggregate was increased. The average electrical resistance between adjacent cells in a representative group of 10 cell aggregates (3–6 cells/aggregate) was $300 \pm 150 \text{ M}\Omega$.

To provide evidence that the connexins forming the gap junctions were present in primary cells and were not functional solely in passaged cells, we made measurements on cells released from synovial tissue by enzyme treatment. In each instance, the results were indistinguishable from those obtained from passaged cells (Fig. 2).

Evidence that functional gap junctions occurred between SLCs was sought by attempting to patch SLCs in tissue explants. In most instances, the presence of matrix proteins and other substances on the synovial surface prevented giga-seal formation. In five instances, however, involving two cells from an osteoarthritis (OA) patient, one cell from a second OA patient, and two cells from a rheumatoid arthritis (RA) patient,

acceptable giga-seals were formed and it was therefore possible to ascertain whether the patched cell formed gap junctions. In all five cases, long time-constants for decay of the current were observed, indicating that the patched cell was part of a gap-junction network (Fig. 3).

Estimation of network size

A potentially important issue in evaluating the physiological role of GJIC in SLCs is the ability to determine the size of the cell networks that can be formed. There are no known direct methods for measuring the network size of cells coupled by gap junctions in non-excitable cells. Consequently, we developed a method based on a calibration curve obtained using HIG-82 cells.

The transient current response to a 2-mV step was measured in aggregates of 1–40 HIG-82 cells and fitted, using the method of least squares, to a curve consisting of two exponentials. The time constant associated with the slower of the two exponential terms was found to be more sensitive to the number of cells in the aggregate (N) and was therefore used to describe the relation between N and τ . Measurements on progressively larger aggregates led to correspondingly larger values of τ (Fig. 4).

We could conclude that each cell in the HIG-82 aggregates formed a gap junction because, in 50 experiments, we never found a patched cell in an aggregate that exhibited a time constant similar to that of a single, isolated cell. Taken together with the time-constant measurements (Fig. 4) and with theoretical analyses of the electrical behavior of large cell aggregates (Kolomytkin et al., 1997), the evidence indicated that each HIG-82 cell in aggregates at least as large as 40 cells was connected by gap junctions, thereby forming one syncytial structure. Using Figure 4, we found that the τ of SLCs in the gap-junction networks present in

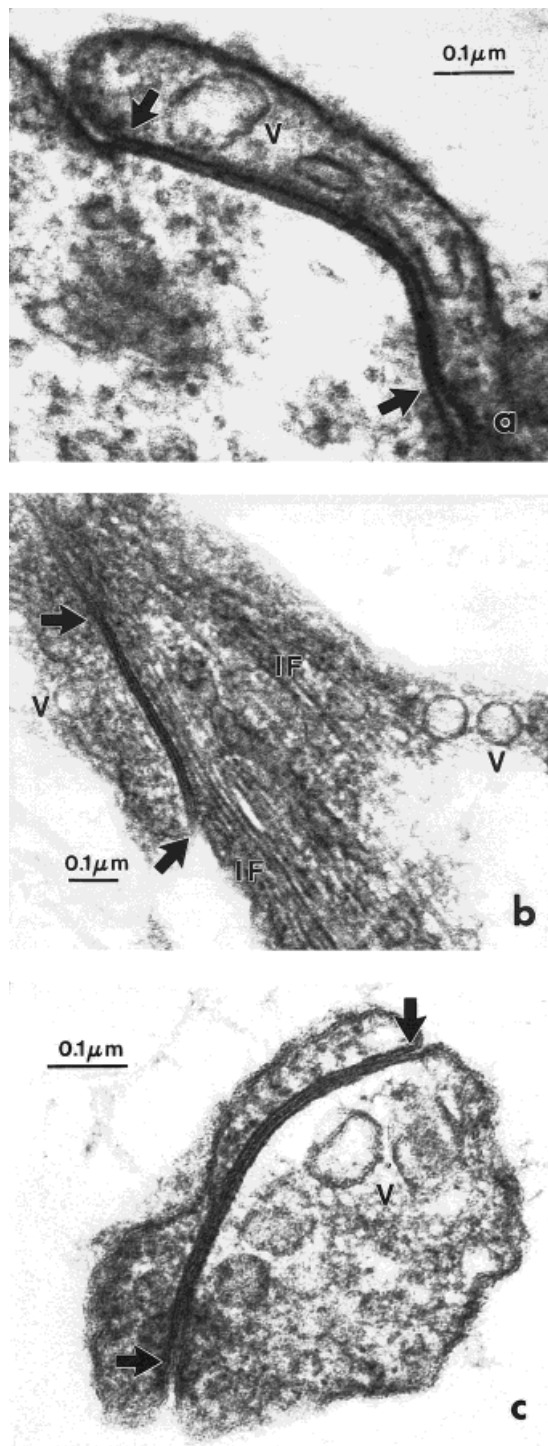


Fig. 8. **a:** Gap junction from a normal synovial biopsy. Vesicles (V) are closely related to the gap junction; 150,000 \times . **b:** A gap junction from an osteoarthritic synovial biopsy. Transmembrane connexons are evident along the gap junction. Intermediate filaments (IF) extend along the longitudinal axis of one of the cell processes. Vesicles (V) are present in both cells, some near the gap junction; 90,000 \times . **c:** A gap junction from a rheumatoid arthritic synovial biopsy. The connexons are evident along some areas of the junction. Vesicles (V) are aligned along the junction; 150,000 \times .

the human synovial biopsies that were successfully patched was greater than the τ from an aggregate of 40 synovial cells measured in vitro, suggesting that the networks in the biopsies were larger than 40 cells.

Dye transfer

We regularly observed dye coupling in cultured cells and in SLCs within a few minutes after dye was injected into the impaled cell (Fig. 5). Comparison of fluorescent images with those obtained under ordinary illumination showed that lucifer yellow diffused from the injected cell into neighboring cells. We were certain that the recorded images were of intracellular lucifer yellow because, under the conditions of our measurements, any leakage of dye dissipated and was removed from view within a few seconds. In each instance, coupling occurred to only a few neighboring cells (more than 10 experiments).

Immunoblots

To determine whether synovial cells expressed connexin43 protein, we performed immunoblots on membrane preparations using antibodies against a unique connexin43 sequence. The antibody recognized a synovial-cell protein that migrated with an effective mass of about 45 kDa (Fig. 6) rather than 43 kDa, which is characteristic of connexin43 proteins obtained from the heart (Nagy et al., 1997).

Immunohistochemistry

Connexin43 was found in cultured synovial cells and synovial tissue (Fig. 7). Punctate staining was seen along intercellular contacts between cultured cells and in the synovial membrane. Specificity of the staining procedure was established by the absence of a fluorescent label in the control sections, which were not treated with primary antibodies.

Electron microscopy

Regular 2-nm intermembrane gap separations characteristic of gap junctions were seen in biopsies from both arthritic and non-arthritic patients (Fig. 8). The intramembrane hemichannels of individual gap-junction hemichannels could frequently be resolved on the basis of periodic density changes (Fig. 8, B and C). The gap junctions were about 1 μ m in length and usually occurred in cell processes and in proximity to regions containing dense accumulations of intermediate filaments and vesicles.

DISCUSSION

More than 250 cells in aggregates of 2–20 cells were patched in passage 2–10 cells from 19 patients. In each case, after application of a standard step voltage, a lengthened current decay was observed as compared with the decay seen from isolated cells ($N = 50$). The results could be explained only under the assumption that the patched cell formed gap junctions with neighboring cells. Because every patched cell was found to have formed gap junctions with neighboring cells, we concluded that every aggregated synovial cell formed gap junctions with at least some cells in its aggregate.

The gap junctions measured in passaged cells could have resulted from overexpression of connexins or activation of gap-junction channels caused by conditions

unique to the cell-culture environment. To partially address this question, we analyzed for the presence of gap junctions in primary cell cultures and found that cells released from synovial tissue and allowed to aggregate on the bottom of the petri dish formed gap junctions in every instance. These observations did not prove that gap junctions actually existed in tissue, but they made it seem less likely that gap-junction formation was an artifact induced by the culture conditions because, if so, it would be necessary to postulate that gap junctions were expressed, assembled, or activated while the synovial cells were being released from tissue and settling on the bottom of the petri dish.

To further assess the physiological significance of intercellular gap junctions, we attempted to observe gap junctions in synovial tissue explants. As expected, it was difficult to form giga-seals with cells in the tissue because of the presence of matrix proteins and other substances on the synovial surface. In more than 90% of the attempts, it was not possible to form a giga-seal. However, in five cases giga-seals were formed; they exhibited resistances of 1–2 G Ω and were stable for about 10 minutes, compared with resistances of about 10 G Ω and stability for several hours in cultured synovial cells. In the instances where acceptable giga-seals were made on the SLCs, we found that the patched cell formed gap junctions with neighboring cells, as assessed on the basis of the current response (Fig. 3). If it is assumed that the likelihood of forming patches and the presence of gap junctions were independent phenomena, then the long time constants observed (Fig. 3) would indicate that SLCs typically formed gap junction networks. The dye-transfer results were consistent with this interpretation. They established that the synovial cells were coupled in such a way that the cells could exchange small signaling molecules, thereby providing a basis for metabolic interaction.

Although our data indicated that synovial cells in culture and in tissue were dye coupled, the method was not capable of revealing the extent of the coupled networks because lucifer yellow does not penetrate all gap junctions and transfer of the dye between cells cannot be detected if the coupling conductance is below 1–2 nS (Vaney, 1991; Dermitezel and Spray, 1993). The electrophysiological data (Figs. 3 and 4), in contrast, suggested that SLCs formed networks that exceeded 40 cells.

Cells and tissues both stained positive for connexin43 (Fig. 7). The images contained punctate structures, some of which were located at boundaries between cells; discrete staining at such locations is the expected result for inferring the presence of gap junctions (van Rijen et al., 1998). Western blots of protein fractions prepared from cell membranes were positive for a protein band containing a connexin43 peptide sequence (Fig. 6). We did not fully address the question of whether other connexins were also present, but preliminary studies suggested that connexin26 and connexin32 might also form in gap junctions between synovial cells. Thus the evidence suggests, but does not prove, that connexin43 mediated the observed coupling.

The intermediate filaments that we observed did not make contact with the gap junctions but were seen in both longitudinal and cross profiles near the gap junctions,

which suggests that they play some role in forming or stabilizing the gap-junction channels. Vimentin intermediate filaments are a major constituent of SLCs (Meek et al., 1991).

The specific function of gap-junction intercellular communication in synovial metabolism is presently unknown. Nevertheless, it is reasonable to anticipate that the presence of gap-junction channels between synovial cells serves a physiological purpose. One possibility is well supported by the known ability of synovial cells to respond to a broad range of signaling agents (van den Berg et al., 1998). The susceptibility of gap-junction channels to regulation by protein kinases and other components of signal transduction pathways (Burt, 1990; Musil et al., 1990; Pelletier and Boynton, 1994; Morley et al., 1995) suggests a mechanism by which the nuclear events triggered by ligand-receptor interactions could be coordinated within a cell network so that the overall response of the tissue would be optimal. By learning more about gap-junction intercellular communication in synovial cells, it should be possible to gain a deeper insight into how these cells execute their characteristic functions and, perhaps, into an understanding of the pathophysiological alterations that occur in the context of joint disease.

We conclude that formation of gap-junction channels capable of mediating ionic and molecular communication is a regular feature of synovial cells, both in tissue and in culture. The gap junctions contain connexin43 protein and may also contain other proteins.

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